Scientific Papers. Series A. Agronomy, Vol. LXIV, No. 1, 2021
ISSN 2285-5785; ISSN CD-ROM 2285-5793; ISSN Online 2285-5807; ISSN-L 2285-5785

MICROBIAL AND ENZYME DECOMPOSITION OF PROTEINS TO RECLAIMED SUBSTRATES - VEGETATION EXPERIMENT WITH *Lolium perenne*

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Abstract

Vegetation experiment with Lolium perenne L. was carried out on reclaimed substrates from humus depot, tailings pond and mine, as applied vermiculite, mineral fertilization and liming. Microbial biomass nitrogen shows the highest values after the addition of 10% vermiculite, simultaneous application of fertilization and liming and self-fertilization, compared to controls, without ameliorants. Activities of the studied enzymes urease and protease follow the increase of the concentration of vermiculite, they are also higher in the samples with self-fertilization and the combined application of fertilization and liming. The values of microbial biomass nitrogen and enzymatic activities are highest in the variants with substrates from the mine. Microbial nitrogen biomass strongly correlates with urease activity and moderately with protease activity.

Key words*: reclamation, vermiculite, microbial biomass nitrogen, urease, protease.*

INTRODUCTION

Changes in soil microbiological properties precede chemical and physical changes, which take place more slowly over time. Microbial biomass and enzymatic activities can serve as sensitive early indicators of the dynamics of organic matter transformation, nutrient cycles, pollution stress and soil regeneration processes (Burns et al., 2013; Perez-de-Mora et al., 2012; Kennedy et al., 1993; Islam et al., 2000; Avidano et al., 2005). The creation of a sustainable ecosystem through biological reclamation of mine embankments and their inclusion in the surrounding landscape is essential (Petrov, 2019). Conducting liming, mineral and organic fertilization, addition of vermiculite and other activities in growth substrates for biological reclamation affect the quantity and composition of microorganisms in them, and respectively the values of microbial biomass and enzymatic activities of microbial origin. In previous studies of the same (Malcheva, 2021) and similar (Stefanova and Petrov, 2019) variants was established that non-spore-forming bacteria occupy a major share in the total microflora of variants of humus depot, tailings and mine , increasing microbial biomass carbon and the activity of enzymes catalase, cellulase, amylase and invertase (Malcheva, 2020).

In recent years, vermiculite has been widely used for the adsorption of metal ions, including heavy metals (Covelo et al., 2007; Abollino et al., 2008; Panuccio et al., 2009; Malandrino et al., 2006, 2011; Sis and Uysal, 2014; Kebabi et al., 2017; De Freitas et al., 2017). In addition, vermiculite significantly improves the drainage properties of substrates, increases porosity and crumbliness, prevents soil compaction and protects roots from sudden temperature fluctuations (Zheleva et al., 2019; Malcheva, 2020). Narendrula-Kotha and Nkongolo (2017) report that total microbial biomass has increased significantly 35 years after liming of regenerated mining deposits, but species microbial diversity remains statistically unchanged. Fuentes et al. (2006) also found that microbiological activity and nitrification increase in soils after liming due to the correction of soil acidity.

The addition alone or in combination of post-flotation lime, mineral fertilisation, sewage sludge, and mineral wool to the reclaimed soil-less substrate stimulates the activity of the enzymes catalase, urease and protease (Joniec, 2018). The increase in urea-N losses, possibly as a consequence of a higher urease activity,

was compensated for by the increase in N immobilized in the biomass (Roscoe et al., 2000). The last cited authors found that the microbial biomass nitrogen content explained 97% and 69% of the variation in urease activity in the upper and deeper soil layer, respectively.
Purpose of the present study was to determine the effect of the application of vermiculite, mineral fertilization and liming on the accumulation of microbial biomass nitrogen and the degree of enzymatic degradation of proteins in reclaimed substrates with *Lolium perenne* L.

MATERIALS AND METHODS

The vegetation experiment began in March 2019 in greenhouse conditions. In plastic containers with a capacity of 1.5 l was poured 1 kg of substrate with the appropriate amount of vermiculite (5%, 10% and 20%). Mineral fertilization and liming are applied. Liming was used to neutralize the substrates. The experiment lasted 6.5 months. The following substrates were used: soil from the humus depot near tailings, tail from tailings (tail) and substrate from a copper mine (mine). Ryegrass seeds are sown with a higher density, and after the germination, the plants, are thinned to a density of 50 plants in a container. On the 58th day after the beginning of the vegetation experiment, liming was done in the mine substrates, and seven days later (64 days from the beginning of the vegetation experiment) fertilization was carried out (rates: N150P150K150 kg/ha) of all variants, without controls. The substrates were maintained at constant humidity. Two replicates were made for each variant of the study: Microbiological and enzymatic analyzes were performed on the 30th and 95th day of the experiment, before and after liming and fertilization. Microbiological and enzymatic analyzes were performed on the 30th and 95th day of the experiment, before and after liming and fertilization. The general scheme of the vegetation experience is presented in Table 1.
Microbial biomass nitrogen (MBN), urease and protease activity were analyzed using the following methods: Biomass N was calculated (Brookes et al., 1985) using the equation: biomass N = 1.85 EN, where EN = (total N from fumigated soil) - (total N from unfumigated soil). Total N in the extracts was measured by Kjeldahl digestion (Brookes et al., 1985); Spectrophotometric method was used for determination of urease activity of substrates (Kandeler and Gerber, 1988); protease activity of substrates was determined by the method Khaziev (1976): in the substrates are placed photoplates with gelatin coating with dimensions of 10/50 mm and every 15 days the percentage of the degraded area is reported with a reference net.

Table 1. Variants of the vegetation experience

Embankments from mine (M):	**Soil from humus depot (SD):**	**Tail from tailing (T):**
M-0 - Without vermiculite and without fertilizing; M-I - Without liming (L), without fertilizing (F) (5%, 10%, 20%); M-II - Without liming, with fertilizing (5%, 10%, 20%); M-III - With liming, without fertilizing (5%, 10%, 20%); M-IV - With liming, with fertilizing.	SD-0 - Without vermiculite and without fertilizing; SD-0F - Without vermiculite and with fertilizing; SD-I - Without fertilizing (5%, 10%, 20%); SD-II - With fertilizing (5%, 10%, 20%).	T-I - Without fertilizing (5%, 10%, 20%); T-II - With fertilizing (5%, 10%, 20%).

Correlation analysis was applied to determine the relationship between microbial biomass nitrogen and enzymatic activities using the software product MS Excel 2010.

RESULTS AND DISCUSSIONS

On 30th day of the study, microbial nitrogen biomass had higher values in the samples with mining substrates, followed by samples with tail and those from the soil depot, which is located near tailings (Figure 1).
The addition of vermiculite increases the values of biomass nitrogen of microbial origin. The best results are establish when adding 10% vermiculite. Similar results were obtained on day 95 of the study (Figure 2).

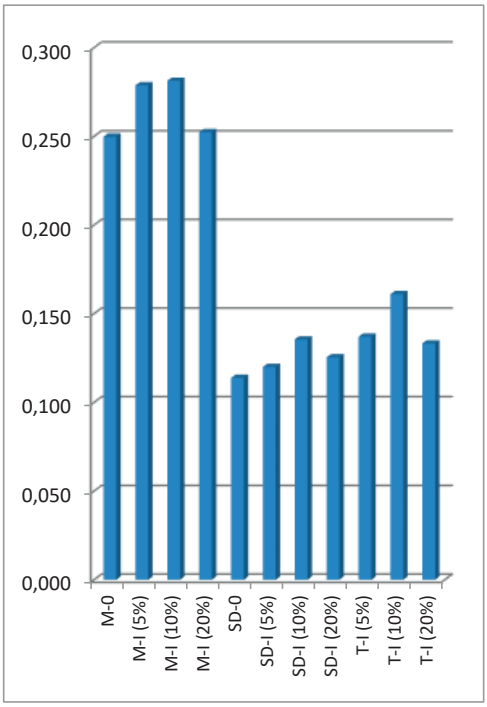

Figure 1. Microbial biomass nitrogen, 30th day (mg N/g soil)

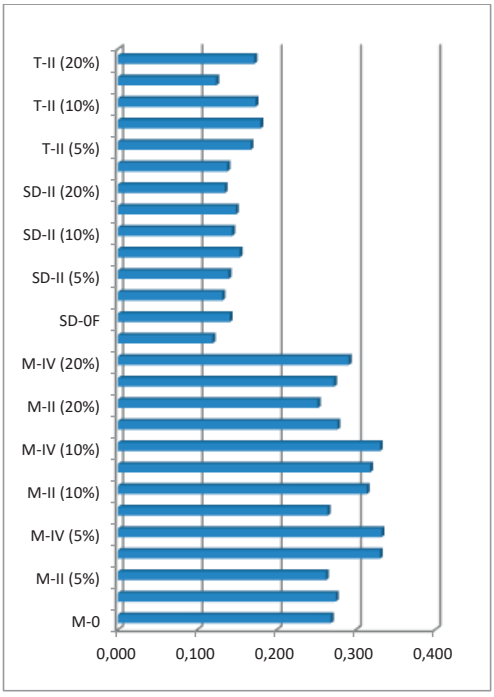

Figure 2. Microbial biomass nitrogen, 95th day (mg N/g soil)

Combined application of liming and fertilization in the mine variants as well as the fertilization in the "SD" and "T" variants increase biomass nitrogen of microbial origin to a higher degree, with the exception of: SD-II (10%), SD-II (20%) and T-II (10%). Variants "with liming, without fertilizing" (M-III) give better results than the "without liming, with fertilizing" (M-II), regardless of the vermiculite concentration.

Microbial nitrogen biomass and enzymatic activities depend on the development of microorganisms in the substrates and their activity. In a previous study on the microbiological activity of the same variants (Malcheva, 2021) it was found that the biogenicity of the tested substrates increases with the application of vermiculite, liming and fertilization. The main share in the composition of the total microflora for the two periods of research is occupied by the non-spore-forming bacteria, followed by the bacilli. Actinomycetes and micromycetes are the least represented. On the 30th day of testing, the total amount of microorganisms in the mine variants was lower than in the control, in contrast to the soil depot and tailings variants. With increasing time of exposure to vermiculite (95th day of the experiment) and after individual and combined application of liming and fertilization, the biogenicity was higher in the mine variants compared to those in the soil depot and those with tailings. This trend shows the importance of vermiculite for the adsorption of heavy metals, especially depending on the duration of action, as well as for improving the physico-chemical properties of substrates. The pH values increase after liming in the mine variants, which, together with the application of fertilization, also contributes to increasing the development of the microflora in the substrates and their activity. Increase in the values of microbial biomass and microbiological activity after liming of regenerated mining deposits and soils was established by other authors (Fuentes et al., 2006; Narendrula-Kotha and Nkongolo, 2017). The introduction of mineral fertilizers increases the amount and activity of microorganisms in the soil (Hart and Stark, 1997; Forge and Simard, 2001; Meena et al., 2014; Bogdanov et al., 2015; Plamenov et al., 2016). Other authors find out that this increase is in

short term, and is often followed by reduction of the microbial biomass and activity (Ohtonen, 1992; Smolander et al., 1994; Périé and Munson, 2000). Vegetation type and litter quality seem to be more important for soil microbial activity than the substrate quality on the reclaimed sites (Stefanova and Petrov, 2019).

In general, the total amount of microorganisms is higher with the addition of 10% and 20% vermiculite. The highest values of trace elements, especially Cu and As, were found in the mine variants (Zheleva et al., 2019). This determines the lower total amount of microorganisms on the 30th day of the study (before liming and fertilization) in these variants, but not their lower activity (Malcheva, 2021). In general, the increase in biogenicity, MBN values and enzymatic activities in the addition of vermiculite in all variants proves the effect of the mineral for purification of heavy metal substrates.

In general, on the 30th day from the start of the experiment (before liming and fertilization) a decrease in urease values was established with an increase in the amount of vermiculite, except for M-20% (Figure 3).

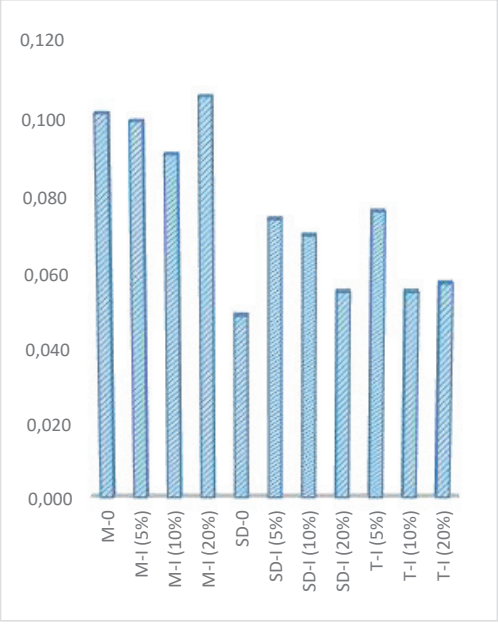

Figure 3. Urease activity of soil microorganisms, 30th day, mg N/g soil

The activity of the enzyme remains lower than that of the control. Probably initially the increasing amount of vermiculite stresses the microorganisms and suppresses their enzymatic activity. This conclusion is confirmed by the results of the 95th day (after liming, fertilizing and mowing) - increasing the activity of the enzyme with increasing the amount of vermiculite (Figure 4). A similar trend was found in the study of the enzymes cellulase, amylase and invertase, while the catalase activity increased with increasing amount of vermiculite for both study periods (Malcheva, 2020).

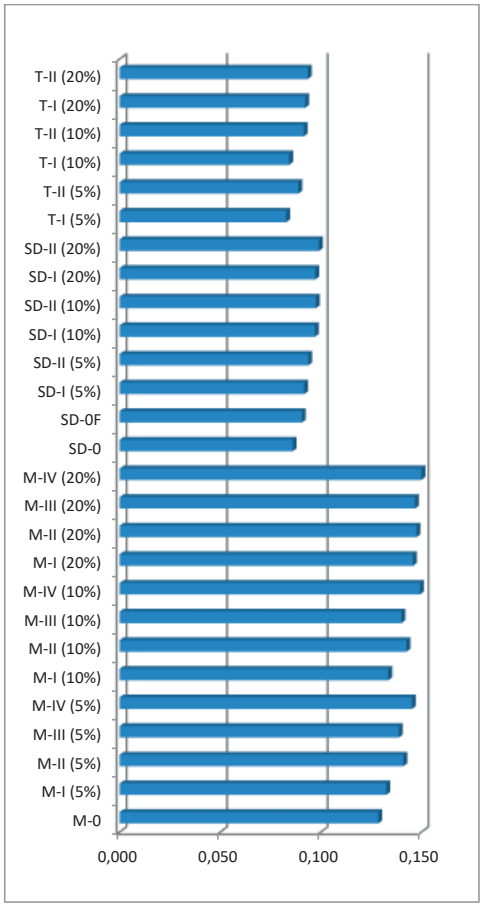

Figure 4. Urease activity of soil microorganisms, 95th day, mg N/g soil

Urease activity is highest in the mine variants, followed by the SD and T variants. The combined application of fertilization and liming at the mine increases of the enzyme activity to a higher degree. The "without liming, with fertilizing" (M-II) variants showed better results than with "liming, without fertilizing" (M-III), regardless of the

vermiculite concentration. In the other variants - "SD" and "T" fertilization increases the urease activity, as the enzyme values increase with increasing concentration of vermiculite. The lowest and closer to those of the control samples are the values of urease in the substrates without liming and fertilization.

The protease activity of the studied variants follows the trends in urease - a higher percentage of degraded area on the 95th day of the study compared to the 30th day, as well as higher values of the enzyme with increasing vermiculite concentration, mainly after liming and fertilizing (Figures 5 and 6).

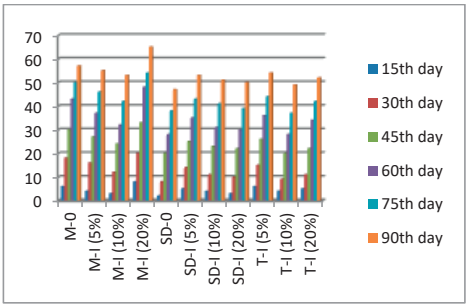

Figure 5. Protease activity of soil microorganisms, 30th day (% degraded area)

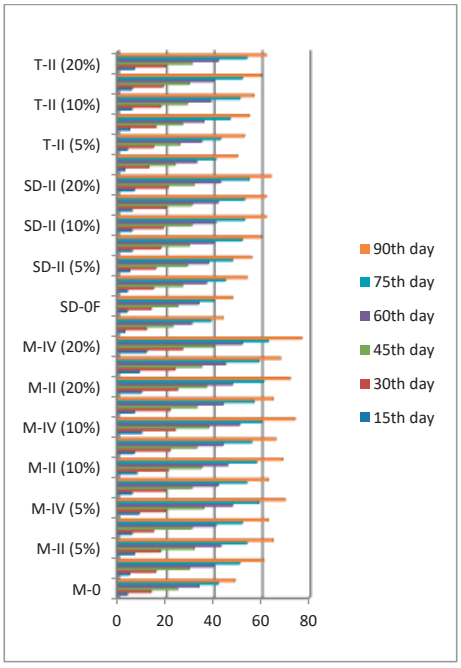

Figure 6. Protease activity of soil microorganisms, 95th day (% degraded area)

Protease values are highest with the addition of 20% vermiculite. The order of magnitude of the protease activity is: M>SD>T. The "without liming, with fertilizing" (M-II) variants showed better results than with "liming, without fertilizing" (M-III), regardless of the vermiculite concentration.

Some researchers have studied the influence of fertilization on the fertility of soil (Jia et al., 2001; Liu, 2004; Malcheva et al., 2016) and growth substrates for reclamation (Malcheva, 2020) by exploring soil enzymatic activity. The addition alone or in combination of post-flotation lime, mineral fertilisation, sewage sludge, and mineral wool to the reclaimed soil-less substrate stimulates the activity of the enzymes catalase, urease and protease (Joniec, 2018).

The correlation analysis is presented in the following Figures 7 and 8.

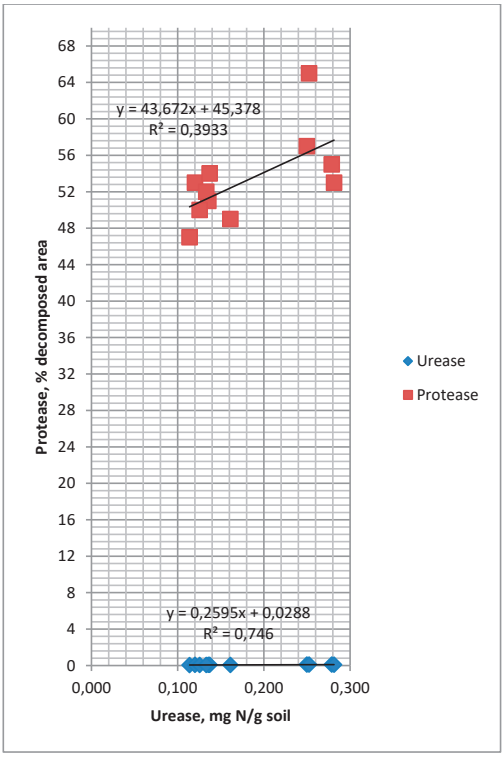

Figure 7. Dependence between MBN and enzyme activities, 30th day

Two times as strong a correlation was found between MBN and urease activity as compared to MBN and the protease activity of soil

microorganisms. These dependencies were higher on day 95 of the study.

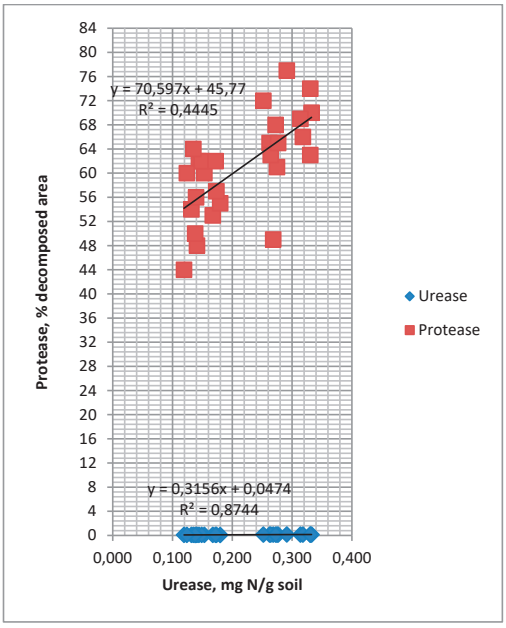

Figure 8. Dependence between MBN and enzyme activities, 95th day

CONCLUSIONS

Microbial biomass nitrogen values increased from the 30th to the 95th day of the study. For both study periods, MBN values were highest in the samples with mining substrates, followed by the samples with tail and those from the soil depot. Biomass nitrogen of microbial origin increases the most with the addition of 10% vermiculite. The best results for this indicator are obtained with the combined application of liming and fertilization in the variants of the mine, as well as fertilization in the variants from the soil depot and those with tail. Variants "with liming, without fertilizing" show better results than "without liming, with fertilizing", regardless of the vermiculite concentration.

Urease activity is highest in the variants from the mine, followed by the variants from the soil depot and those with tail. Before liming and fertilizing (30th day), a decrease in the enzyme activity with an increase in the amount of vermiculite was found. However, after liming, fertilizing and mowing (95th day), urease activity increases with increasing amount of vermiculite in all variants. The best results are obtained by adding 20% vermiculite. Combined application of fertilization and liming in the mine, as well as fertilization in the other variants, increases the activity of the enzyme to a higher degree. "Without liming, with fertilizing" options show better results than "with liming, without fertilizing", regardless of the concentration of vermiculite.

Protease activity increases to a higher degree also after liming and fertilization, as well as with increasing the concentration of vermiculite - the best results are obtained by adding 20% vermiculite. The values of the enzyme are the highest in the variants from the mine, followed by the variants from the soil depot and those with tail. Variants "Without liming, with fertilizing" show better results than "with liming, without fertilizing", regardless of the concentration of vermiculite.

In general, the applied activities - liming, mineral fertilization and addition of vermiculite increase the values of biomass nitrogen of microbial origin and the studied enzyme activities compared to control samples. A strong correlation is establish between microbial biomass nitrogen and urease activity. While between MBN and protease activity the dependence is moderate. These dependencies increase with the duration of the study.

ACKNOWLEDGEMENTS

The variants for this study were developed on the project №937/2019.

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