

Extracellular protease-producing actinomycetes and other bacteria in cultivated soil

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Abstract. The occurrence and properties of extracellular protease-producing actinomycetes and other bacteria in cultivated soil were studied. Experimental soils consisted of three mineral soil samples and one *Sphagnum* peat sample from a greenhouse. The mineral soil samples represented arable, pasture and uncultivated soils.

From experimental soils, 240 bacterial strains were isolated, 68 strains thereof were proteolytic. A greater number of proteolytic strains originated from pasture soil than from the other soils. Actinomycetes accounted for 70 % of the proteolytic strains isolated from pasture soil. Several proteolytic bacteria were isolated also from peat, but only few of them were typical actinomycetes. Many strains with high extracellular protease activity proved to be fermentative bacilli.

Production of oxidase enzymes, significant in the humification processes, occurred frequently among strains isolated from pasture soil and peat. The ability to produce dark melanoid pigments was a frequently noted characteristic of the proteolytic actinomycetes.

Index words: soil proteases, soil phenoloxidases, soil oxidases, humus polymerization

Introduction

Transformation of organic matter in soil is partially catalyzed by enzymes found outside the living soil organisms (SKUJINS 1967). Many soil microorganisms release extracellular enzymes into the soil, and these enzymes are involved in the decomposition of plant, animal and microbial residues. Proteases are enzymes

which hydrolyze residues containing peptide bonds. Energy and nutrients are formed in the degradation process of organic residues, and the degradation products are also involved in the formation of humic matter.

Soil is a very complicated environment and enzymes acting therein are supposed to tolerate numerous environmental factors. However, it is well known that part of the enzymes remain

in the soil in an active state for a certain period (SKUJINS 1967). The persistence of extracellular proteases in soil is enhanced by their adsorption to soil organic matter and clay particles. Ionically bound proteases might represent readily mobilizable reserves, while covalently bound proteases form a more stable but immobilized reserve (LADD 1972).

The composition and abundance of soil microbial flora producing extracellular proteases are affected by environmental factors such as moisture, temperature, aeration, acidity and type and content of organic matter. These factors are remarkably affected by cultivation. In arable soil, various measures related to cultivation bring about considerable fluctuations in environmental conditions. In view of soil microbes, regularly repeated ploughing is a radical action, while in pasture soil which is not tilled every year, undisturbed development of microbial flora may continue. When decomposable organic residues occur in abundance in soil, microbial flora proliferates in the presence of favourable environmental conditions. In this respect, excess acidity is a

usual limiting factor. Well humified soil organic matter is not primarily important as a source of energy, but it has favourable effects on the environment, e.g. increasing the water-holding capacity of the soil.

The aim of this study was to investigate the occurrence and properties of extracellular protease-producing actinomycetes and other bacteria in cultivated soil.

Material and methods

Experimental soils. For isolation of proteolytic soil organisms, four soil samples were collected. Three of the samples representing arable, pasture and uncultivated soil were from the surface layer of mineral soils and one sample was a *Sphagnum* peat sample from a greenhouse. The pH of the soil was measured in a soil — 0.01 M CaCl₂ suspension (1 : 2.5) (RYTI 1965). The organic carbon content of the samples was determined using a modification (GRAHAM 1948) of ALTEN's wet combustion method. The samples, by increasing pH, were as follows:

| Sample No. | Origin | Soil class | pH | Org.C, % |
|------------|-------------------|----------------------|-----|----------|
| 1 | Uncultivated soil | Muddy clay | 3.5 | 3.1 |
| 2 | Arable soil | Coarser finesand | 5.0 | 3.1 |
| 3 | Greenhouse peat | <i>Sphagnum</i> peat | 5.7 | 43.4 |
| 4 | Pasture soil | Coarser finesand | 6.0 | 4.9 |

Isolation of soil organisms. Samples of 5 g undried soil were dispersed in 100 ml of sterilized phosphate buffer solution (pH 7.2) using a sterile Ultra Turrax K homogenizer. Three successive hundredfold dilutions of soil suspensions were prepared with sterilized phosphate buffer solution, shaken by hand, and five replicates of 1 ml and 0.1 ml of the two latter dilutions were plated in the following medium: soluble starch, Bacto peptone and yeast extract 0.5 g each, glycerol 1 ml, K₂HPO₄ 0.2 g, MgSO₄·7H₂O 0.05 g, 0.01 % FeCl₃ water solution 4 drops, agar 15 g, soil extract 250 ml, distilled water 750 ml, actidione 20 mg, pH 6.9. The medium was distributed in 10 ml portions into test tubes and sterilized at 120°C for 20 minutes. Ten grams

of milk powder was dissolved in 100 ml distilled water and sterilized at 118°C for 15 min. and supplied into the medium (10 % v/v). The plates were incubated at room temperature for one week and counted after two and six days. The criterion of identification of caseolytic organisms was a clear zone surrounding the colony. For isolation of actinomycetes, five replicates of 1 ml of the three dilutions were plated in chitine agar (SKERMAN 1969). The plates were incubated at room temperature for one week and counted after incubation.

Testing of physiological characteristics. Proteolytic and chitinolytic colonies were transferred to test tubes containing 2.5 ml semisolid medium (7 g agar per 1000 ml)

which was prepared in the same way as the medium for isolation of soil organisms except that it contained no actidione. The cultures were incubated at room temperature until good growth was found. Thereafter the strains were transferred to liquid media: tryptone and yeast extract 0.5 g each, KH_2PO_4 0.4 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05 g, NaCl 0.1 g, FeCl_3 0.01 g, soil extract 250 ml, distilled water 750 ml, pH 6.8, sterilized at 120°C for 20 min. The cultures were incubated at 28°C until good growth was found and then the strains were transferred with a multipoint inoculator to the media used for testing physiological characteristics.

The ability to denitrify was indicated by gas bubbles in the test tubes containing the liquid medium described above supplied with 7 g agar and 1 g KNO_3 per 1000 ml and closed with vaspar. The utilization of glucose was tested with OF-medium (HUGH and LEIFSON 1953). The oxidase activity was indicated by red colour brought about by 0.5 % (w/v) water solution of dimethyl-p-phenyldiamine-hydrochloride (KLINGE 1960) in liquid medium cultures containing tryptone and yeast extract 5 g each and agar 7 g per 1000 ml. The production of melanine was noted in this medium. The proteolytic properties of the strains were tested with litmus milk: 20 ml 2 % solution of litmus, 1000 ml skimmed milk, sterilized at 114°C for 15 min. All tests were checked after one week of incubation at room temperature. After five days of incubation, growth was checked in three successive passages in synthetic medium with citrate as the sole carbon source (EKLUND 1970).

Determination of protease activity. Proteolytic strains were transferred to slants: tryptone 5 g, glycerol 1 ml, yeast extract 0.5 g, KH_2PO_4 0.2 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05 g, FeCl_3 0.01 % water solution 4 drops, agar 15 g, distilled water 1000 ml, pH 6.9, sterilized at 121°C for 15 min. The ability of strains to utilize casein was retested, and the strains showing good proteolysis were chosen for protease production. For cultivation of inoculum, strains were transferred to test tubes contain-

ing 2 ml of yeast extract-malt extract liquid medium (PRIDHAM et al. 1956—1957, ref. SHIRLING and GOTTLIEB 1966) and incubated for two days. Inocula were transferred to 250 ml conical flasks containing a medium presented by EKLUND et al. (1971) and shaken at 28°C (200 rpm). Samples for the determination of protease activity were taken after 3, 4 and 5 days of incubation.

Protease activity was determined according to EKLUND et al. (1971). The growth solutions were allowed to hydrolyze casein at 40°C and pH 8.0. The reaction time was 20 min for the samples incubated for three days and 10 min for the samples incubated for four or five days. The reaction was stopped by precipitating the unhydrolyzed casein with 5 % trichloroacetic acid. The content of tyrosine produced in casein hydrolysis was determined in the filtrates by measuring the absorbances of the assayed solutions and blanks at the wavelength 280 nm, using a Beckman DB spectrophotometer. One enzyme unit (EU) is the amount of enzyme which releases hydrolysis products at a rate equivalent to 1 meq of tyrosine per minute under the conditions specified (JÖNSSON 1969).

Results

The number of soil organisms grown on soil extract-milk agar plates are given in Table 1.

Table 1. Number of proteolytic and other soil organisms.

| Soil sample No. Origin | Organism | Number of organisms (g^{-1} of soil) $\times 10^6$ | |
|---------------------------|-------------|---|----------------------------------|
| | | After 2 days of incubation | After 6 days of incubation |
| 1 Uncultivated soil | Proteolytic | — | 0.48 |
| | Total | — | 0.56 |
| 2 Arable soil | Proteolytic | — | 0.16 |
| | Total | — | 0.88 |
| 3 Greenhouse peat | Proteolytic | 3.76 | 30.0 |
| | Total | 7.76 | 115.5 |
| 4 Pasture soil | Proteolytic | 1.44 | 48.0 |
| | Total | 9.24 | 143.6 |

After two days of incubation, growth was found only among the organisms isolated from greenhouse peat and pasture soil. Nearly 50 % of the colonies derived from peat and 16 % of those derived from pasture soil were proteolytic. After six days of incubation, the percentages of proteolytic colonies isolated from these soil samples were 26 and 33, respectively. After six days of incubation, growth was found also on plates representing other soil samples, but the numbers of colonies were much smaller. The percentages of proteolytic colonies isolated from uncultivated and arable soil samples were 86 and 18, respectively.

After seven days of incubation, growth was found on all chitine agar plates (Table 2). The

Table 2. Number of actinomycetes and other soil organisms counted on chitine agar plates.

| Soil sample No. Origin | Organism | Number of organisms (g ⁻¹ soil) × 10 ⁶ after 7 days of incubation |
|---------------------------|--------------------------|---|
| 1 Uncultivated soil | Typical actinomycetes | 0.035 |
| | Total | 0.055 |
| 2 Arable soil | Typical actinomycetes | 0.54 |
| 3 Peat | Typical actinomycetes | — |
| | Other* | 3.50 |
| 4 Pasture soil | Typical actinomycetes | 13 |

* mainly nocardioforms and mycobacteria

colonies grown on plates representing peat and pasture soil were more numerous than those representing other soil samples. All the colonies isolated from arable and pasture soil samples and 64 % of those from the uncultivated soil sample were actinomycetes, while typical actinomycete colonies were not found on plates representing peat. However, the bacteria grown on these plates included *Nocardia* and *Mycobacterium* strains.

The number of bacterial strains isolated from soil extract-milk agar plates was 124. The majority of the strains originated from peat and pasture soil samples (Table 3). Actinomycetes were isolated mainly from pasture soil. On the basis of physiological tests, proteolytic strains were isolated from all soils, but oxidase-producing and denitrifying strains only from peat and pasture soil (Table 3). The ability to produce melanine was associated exclusively with strains originating from pasture soil. The glucose utilization was mainly fermentative among strains originating from uncultivated and arable soil samples, but mainly oxidative among strains originating from peat. Strains originating from peat exhibited the poorest ability to grow in synthetic medium.

The number of strains isolated from chitine agar was 116. The proportion of actinomycetes was greatest among strains originating from pasture soil and lowest among those originating from peat (Table 4). Proteolytic and oxidase-producing strains originated more fre-

Table 3. Properties of all strains isolated from soil extract-milk agar plates.

| | Soil sample | | | |
|----------------------------------|-------------------|-----------------------|-----------|--------------|
| | 1 Uncultivated | 2 Arable | 3 Peat | 4 Pasture |
| Number of strains | 6 | 6 | 46 | 66 |
| | | % of isolated strains | | |
| Typical actinomycetes | — | — | — | 26 |
| Proteolytic | 33 | 67 | 15 | 23 |
| Oxidase-producing | — | — | 72 | 56 |
| Melanine-producing | — | — | — | 12 |
| Fermentative glucose utilization | 66 | 67 | 5 | 26 |
| Oxidative » » | 17 | 33 | 72 | 32 |
| Growth in synthetic medium | 67 | 100 | 40 | 58 |
| Denitrifying | — | — | 5 | 6 |

quently from peat and pasture soil than from the other soil samples. Melanine-producing strains were isolated from all soil samples. The majority of strains were able to grow without organic nitrogen, while the proportion of denitrifying strains was low. Oxidative utilization of glucose was more common than fermentative utilization.

The number of proteolytic strains was 68, 30 of which were isolated from soil extract-milk agar plates (Table 5) and 38 from chitine agar plates (Table 6). The majority of proteolytic strains originated from pasture soil, actinomycetes accounting for 70 %.

The protease activity of 30 strains showing good proteolysis was determined (Fig. 1). Of

Table 4. Properties of strains isolated from chitine agar.

| | Soil sample | | | |
|----------------------------------|-------------------|-----------------------|-----------|--------------|
| | 1 Uncultivated | 2 Arable | 3 Peat | 4 Pasture |
| Number of strains | 45 | 16 | 13 | 42 |
| | | % of isolated strains | | |
| Typical actinomycetes | 47 | 50 | 23 | 74 |
| Proteolytic | 13 | 19 | 54 | 50 |
| Oxidase-producing | 9 | 19 | 46 | 33 |
| Melanine-producing | 13 | 31 | 8 | 30 |
| Fermentative glucose utilization | 64 | 6 | 8 | 26 |
| Oxidative » » | 7 | 75 | 92 | 41 |
| Growth in synthetic medium | 76 | 63 | 85 | 67 |
| Denitrifying | 7 | 6 | 8 | — |

Table 5. Properties of proteolytic strains isolated from soil extract-milk agar plates.

| | Soil sample | | | |
|----------------------------------|-------------------|-----------------------|-----------|--------------|
| | 1 Uncultivated | 2 Arable | 3 Peat | 4 Pasture |
| Number of strains | 2 | 4 | 8 | 16 |
| | | % of isolated strains | | |
| Typical actinomycetes | — | — | — | 63 |
| Oxidase-producing | — | — | 75 | 25 |
| Melanine-producing | — | — | — | 31 |
| Fermentative glucose utilization | 100 | 75 | 25 | 31 |
| Oxidative » » | — | 25 | 63 | 50 |
| Growth in synthetic medium | 100 | 100 | 50 | 88 |
| Denitrifying | — | — | — | 6 |

Table 6. Properties of proteolytic strains isolated from chitine agar.

| | Soil sample | | | |
|----------------------------------|-------------------|-----------------------|-----------|--------------|
| | 1 Uncultivated | 2 Arable | 3 Peat | 4 Pasture |
| Number of strains | 7 | 3 | 7 | 21 |
| | | % of isolated strains | | |
| Typical actinomycetes | 43 | — | 14 | 76 |
| Oxidase-producing | — | 33 | 43 | 24 |
| Melanine-producing | 43 | — | — | 48 |
| Fermentative glucose utilization | 43 | — | 14 | 14 |
| Oxidative » » | 29 | 100 | 86 | 67 |
| Growth in synthetic medium | 57 | 100 | 86 | 81 |
| Denitrifying | 14 | 33 | 14 | — |

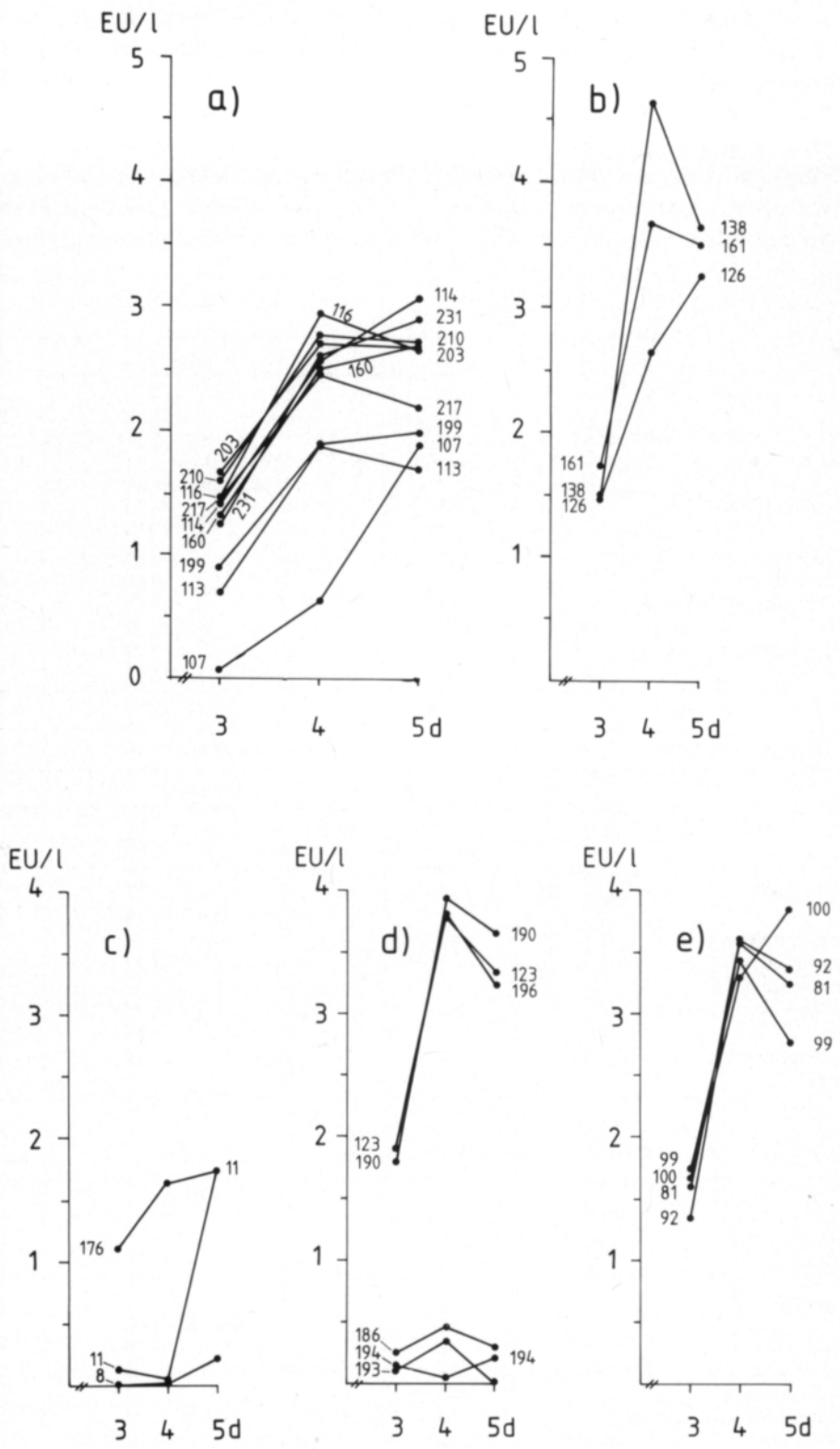


Fig. 1. Protease activity of actinomycetes (a) and other bacteria in uncultivated soil (b), arable soil (c), greenhouse peat (d) and pasture soil (e).

these strains, 11 were actinomycetes originating chiefly from pasture soil (Fig. 1a). Many actinomycetes strains showed moderate protease activity. The bacterial strains from uncultivated soil showing high protease activity were all *Bacillus cereus* strains (Fig. 1b). Protease activity of strains originating from arable soil was relatively low (Fig. 1c). Two of these strains were bacilli (strain 8 *Bacillus cereus* var. *mycoides*, strain 11 *Bacillus cereus*) (Fig. 1c). Strains from peat represented both low and high protease activity (Fig. 1d). Three strains showed high protease activity: strain 123 was *Bacillus subtilis*, 190 and 196, not being identified, produced melanine and their utilization of glucose was oxidative. The four bacterial strains from pasture soil showing high protease activity were *Bacillus coagulans* strains, except strain 92 (Fig. 1e). Figure 1 shows that protease activity tended to be highest on the fourth day of protease production.

Discussion

In this study, pasture soil was superior to arable soil in terms of number of proteolytic bacteria, especially actinomycetes. This is in accordance with observations that protease activity is higher in pasture soil than in arable soil (LADD 1972) and availability of organic nitrogen is better in pasture soil than in tilled soil (HUNTJENS 1972). Because the proteolytic activity is concentrated in the surface layer of soil (HOFFMANN and TEICHER 1957), the disturbing effect of tillage on microbial flora may arise from the fact that surface soil is mixed into deeper layers. Increased proportions of actinomycetes in the microbial flora of grassland soil, as compared to that of tilled soil, have been reported also by WIERINGA (1958) and WOLDENDORP (1963). Furthermore, according to SPEIR et al. (1982), the number of actinomycetes and other bacteria increases with pasture age.

Soil pH is an important factor in the maintenance of soil microbial flora. Generally, casein hydrolysing activity is low in acid soils

(AMBROŽ 1965). In this study, the number of proteolytic bacterial strains was greater in peat and pasture soil than in the other soils with lower pH.

The abundance of proteolytic actinomycetes in pasture soil may partly be explained by the pH level of the soil (6.0) which was higher than the pH of the other soils examined. In greenhouse peat, however, typical actinomycetes were uncommon despite its high pH level (5.7). The low pH (3.5) of uncultivated muddy clay soil might explain the minute number of proteolytic actinomycetes and other bacteria as well. However, actinomycetes in general are known as active degraders of microbial biomass. Therefore, they may also reflect an rich microbial flora in pasture soil, as compared to the other soils investigated.

The richer proteolytic flora in peat and pasture soil, as compared to that of the other experimental soils, may also be related to the higher content of organic matter. This is supported by the observations of HOFFMANN and TEICHER (1975). They reported fivefold proteolytic activity in a peat soil (pH_{KCl} 6.0) as compared to that in a sand soil (pH_{KCl} 5.8). According to them, proteolytic activity in three sand soils with nearly the same pH (6.7–6.9) increased with increasing content of organic matter.

In this study, many strains with high protease activity were bacilli. The ability to utilize both inorganic and organic nitrogen might enhance the maintenance of bacilli in different soils (MISHUSTIN and MIRSOEVA 1968). The ability to form endospores may enable bacilli to survive in an inactive state in acid soils (GOODFELLOW et al. 1968, HOLDING et al. 1965).

Oxidase enzymes, especially peroxidases and phenoloxidases, play an important role in humification processes (SKUJINS 1967). Oxidase-producing bacteria have also been found in fertile soil (SUNDMAN 1970). Various organisms, e.g. common rhizosphere organisms like pseudomonads (EKLUND 1970), are included in oxidase producers. In this study, several strains isolated from peat and pasture

soil were capable of producing oxidase enzymes.

Production of dark melanoid pigments resembling humic polymers is often found in cultures of fungi, actinomycetes and other bacteria (VON PLOTHO 1950, KÜSTER 1952, 1955, MARTIN and HAIDER 1969, 1971, EKLUND 1970, HUNTJENS 1972). In this study, the production of melanoid pigments seemed to be associated chiefly with the strains isolated from pasture soil. Especially actinomycetes, which accounted for 70 % of the proteolytic strains isolated from pasture soil, were melanoid producers. This is in accordance with the observation of FLAIG and KUTZNER (1960) that actinomycetes which produce melanoid pigments are more numerous in grassland soil than in tilled soil of the same type.

In soil, the phenolic compounds occur together with other oxidable compounds like proteins and carbohydrates, all involved in

metabolic processes including formation of humic material. In the formation of humic material, metabolism of proteins and phenolic compounds are interrelated in such a way that amino acids released by the action of extracellular proteases react with phenols in the presence of phenoloxidases (e.g. HAIDER et al. 1965). Phenoloxidases also catalyze polymerization reactions of phenolic and humic compounds.

Presence of proteolytic actinomycetes in abundance in pasture soil may also be an indication of rich microbial biomass in the degradation of which actinomycetes hold a key position (WEBLEY and JONES 1971). The results confirm from a certain microbial point of view the favourable influence of pastures (and leys in general) on the microbial activity in soil closely associated with mobilization of nitrogenous compounds and a restoring effect on the stable organic matter in soil.

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Ms received November 6, 1985

SELOSTUS

Ekstrasellulaarisia proteaaseja tuottavat sädesienet ja muut bakteerit viljelysmaassa

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Ekstrasellulaariset proteaasit ovat entsyymejä, jotka maassa osallistuvat tyrellisten orgaanisten jätteiden hajotukseen. Proteaaseja tuottavan mikrobiston koostumus ja runsaus riippuu ympäristökijöistä kuten kosteudesta, lämpötilasta, ilmvuudesta, happamuudesta ja orgaanisen aineksen määrästä ja laadusta. Viljelytoimenpiteiden kuten muokkauksen avulla näihin ympäristökijöihin voidaan vaikuttaa. Tarkoituksena oli tutkia proteolyttisten bakteerien määrää, laatua ja fysiologisia ominaisuuksia laidunmaassa ja säännöllisesti muokatussa pelto- maassa. Lisäksi koemaina olivat orgaanista maata edustava kasvuturve ja viljelemätön hapan liejusavi. Koemaista

eristetyistä 240 bakteerikannasta 68 oli proteolyyttistä. Eniten proteolyyttisiä kantoja eristettiin laidunmaasta, jossa näistä kannoista 70 % oli sädesieniä. Myös turpeesta eristettiin runsaasti proteolyyttisiä bakteerikantoja, joiden joukossa ei kuitenkaan juuri ollut tyypillisiä sädesieniä. Useat kannat, joiden proteaasiaktiivisuus oli voimakas, osoittautuivat fermentatiivisiksi bacilluksiksi. Laitumen ja turpeen bakteerikannoille oli tavallista kyky muodostaa oksidaasientsyymejä, joilla on merkitystä humifioitumisprosesseissa. Tutkituille proteolyyttisille sädesienille oli tyypillistä kyky tuottaa tummia melanoidipigmenttejä.