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Effects of Soil Storage at Freezing Temperatures on Soil Enzymatic Activities

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Abstract: Soil enzyme activities are good indicators of soil health. It has been hypothesized that soil storage before analysis might affect microbial function. The objective of the present study was to determine if soil storage at -20 and -80°C affects soil enzymatic activities. Soil samples were collected and stored for four weeks at -20 and -80°C. Activities of nine enzymes were measured in fresh samples and every two weeks during storage. Overall, nine enzymes were targeted including β-Glucosidase (BG), Cellobiohydrolase (CBH), β-N-Acetylglucosaminidase (NAGase), Aryl Sulfatase (AS), Acid Phosphatase (AP), Alkaline Phosphatase (AlP), Glycine Aminopeptidase (GAP), Leucine Aminopeptidase (LAP) and Peroxidase (PER). With the exception of GAP and LAP, no significant differences were observed between samples stored at -20°C for 2 weeks compared to controls. Storage at -80°C for two weeks resulted in a decrease in all the enzyme activities except for PER, BG, and LAP. With the exception of PER, storage at -20 and -80°C decreases the activities of all the enzymes tested after four weeks of storage. These changes varied with specific enzyme targeted. Further studies should be conducted to determine how these low storage temperatures affect microbial diversity and abundance.

Keywords: Microbial Enzyme Activity, Soil Storage, Freezing Temperatures, Climate Changes

Introduction

Soil microorganisms play a key role in soil quality and climate feedback because they are responsible for the production and consumption of greenhouse gases including carbon dioxide (Jansson and Hofmockel, 2020). Soil microbes sequester carbon and other nutrients and they emit these nutrients in the form of gases including carbon dioxide and methane (Tang *et al*., 2022). The carbon used in these processes comes from a variety of sources and is stored in carbon pools in soil (Zhang *et al*. 2020b; Tang *et al*., 2022). Microbes and their enzymes have a more difficult time accessing the carbon in the pools when it is unstable which occurs under freezing conditions (Tang *et al*., 2022). This is just one of the many ways scientists can track changes in climate using soil microbes.

Temperature is one of many parameters that influence enzyme activity (Peterson *et al*., 2007). Cold temperatures can destabilize tertiary structures in the enzyme (Privalov, 1990) and higher temperatures can cause heat denaturation (Peterson *et al*., 2007). Every enzyme performs optimally and remains stable under specific temperature ranges (Chua *et al*., 2017). When temperatures remain warm for longer periods or rise slightly, soil nutrient availability and microbial activities can increase (Xiao *et al*., 2018). Some enzymes are inactivated when temperatures drop below freezing and their activity is only restored when returned to normal room temperature (Privalov, 1990). Contrary to this, some enzymes have exceptional tolerances to cold and they can remain active under cold temperatures (Wallenius *et al*., 2010). Because enzymes react differently to different temperatures, it is important to analyze them individually (Poulsen *et al*., 2021).

Analysis of soil enzymatic activities from fresh soil is not always possible because of different constraints. Currently, there are no standard (reference) methods for soil storage. There are many ways to store samples and the most common practices are freezing samples or air-drying samples (Wallenius *et al*., 2010). Air-drying is a universally accepted practice for storing soil samples because the dried soil undergoes minimal microbial and chemical reactions that allow for stable analysis conditions over time (Obalum, 2017). However, because the dried soil cannot reabsorb water efficiently during soil

preparation, the concentration of solutes increases compared to the original sample. This can alter the outcome of the analysis (Obalum, 2017). It is for this reason that some researchers recommend freezing the samples instead to allow fewer changes in the microbial community present in soil over time (Poulsen *et al*., 2021). Investigations of microbial activities revealed that most enzyme activities inconsistently decreased over time at 4°C storage (Moy and Nkongolo, 2023). Freezing can affect soil analysis results, but frozen samples over time appear to maintain consistent results (Poulsen *et al*., 2021). When freezing soil samples, it is important to determine the best soil storage temperature because some enzymes only provide stable results at certain temperatures, namely -20 and -70°C (Wallenius *et al*., 2010). Knowing which enzymes are able to withstand colder temperatures has applications in protein folding investigations and biotechnology. The results of several studies are still inconsistent and more investigations are warranted (Wallenius *et al*., 2010; Chua *et al*., 2017).

The present study focuses on the effects of exposure to cold temperatures, -20 and -80°C, on nine different soil enzymes. These enzymes include β-glucosidase (BG), Cell-Biohydrolase (CBH), β-n-acetylglucosaminidase (NAG), Arylsulfatase (AS), Acid Phosphatase (AP), Alkaline Phosphatase (ALP), Glycine Aminopeptidase (GAP), Leucine Aminopeptidase (LAP) and Peroxidase (PER).

It is hypothesized that when soil samples are stored in the -80°C freezer, they will be able to maintain consistent activity levels over time because the microorganisms will be frozen and inactive until the samples thaw and are ready for testing. The effects of storing at -80°C will be more pronounced compared to -20°C. Therefore, the objective of this study is to investigate how each enzyme responds to soil freezing at -20 and -80°C during storage.

Materials and Methods

Sample Collection and Storage

Soil was sampled from the City of Greater Sudbury close to Kingsway Avenue, coordinates, 46°29’54”N 80°58’14”W. Ten (10) soil samples per replication (three replicates) were collected from the organic layer (0-5 cm in depth) resulting in a total 60 subsamples. The samples were sieved in a 1mm mesh to remove debris, rocks, and plants. Fresh samples were used as a control and enzymatic activities were measured immediately. Sub-samples of each were replicated at -20°C for two and four weeks. Other sub-samples of the three replicates were stored at -80°C for the same period (2 and 4 weeks). Hence, enzymatic activity was performed every two weeks.

Soil Samples Characterization

Soil pH was measured in water as described in Narendrula-Kotha and Nkongolo (2017). Organic matters were determined using the Loss on Ignition (LOI) analysis at Tesmark Inc. (Sudbury). Bioavailable metal analyses were performed as described in Nkongolo *et al*. (2013; 2022); Narendrula-Kotha and Nkongolo (2017).

Enzyme Analysis

Enzymatic activity was performed as described by Moy and Nkongolo (2022; 2023) using purified enzymes. Nine different enzymes were selected for analysis based on their ability to catalyze reactions, as well as their functions involving geochemical processes (Table 1). They include β-Glucosidase (BG), Cellobiohydrolase (CBH), β-N-Acetylglucosaminidase (NAGase), Aryl Sulfatase (AS), Acid Phosphatase (AP), Alkaline Phosphatase (ALP), Glycine Aminopeptidase (GAP), Leucine Aminopeptidase (LAP) and Peroxidase (PER). For one replicate, 4 g of soil was allocated to measure the dry weight. 4 g of soil was mixed with 40 mL of 50 mM sodium acetate buffer. The mixture was vortexed for 1 min. A total of 500 μL of each enzyme substrate was added to the 1.5 mL tubes and 500 μL of sodium acetate buffer was added to an additional 1.5 mL tube for control 1. Then, 900 μL of buffer was added to a 2 mL tube for control 2. A total of 500 μL of the soil mixture was added to each 1.5 mL tube containing the enzyme and control 1. Then, 900 μL of the soil mixture was added to control 2. This process was repeated two more times for the other two replicates. Ten (10 uL) of 0.3% H_2O_2 was added to the 2 mL tube containing the buffer and the soil mixture. The 1.5 mL tubes containing POD, PPO, and control 1 were stored on a rotating wheel at 4°C for 2 h. The other 1.5 mL tubes containing the enzymes and the 2 mL tube containing the buffer were incubated in a rotary shaker at 25°C for 2 h. All of the tubes underwent centrifugation at 3400 rpm for 3 min and 30 sec. A total of 100 μL of supernatant from each tube was added to a corresponding triplicate on a 96-well plate. Then, 5 μL of 1 m NaOH was added to all of the wells except those containing PPO, POD, control 1, and 3 separate replicates of control 2. A 96-well microplate was read at an absorbency of 405 nm using the FLUOstar OPTIMA FL (BMG LABTECH) for enzymes that used PNP and p-nitroanilide substrates. Another 96-well microplate was read at 450 nm for enzymes that used peroxidase as a substrate. Absorbencies for substrate and sample controls were performed to correct the absorbencies. For the cold temperature storage, this entire process was done twice in correspondence to limed and untreated sites. For temperature sensitivity, this process was done once for the limed site”.

Table 1: Enzymes and their respective substrates and functions in soil ecosystems

Enzyme	Substrate	Functions
β-glucosidase (BG)	*pNP β-D-glucopyranoside	-Cellulose degradation -Carbon cycling
Cellobiohydrolase (CBH)	*pNP-β-D-cellobioside	-Cellulose and some beta 1,4 glutans degradation -Carbon cycling
β-N-Acetylglucosaminidase Aka Chitinase (NAG)	*pNP-N-acetyl-β-D-glucosaminide	-Degradation of chitin -Carbon/nitrogen cycling
Acid Phosphatase (AP)	*pNP phosphate (buffer pH 5.0)	-Phosphate production -Phosphorus cycling
Alkaline Phosphatase (ALP)	*pNP phosphate (buffer pH 9.0)	-Produces ester-bound phosphate
Arylsulfatase (AS)	*pNP sulfate	-Sulfate production -Sulfur cycling
Glycine Aminopeptidase (GAP)	Glycine-p-nitroanilide	-Degrade amino acids into peptide -Nitrogen cycling
Leucine Aminopeptidase (LAP)	L-Leucine-p-nitroanilide	-Degrade leucine and other hydrophobic amino acids -Nitrogen cycling
Peroxidase (PER)	L-3,4-dihydroxyphenylalanine (DOPA)	-Polyphenols degradation -Carbon cycling

*pNP represents 4-nitrophenyl

Statistical Analysis

The Shapiro-Wilk test ($p<0.05$) was conducted using the SPSS program to test the normality of the data. ANOVA tests followed by Tamhane's T2 post hoc tests were performed to compare differences among enzymatic activities for the different treatments including storage temperatures (-20 and -80°C) and storage time (2 and 4 weeks).

Results

Soil pH, Organic Matter, and Bioavailable Metals

The mean pH of fresh soil samples was 6.2. Soil moisture and organic matter content were 10.9 and 10.6%, respectively. The bioavailable amounts of copper, nickel, and zinc were low (<2 mg/kg on average).

Enzymatic Activities

Significant differences were found between various storage temperatures and durations for all enzymes analyzed except for peroxidase. As seen in Fig. 1, peroxidase did not display any significant changes in activity levels when stored at -20 or -80°C compared to the fresh samples, or between the samples stored for 2 weeks and 4 weeks.

With the exception of GAP and LAP, no significant differences were observed between samples stored at -20°C for 2 weeks compared to controls (Figs. 2-3). Soil samples stored at -80°C showed a significant decrease in microbial activity compared to the fresh samples for all the enzymes tested except PER.

Overall, variations in enzymatic activities were observed during storage under different conditions. Specifically, a significant decrease of GAP activities was observed also after 2 weeks of storage at -80°C while LAP activities decreased in samples stored at -80°C only after 4 weeks. BG activities decreased in storage at -20 and -80°C only after 4 weeks of storage. CBH activities also decreased in samples stored at -20°C after 4 weeks. This decrease was observed in samples stored at -80°C after 2 and 4 weeks. Likewise, NAG, AP, and ALP activities decreased after storage at -20°C after 4 weeks and at -80°C after 2 and 4 weeks (Figs. 4-8). This is documented in the variations in response to storage at different temperatures. In fact, AS activities decreased after 4 weeks of storage at -20°C and only after 2 weeks at -80°C (Fig. 9).

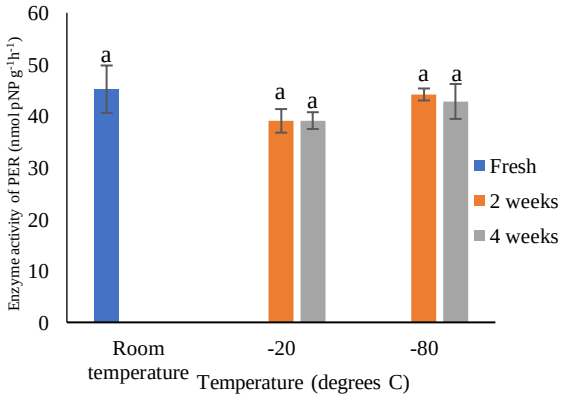

Fig. 1: Activities of PER at different sampling dates and storage conditions. Means with the same letters are not significantly different ($p\geq0.05$). Room temperature represents fresh samples

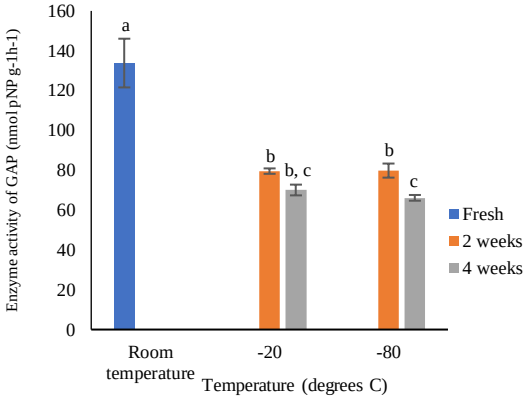

Fig. 2: Activities of GAP at different storage periods (2 and 4 weeks) and temperatures (-20 and -80 °C). Means with different letters are significantly different (p≤0.05). Room temperature represents fresh samples

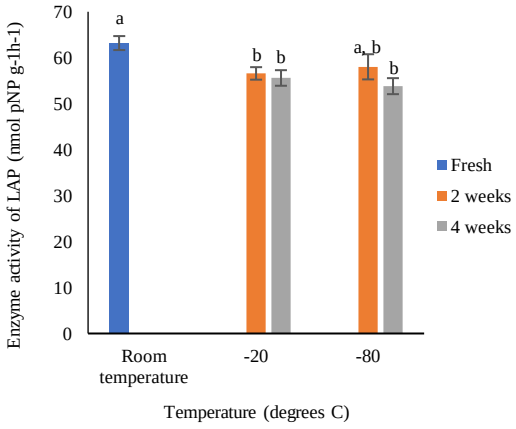

Fig. 3: Activities of LAP at different storage periods (2 and 4 weeks) and temperatures (-20 and -80°C). Means with different letters are significantly different (p≤0.05). Room temperature represents fresh samples

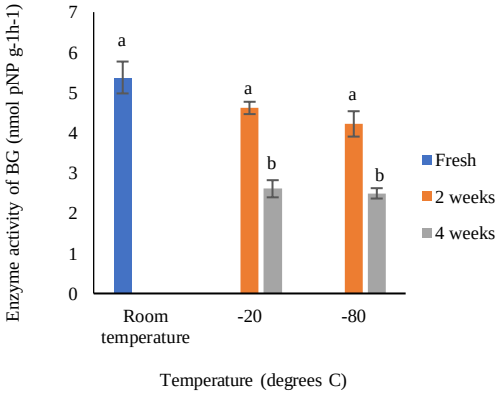

Fig. 4: Activities of BG at different storage periods (2 and 4 weeks) and temperatures (-20 and -80°C). Means with different letters are significantly different (p≤0.05). Room temperature represents fresh samples

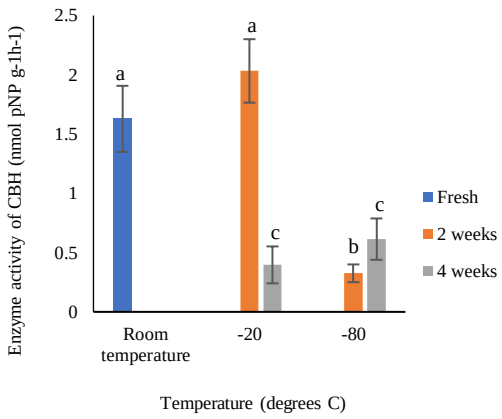

Fig. 5: Activities of CBH at different storage periods (2 and 4 weeks) and temperatures (-20 and -80°C). Means with different letters are significantly different (p≤0.05). Room temperature represents fresh samples

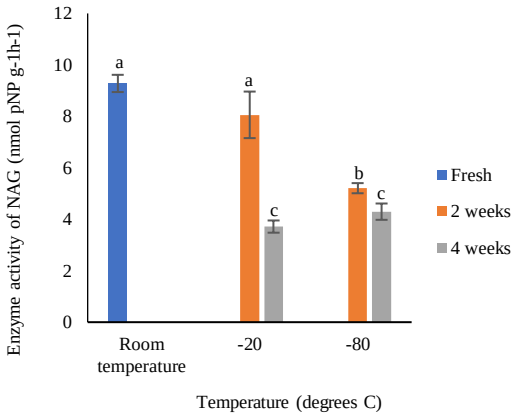

Fig. 6: Activities of NAG at different storage periods (2 and 4 weeks) and temperatures (-20 and -80°C). Means with different letters are significantly different (p≤0.05). Room temperature represents fresh samples

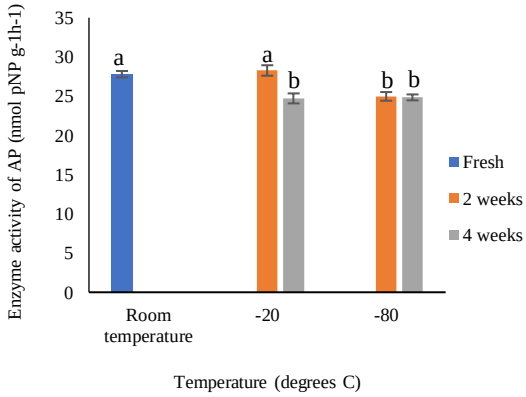

Fig. 7: Activities of AP at different storage periods (2 and 4 weeks) and temperatures (-20 and -80°C). Means with different letters are significantly different (p≤0.05). Room temperature represents fresh samples

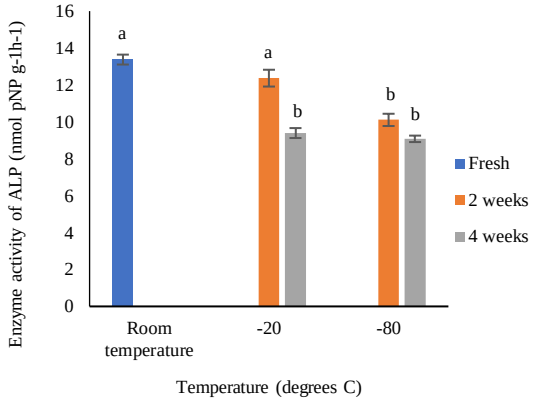

Fig. 8: Activities of ALP at different storage periods (2 and 4 weeks) and temperatures (-20 and -80°C). Means with different letters are significantly different (p≤0.05). Room temperature represents fresh samples

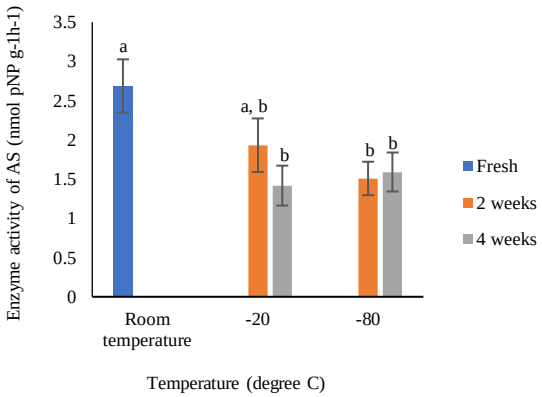

Fig. 9: Activities of AS at different storage periods (2 and 4 weeks) and temperatures (-20 and -80°C). Means with different letters are significantly different (p≤0.05). Room temperature represents fresh samples

Discussion

A previous study on the samples from the same areas stored at 4°C revealed that activities of BG, NAGase, AP, ALP, and AS decreased significantly after two weeks of storage at 4°C and then remained unchanged (Moy and Nkongolo, 2023). The responses of these enzymes after storage at -20 and -80°C showed different patterns during storage.

Effects of Storage at -80°C

Storage at -80°C resulted in significant decreases of all enzymes with the exception of PER. A study by Poulsen *et al.* (2021) also found that freezing at extreme temperatures did alter enzyme activity when compared to fresh samples but frozen samples showed consistent activity over time. Another study by Lane *et al.* (2022) found that enzymes can maintain consistent activity levels when frozen at -80°C. They conclude that AP and BG are best stored at this temperature. The differences between the fresh samples and the samples stored at -80°C could be ascribed to cold denaturation (Georlette *et al*., 2004). Some enzymes become inactive at a certain temperature but can be restored when returned to room temperature (Privalov, 1990). Cold denaturation occurs through the hydration of polar and non-polar groups of proteins (Georlette *et al.* 2004) and causes the protein structures to become unstable (Privalov, 1990). This would result in the lower activity rates displayed in the samples stored at -80°C. It is also possible that the frozen samples needed more time to adjust to room temperature to allow their activity levels to return to their state prior to freezing (Privalov, 1990).

Freeze-thaw cycles can influence enzyme activity both in the field environment and in frozen samples (Poulsen *et al.* 2021). When temperatures drop below 0°C, ice crystals can form within and around the enzymes causing them to denature and slowing their activity (Miura *et al*., 2019). However, Miura *et al.* (2019) found that most enzymes in their study were able to recover after 3 days of thawing. Their study showed that samples frozen at -5°C recovered fully and samples stored at -20°C saw a 10% reduction in activity. It appears that as the temperature at which the samples were stored decreases, the difference between the fresh and the frozen samples increases, which could explain why the samples stored at -80°C in this study with the exception of PER, significantly varied from the fresh samples.

Effects of Storage at -20°C

Most enzyme activities remained unchanged during storage at -20°C for 2 weeks compared to controls. Chua *et al.* (2017) found that some enzymes are stable with consistent activity at -20°C for up to 30 days including one of the enzymes ALP used in the present study. However, enzyme activity is still temperature and storage-dependent and varies for different enzymes (Wallenius *et al*., 2010; Chua *et al*., 2017). This could explain why GAP and LAP did not follow the same trend as the other enzymes. Miura *et al.* (2019) found that soil samples stored at -20°C and then thawed showed a 10% reduction in enzyme activity after 24 h of storage. The slight discrepancy between Miura *et al.* (2019) results and the data reported in this study might be ascribed to physio-chemical differences in soil samples.

Ideal storage temperatures depend on the natural conditions of particular soil (Lane *et al*., 2022). Because Sudbury experiences cold winters with temperatures reaching below -20°C, the enzymes would be adapted to withstand that temperature. However, it should be noted that the majority of the enzymes that showed no significant difference between fresh samples and the samples stored at -20°C for 2 weeks did show significant differences after 4 weeks at -20°C with the exception of BG. Soil enzymes can display strong seasonality (Zhang *et al.* 2020a).

Because the samples were collected during the summer, the enzymes would not have had time to adjust to the cold temperature and therefore will be significantly affected during storage for 4 weeks in the freezer (Makarov *et al*., 2017; Broadbent *et al*., 2021). The longer soil samples are stored, the more microorganisms will die and the living cells will release organic osmolytes as they struggle to stay alive under the cold temperature stressor (Makarov *et al*., 2017). Organic osmolytes are small solutes used by organisms that are water-stressed in order to maintain cell volume and function (Yancey, 2005). More studies still need to be performed to determine the impact of additional organic osmolytes being released but it is clear that organisms do this when under stress and it is physiologically expensive (Kakumanu *et al*., 2019).

Effects of Storage Time and Temperature on PER

The peroxidase enzyme did not follow the expected pattern of maintaining activity levels constant when stored at -80°C compared to -20°C. No changes in PER activities were observed after storage at -20 and -80°C compared to the fresh samples. Darwesh *et al.* (2019) reported that immobilized peroxidase is much more stable than the free enzyme and it is able to be stored for up to 90 days at 4 and 25°C. It is possible that the soil samples contained immobilized peroxidase and that is why there were no significant differences among the test groups. Another study by Wallenius *et al.* (2010) found that sensitivity to storage time and temperature was enzyme-dependent. PER is used by some plants to acclimatize to the cold and may be involved in the prevention of oxidative damage (Shahidul Haque *et al*., 2014). Because PER is used by plants subjected to lower temperatures, the enzyme would need to remain active at lower temperatures (Shahidul Haque *et al*. 2014). This might explain the consistent results in this experiment.

Effects of Storage Time and Temperature on GAP and LAP

Peptidase enzymes, GAP, and LAP showed significant differences between the fresh samples and all the frozen samples. A study in Mongolia found that LAP is adapted to cold (Otgonsuren *et al*., 2020) which could explain why it maintained consistent results across all four frozen sample conditions. Another study found that LAP showed almost no increase in relative temperature sensitivity when subjected to lower temperatures (Koch *et al*., 2007). Few studies have been conducted on the effect of cold temperatures on GAP. This study shows that GAP and LAP responses to cold temperatures were similar. Wallenstein *et al.* (2009) suggested that soil microorganisms continue to produce peptidase when the soil is frozen if that soil experiences cold temperatures naturally at some point during the year. Further research on the effects of cold on peptidase enzymes (GAP and LAP) needs to be conducted to determine why they appear to be cold-resistant.

Effects of Storage Time at -20°C on BG

BG was the only enzyme other than PER that maintained consistent results between the fresh sample and the samples stored at -20°C over a two and 4-week period, respectively. Studies suggest that soil microorganisms still produce BG when the soil is frozen if that soil is accustomed to colder temperatures (Wallenstein *et al*., 2009). Because the Sudbury climate has cold winters, the soil is accustomed to cold temperatures. This could explain why the activity of BG remained consistent at -20°C. When temperatures drop below freezing, ice crystals can denature enzymes and slow their activity, but BG does not appear to be greatly affected by this phenomenon (Miura *et al*., 2019). Although BG was able to maintain consistent results for the full 4 weeks in this investigation, other studies recommended storing samples at -80°C when testing for BG activity if samples have to be stored for over 4 weeks (Lane *et al*. 2022). This was reflected in the results of this study as the samples stored at -80°C showed consistent results between 2 and 4 weeks of storage for BG activities.

Significance of Results in Relation to Climate Change and Real-World Applications

Although the conditions of this experiment did not match the conditions soils are subjected to in the natural environment, the results still provide valuable insight into how climate variability and change can affect soil ecosystems. Soil enzymes have the capability to illustrate the effects of climate change on terrestrial ecosystems (Zuccarini *et al*., 2020). Knowing which enzymes are able to withstand the cold is important for understanding protein folding mechanisms (Georlette *et al*. 2004). Although climate change is often associated with global warming, it is also increasing the degree of cold in environments (Abbass *et al*., 2022). Some areas are experiencing higher levels of precipitation, including snowfall (Abbass *et al*., 2022) while others are exposed to lower levels of precipitation (Jansson and Hofmockel, 2020). Without adequate snow cover, soil is left without insulation and experiences harsher winters as a result (Broadbent *et al*., 2021). The soil enzymes display strong seasonality (Zhang *et al*., 2020a), and being subjected to colder weather sooner and more frequently as a result of less snow, changes their activity patterns (Broadbent *et al*., 2021).

Knowing which enzymes are resistant to cold conditions will be critical for helping the agricultural sector determine when to plant crops (Celestina *et al*., 2019). It will also provide useful information on microbial diversity (Jansson and Hofmockel, 2020). With a growing global population, it is important to know how to best manage our agricultural land to provide the best food security possible.

This study showed that PER is resilient to cold freezing temperatures for at least 4 weeks. However, it is unclear if the PER in this study was present in an immobilized form, as immobilized PER is significantly more stable under temperature changes compared to mobile PER (Darwesh *et al*., 2019). PER plays, a major role in the degradation of phenolic compounds and as a result plays a role in bioremediation (Darwesh *et al*., 2019). Phenolic compounds are noxious pollutants and many are produced by industrial activities (Satapathy *et al*., 2021). There are currently no efficient or economical ways to treat wastewater containing phenolic compounds. However, because of their harmful properties, it is recommended to remove them from the environment (Satapathy *et al*., 2021). Some studies showed that PER activities increase as temperatures drop (Shahidul Haque *et al*., 2014). This information could be used to better treat areas polluted with phenolic compounds.

Furthermore, PER may be involved in the prevention of oxidative damage in plants during cold weather (Shahidul Haque *et al*., 2014). Extreme conditions like cold temperatures can alter the metabolism of reactive oxygen and nitrogen species and when the concentration of these species is too high, they exceed the capacity of antioxidant defense enzymes, which can result in cell death (Chaki *et al*., 2020). Because PER is acclimatized to the cold, it can help defend the plants (Shahidul Haque *et al*., 2014) and possibly increase crop productivity (Chaki *et al*., 2020).

As previously mentioned, soil and the enzymes within it play a major role in biogeochemical cycles, namely the carbon and nitrogen cycles (Jansson and Hofmockel, 2020; Zhang *et al.* 2020a). With soils experiencing new patterns in freeze-thaw cycles, it is unclear if the nutrient cycles will cause soil to become a better carbon sink or a large source of greenhouse gas emissions (Jansson and Hofmockel, 2020). The carbon pool within soils is more stable when it is under warmer conditions compared to freezing conditions (Tang *et al*., 2022). This could be because, under warm conditions, enzymes are all functional whereas this study and many other reports have shown that under freezing conditions, only certain enzymes are functioning at full capacity. Knowing which enzymes are functional and what their role in the nutrient cycle is will be critical in predicting how much greenhouse gas emissions exit the soil (Tang *et al*., 2022).

The main limitation of this study is that it characterizes soil samples from one ecological area. It is possible that soil physico-chemistry (pH, organic matter content, Cation exchange capacity ...) can influence the variations of soil enzymes during storage. Hence, analysis of soils from different sites is warranted to validate these results.

Conclusion

The objective of the present study was to determine if soil storage at -20 and -80°C impacts soil enzymatic activities. This study shows with the exception of PER, that storage at -20 and -80°C decrease significantly soil enzymatic activities after four weeks. Storage at -20°C does not affect most of the enzymes tested after two weeks. Detrimental effects of storage at -80°C were observed after 2 weeks while enzymatic activities for most enzymes remained unchanged two weeks after storage at -20°C. Further studies are warranted to determine if these storage conditions affect soil microbial diversity and abundance.

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Author's Contributions

Ainsley Lougheed: Designed the work and wrote the manuscript. Collected the data.

Kabwe Nkongolo: Designed the work and wrote the manuscript. Supervised the work and obtained the funding.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

Competing Interests

The authors declare that they have no competing interests.

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