Int. J. Environ. Res., 6(2):467-474, Spring 2012
ISSN: 1735-6865

Assessment of Feather Hydrolysate from Thermophilic Actinomycetes for Soil Amendment and Biological control Application

Gousterova, A.[1], Nustorova, M.[3], Paskaleva, D.[1], Naydenov, M.[2], Neshev, G.[2], Vasileva-Tonkova, E.[1*]

[1] The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bl. 26, 1113 Sofia, Bulgaria

[2] Agricultural University, 12 Mendeleev Blvd., 4000 Plovdiv, Bulgaria

[3] University of Forestry, 10 Kliment Ohridski Blvd., 1156 Sofia, Bulgaria

Received 19 Jan. 2011; Revised 6 Oct. 2011; Accepted 12 Nov. 2011

ABSTRACT: Protein-rich hydrolysate from feather waste was obtained using a mixed culture of selected thermophilic actinomycete strains, and was tested for possible application as soil amendment and biological control agent. For this purpose, a 4 months laboratory experiment was carried out using two types of urban soils (Sofia, Bulgaria): native park soil and anthropogenic soil. The effect of the obtained hydrolysate on some soil parameters (pH, some enzyme activities and microbial activity), seed germination and ryegrass growth, and activity against some plant pathogenic fungi was studied. The results demonstrated that soil enrichment with the organic solution in low concentrations exerted a positive effect on soil urease and microbial activity, seed germination and ryegrass growth, and this trend was better expressed in the anthropogenic soils. Feather hydrolysate showed good activity against plant pathogenic fungi *Fusarium solani*, *Fusarium oxysporum*, *Mucor sp*. and *Aspergillus niger*. Produced antifungal compounds were isolated and partially characterized as amphiphilic peptides. To the best of our knowledge, antifungal peptides produced by *Thermoactinomyces sp*. have not been reported. Therefore, the feather hydrolysate obtained by means of the mixed culture of *Thermoactinomyces* strains has potential to be used as alternative organic amendment for restoration of contaminated soils and for accelerating ryegrass growth. It could successfully used also for as biocontrol agent applicable to crop plant soil.

Key words: Feather hydrolysate, Thermoactinomycetes, Ryegrass growth, Soil microflora, Soil enzymes, Fungicide activity

INTRODUCTION

Feathers constitute over 90% protein; the main component being beta-keratin, a fibrous and insoluble structural protein extensively cross-linked with disulfide bridges, hydrogen bonds and hydrophobic interactions, resulting in the mechanical stability of keratin and its resistance to common proteolytic enzymes such as pepsin, trypsin and papain (Onifade *et al.*, 1998). Feathers are generated in high amounts as byproducts of poultry processing industry, and their accumulation could lead to environmental problems. Limitations of conventional methods for producing readily digestible feather meal impels the use of microbial treatment of recalcitrant feather wastes as ecologically safe, low-cost method that offers mild reaction conditions (Grazziotin *et al.*, 2006; Gupta & Ramnani, 2006; Moritz & Latshaw, 2001; Onifade *et al.*, 1998; Wang & Parsons, 1997). The use of thermophilic microorganisms for keratin waste treatment provides a unique opportunity because of the thermostability of secreted proteolytic enzymes as well as enhancing protein denaturing process at high temperature (Edwards, 1993; Gousterova *et al.*, 2005; Ignatova *et al.*, 1999; Suzuki *et al.*, 2006).

Bioconversion of keratin residues is attracting increasing biotechnological interest since it might represent an alternative way of waste management that could be coupled with the production of valuable products (Brandelli, 2008; Pasupuleti *et al.*, 2010). The simplest and most appropriate application of recycled keratin wastes and other organic wastes is as cheap soil amendments and fertilizers providing organic matter, an important constituent of biologically active and productive soils (Hadas & Kautsky, 1994 Nustorova *et al.*, 2006; Ros *et al.*, 2003; Zheljazkov, 2005). Moreover, using organic amendments may result in a soil with greater resistanse against plant

*Corresponding author E-mail: evaston@yahoo.com

pathogenic organisms and reduced use of fungicides. It is therefore promising to develop ecologically friendly methods for more effective utilization of keratin wastes to obtain new organic amendments and fertilizers for improving quality of agricultural soils.

In a previous work, collection of thermophilic actinomycete strains was screened for their ability to use feather wastes as a sole carbon and nitrogen source (Vasileva-Tonkova *et al.*, 2009). Ecologically safe method was proposed for effective feather wastes utilization by designed mixed culture of selected thermoactinomycete strains, and the obtained protein hydrolysate was characterized. The objectives of the present study were: (i) to evaluate the effect of using feather hydrolysate as soil amendment for restoration of contaminated soils by checking some soil characteristics (pH, protease and urease activity and microbial activity), seed germination and ryegrass growth; (ii) to evaluate the obtained feather hydrolysate as biological control agent by testing antigungal activity against some plant pathogenic fungi, (iii) to isolate and identify produced antigungal compounds.

MATERIALS & METHODS

Chicken feathers were obtained as a waste product from a poultry slaughterhouse in Bulgaria. Raw feathers were autoclaved twice (60 min, 130°C) then dried, grinded and used as a starting material for preparing feather hydrolysate. A mixed culture of selected thermoactinomycete strains was used for preparing prîtein-rich hydrolysate from feather waste. The strains designated by 3H, 8H and M4, were isolated from Antarcticà and were identified by routine morphological, microbiological and biochemical methods as belonging to the genus *Thermoactinomyces* (Bergey's Manual, 1994). Feather hydrolysate (FH) was obtained after 72 h cultivation of a mixed culture of the selected thermoactinomycete strains in mineral salts medium with 0.7% feather waste using a procedure previously described (Vasileva-Tonkova *et al.*, 2009). After removal of undegraded residues, the obtained FH was analyzed for pH, soluble protein content, amino acids content, total glycoside content and antifungal activity.

Soluble protein content was determined by the method of Bradford using human serum albumin as a standard (Bradford, 1976).Water-soluble carbohydrates were determined according to the method of Dubois *et al*. (1956) using glucose as a standard. Amino acids analysis was performed in an amino acid analyzer A-200 (Knauer, Germany) after hydrolysis of the samples in 6 N HCl for 24 h at 105°C. Proteolytic activity of soil samples was determined as casein hydrolyzing activity in CTA units (Committee of Thrombolytic Agents) using a modification of the method of Johnson *et al.* (1969). Urease activity was determined by the method of Zantua and Bremner (1975) with modification using urea as a substrate.

Pot experiment was carried out during 4 months under controlled conditions in plastic vessels of 5 kg each containing 3 kg soils. Two types of urban soils (Sofia, Bulgaria) were used: (i) native park soil taken inside of an urban park, and (ii) anthropogenic soil taken near a high road with intensive traffic (boulevard "Tsarigradsko shose"). For each type of soil, sixteen soil samples were taken at 0 - 15 cm soil layer then mixed and distributed in vessels. Variants with park soil were designated by "V", and variants with anthropogenic soil were designated by "B". Different amounts of FH were added to the soil variants (per g soil): 0.06 ml (variants V1, B1), 0.09 ml (variants V2, B2), and 0.12 ml (variants V3, B3). All hydrolysate inputs were carried out in triplicate. Three vessels without FH addition were also prepared as control samples (variants V0, B0). The soil in each vessel was cropped with 0.2 g ryegrass seeds and the vessels were placed in a room maintained at 28 ± 2°C. The soil humidity was maintained at 60% of the field capacity. During the experiment, seed germination, ryegrass growth, pH and microbial activity of the soil were followed in each variant. The microbiological analysis of the soil samples included determination of the soil microflora by plate dilution frequency technique using specific culture media as described earlier (Gousterova *et al.*, 2008).

Standard agar well diffusion assay was employed for *in vitro* study of antifungal activity of the obtained FH. Two replicates were carried out against each of the following plant pathogenic fungi: *Alternaria sp., Alternaria alternata, Absidia glauca, Fusarium solani, Fusarium oxysporum, Aspergillus niger, Aspergillus terreus, Mucor sp., Colletotrichum acutatum*, *Nectria haematococca*, and *Phytophthora sp*. After incubation, the diameter of the inhibition zone was measured as an indication of antifungal activity.

Antifungal compounds were extracted twice from the protein hydrolysate obtained from feather waste by chloroform/methanol mixture (2:1 v/v) The pooled extracts were evaporated, and the pellets dissolved in a small amount of methanol. The isolated compounds were characterized by thin-layer chromatography (TLC) on silica gel 60 plates (G60, Merck, Germany) using chloroform-methanol-water (65:25:2 v/v/v) as a solvent system, and chromatograms were sprayed with specific detection reagents.

RESULTS & DISCUSSION

The obtained FH (pH 8.2-8.5) contained total soluble protein 2.91 ± 0.25 mg/ ml and total glycosides

0.828 ± 0.12 mg glucose ml^{-1}. It was rich in amino acids including essential and rare ones like serine, cysteine and proline (Fig. 1). As was shown by qualitatively testing on solid media, the solution contained also proteolytic and lipolytic enzymes. Protein hydrolysates as a source of soluble proteins, amino acids and other valuable products help in improving the microflora of the soil thereby facilitating the assimilation of nutrients by plants (Paluszak & Olszewska, 2000). The pH was measured in suspension of soils in distilled water. The treatment of both types of soils with the organic solution and ryegrass cultivation almost not alter soil pH, which remained slightly alkaline to neutral during the experiment (Fig. 2A). This indicated relatively high buffer capacity and anion availability of the soils.

Measurement of enzyme activities such as protease and urease can be sensitive indicator of changes in soil fertility since they are related to the mineralization of important nutrient elements (Tejada *et al.,* 2006). Proteases and ureases in soils play a significant role in nitrogen mineralization, an important process in regulating the amount of plant available nitrogen for plant growth. The results showed an increase in urease activity in FH-amended soils which was better expressed in park soil (about 4-fold higher activity in V3 than in V0) (Fig. 2B). The added material may stimulate soil microbial activity, and root exudates provide nitrogenous substrates, which can induce the synthesis of these enzymes in the soil (Speir *et al.,* 1980; Tejada *et al.,* 2006). Protease activity decreased in both types of soil with low and middle FH-inputs possibly due to the decrease of available proteins and also to decrease of available nutrient for microbial growth (Fig. 2C). Similar results have been observed by Ros *et al.* (2003) in organically amended soil. In soil variants with the high dose of FH-input protease activity remained similar to the control in the park soil (V3), or slightly higher than the control in the anthropogenic soil (B3). The positive effect of organic amendments on soil biological quality is due to the stimulation of microbial growth and/or to the addition of microbial cells or enzymes with the amendment which can counteract the negative effect of some toxic compounds Garcia *et al.,* 1994). Results on the effect of FH on ryegrass seed germination are shown in Fig. 3. In all variants, an initial reduction in the rate of seed germination was observed (on Day 3 after cropping). Then percentage of seed germination increased reaching highest values with the lowest hydrolysate input in both types of soil – 57% in park soil (V1) and 49% in anthropogenic soil (B1). Percentage of seed germination decreased with increasing the amount of the added hydrolysate - about 1.5-fold decrease in the park soil (V3), and about 3-fold decrease in the anthropogenic soil in comparison to the control variants (on Day 10 after cropping). The inhibition in germination of seeds treated with higher amount hydrolysate could be due to some components of the hydrolysate like dyes, salts, phytotoxic organic metabolites, etc. There are reports that high salinity could influence the water relations of the media thereby influencing seed germination (O'Brien *et al.,* 2002).

The effect of FH on plant growth was tested using ryegrass as a model plant. During the experiment, an increase in the growth rate of ryegrass was observed which was different in both types of soil (Fig. 4). In the park soil samples, the organic solution stimulated the growth of ryegrass at all used concentrations. This effect was better pronounced with the lowest concentration of hydrolysate (V1).In the anthropogenic soil samples, different results were obtained. Microbial hydrolysate stimulated the growth

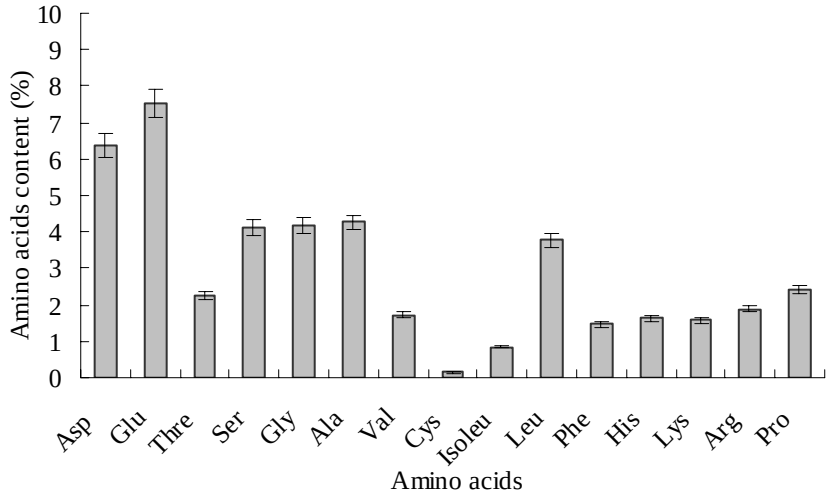

Fig. 1. Amino acids content of feather hydrolysate obtained by means of a mixed culture of thermoactinomycete strains

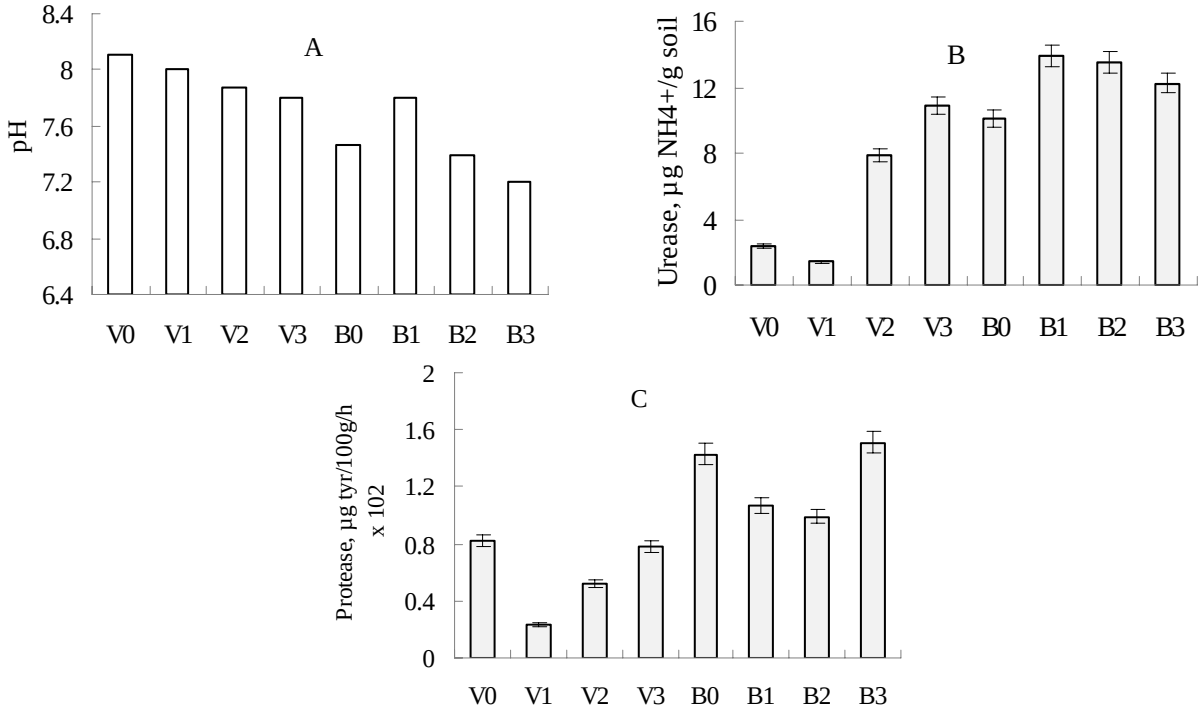

Fig. 2. Dynamics of pH (A), urease activity (B) and protease activity (C) of soil samples withtreated and untreated with feather hydrolysate. Symbols: V.–.variants with park soil; B – variants with anthropogenic soil. V0, B0 – control (without hydrolysate); V1, B1 – with addition of 0.06 ml hydrolysate per g soil; V2, B2 – with addition of 0.09 ml hydrolysate per g soil; V3, B3 – with addition of 0.12 ml hydrolysate per g soil

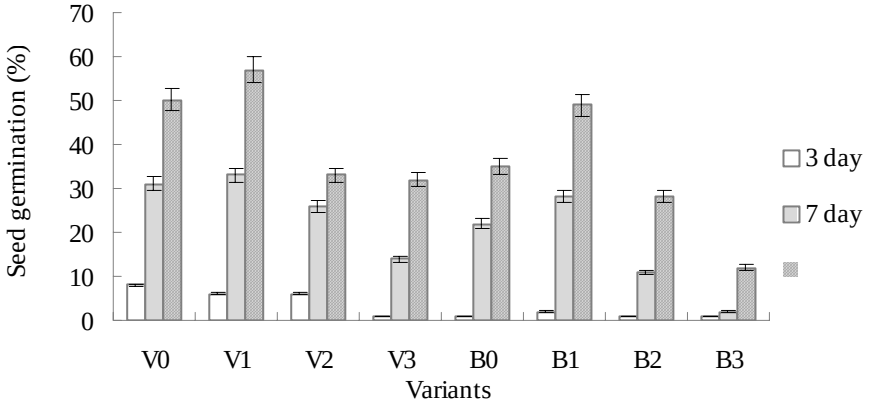

Fig. 3. Dynamics of germination of ryegrass seeds in soil samples treated and untreated with feather hydrolysate. Variants are as in Fig. 2.

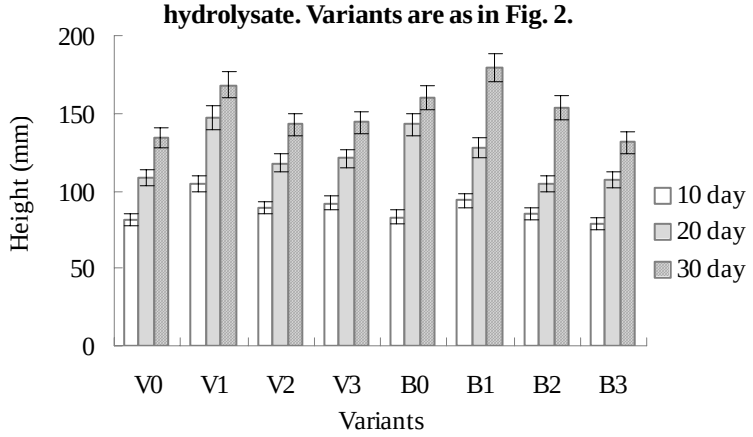

Fig. 4. Dynamics of ryegrass growth (height, mm) in soil samples treated and untreated with feather hydrolysate. Variants are as in Fig. 2.

of ryegrass at concentrations 0.06 and 0.09 ml/g soil (B1, B2) and inhibited the growth at the highest concentration (B3). As in the park soil, stimulation effect was better expressed with the lowest amount of hydrolysate (B1). Stimulation of ryegrass growth is probably due to an increased activity of the soil microorganisms which are primary decomposers of the organic matter providing accessible nutrients for plant growth (Paluszak & Olszewska, 2000).

The dynamics of abundance of heterotrophic microflora in the soil is an important parameter for evaluation of the soil biogeny after application of the organic solution. Soil microbial activity was in correlation with data on seed germination and plant growth. During the soil experimentS, an increase of the total microflora and diversity of microbial populations presented within microbiocenosis was revealed in both types of soil which indicated that decomposition of the organic matter occurred. The microbial activity was higher in the anthropogenic soils than in park soils: in variant B2 with anthropogenic soil total microflora was 3-fold higher than in the control (B0) while in variant V2 with park soil it was about 2-fold higher (Fig. 5A). In soil samples with highest amount of FH (variants V3 and B3) inhibition of the growth of microorganisms was observed in comparison with the control samples probably due to the intensive processes of self-cleaning occurring with the active participation of the soil microorganisms. The microbiological analysis of the soil samples revealed a varied heterotrophic microflora: ammonifying bacteria, bacilli, micromycetes, and actinomycetes. Populations of ammonifying bacteria have been found to predominate in the anthropogenic soil samples indicating the important role of these microorganisms in the processes of self-cleaning of contaminated soils (Fig. 5B) (Ros *et al.*, 2003).

In the course of screening for novel antimicrobial substances, the obtained FH was tested for antifungal activity against some common plant pathogenic fungi. The solution showed activity against four of the test cultures: *Fusarium solani, Fusarium oxysporum* and *Mucor sp.* with zone of inhibition 16 – 20 mm in diameter, and against *Aspergillus niger* with zone of inhibition 10–12 mm in diameter.

The compounds in the organic extracts from FH were initialy chatacterized by TLC. Three spots were visualized after staining with ninhydrin reagent with R_f 0.636, 0.729 and 0.776 indicating compounds containing free amino groups (Fig. 6). No spots were revealed after orcinol/sulfuric acid staining. With iodine vapors no spots were visualized at the same R_f values indicating lack of a lipid moiety of the compounds. The results suggested that the mixed culture of thermoactinomycete strains produced antifungal peptides during growth on feather wastes a sole carbon and nitrogen source.

Several antifungal_peptides produced by bacteria and fungi have been reporded (De Lucca & Walsh, 1999). Actinomycetes have provided many important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive substances including antifungal antibiotics (*Gohar et al.*, 2006; Iznaga *et al.*, 2004; Lim *et al.*, 2000). To the best of our knowledge, the isolation and

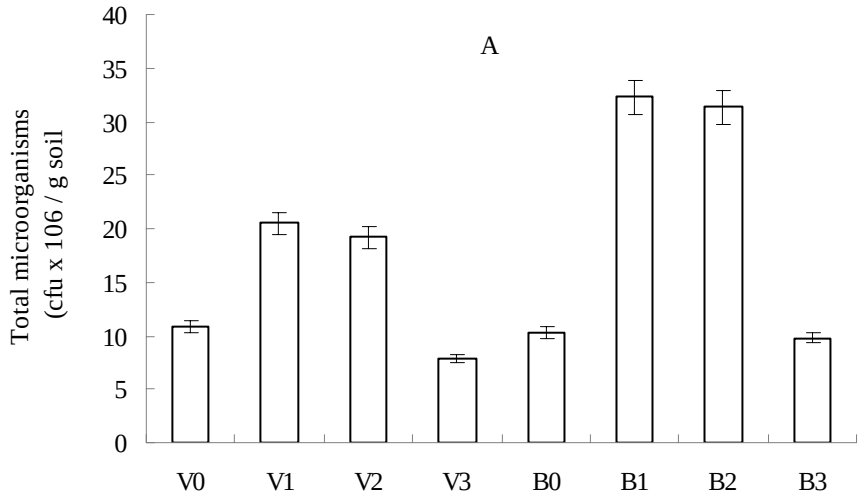

Fig. 5. Total number of microorganisms (A) and number of ammonifying bacteria (B) in soil samples without and supplemented with feather hydrolysate. Variants are as in Fig. 2.

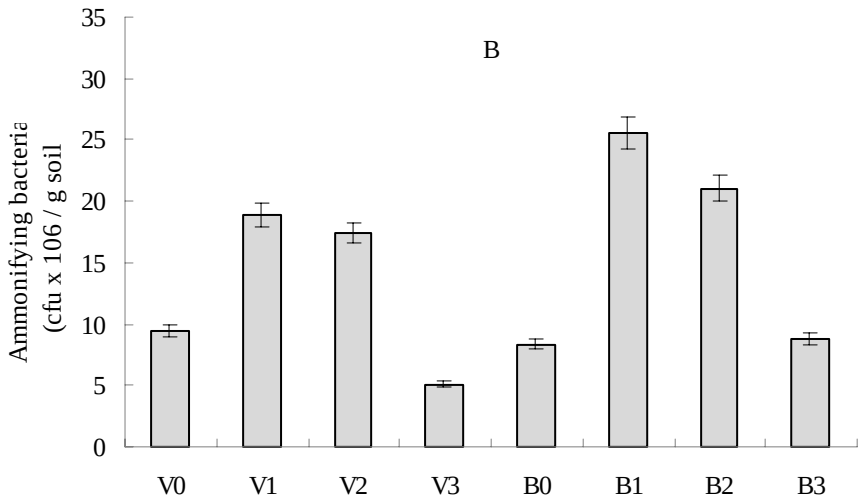

Fig. 5. Total number of microorganisms (A) and number of ammonifying bacteria (B) in soil samples without and supplemented with feather hydrolysate. Variants are as in Fig. 2.

Fig. 6. Thin-layer chromatogram of antifungal peptides produced by a mixed culture of thermoactinomycete strains grown on feather wastes. The organic extract was spotted onto silica gel plates and stained with ninhydrin reagent as described in the text

characterization of antifungalpeptides produced by *Thermoactinomyces sp.* have not been reported until now.

CONCLUSION

The results obtained in this study indicated that the mixed culture of thermoactinomycete strains could not only used to upgrade the nutritional value of recalcitrant feather wastes but is also a potential candidate for the development of low-cost soil organic amendment for restoration of contaminated soils and for accelerating ryegrass growth. It could successfully be used also as biocontrol agent applicable to crop plant soil. This way of treatment of feather wastes could solve both economical and environmental problems at the same time giving value added bioproducts with potential organic farming application.

ACKNOWLEDGEMENT

The authors acknowledge the financial support provided by the National Science Fund of the Bulgarian Ministry of Education, Youth and Science under project VU-L-303.

REFERENCES

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt. Biochem., **72**, 248-254.

Brandelli, A. (2008). Bacterial keratinases: useful enzymes for bioprocessing agroindustrial wastes and beyond. Food Biopr. Technol., **1**, 105-116.

De Lucca, A. J. and Walsh, T. J. (1999). Antifungal peptides: novel therapeutic compounds against emerging pathogens. Antimicrob. Agents Chemother., **43**, 1-11.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, T. (1956). Colorimetric method for determination of sugars and related substances. Analyt. Chem., **28**, 350-356.

Edwards, C. A. (1993). Isolation properties and potential applications of thermophilic actinomycetes. Appl. Biochem. Biotechnol., **42**, 161-179.

Garcia, C., Hernandez, T., Costa, F. and Ceccanti, B. (1994). Biochemical parameters in soils regenerated by the addition of organic wastes. Waste Manag. Res., **12**, 457-466.

Gohar, Y., Beshay, U., Daba, A. and Hafez, E. (2006). Bioactive compounds from Streptomyces nasri and its mutants with special reference to proteopolysaccharides. Polish J. Microbiol., **55**, 179-187.

Gousterova, A., Braikova, D., Goshev, I., Christov, P., Tishinov, K., Vasileva-Tonkova, E., Haertlé, T. and Nedkov, P. (2005). Degradation of keratin and collagen containing wastes by newly isolated thermoactinomycetes or by alkaline hydrolysis. Lett. Appl. Microbiol., **40**, 335-340.

Gousterova, A., Nustorova, M., Christov, P., Nedkov, P., Neshev, G. and Vasileva-Tonkova, E. (2008). Development of a biotechnological procedure for treatment of animal wastes to obtain inexpensive biofertilizer. World J. Microbiol. Biotechnol., **24**, 2647-2652.

Grazziotin, A., Pimentel, F. A., de Jong, E. V. and Brandelli, A. (2006). Nutritional improvement of feather protein by treatment with microbial keratinase. Animal Feed Sci. Technol., **126**, 135-144.

Gupta, R. and Ramnani, P. (2006). Microbial keratinases and their prospective applications: an overview. Appl. Microbiol. Biotechnol., **70**, 21-33.

Hadas, A. and Kautsky, L. (1994). Feather meal, a semi-slow-release nitrogen fertilizer for organic farming. Fertilizer Res., **38**, 165-170.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., and Williams, S. T. (1994). Bergey's Manual of Determinative Bacteriology (9th edn.), Baltimore, Philadelphia, Hong Kong, London, Munich, Sydney, Tokyo: Williams & Wilkins.

Ignatova, Z., Gousterova, A., Spassov, G. and Nedkov, P. (1999). Isolation and partial characterization of extracellular keratinase from a wool degrading thermophilic actinomycete strain Thermoactinomyces candidus. Can. J. Microbiol., **45**, 217-222.

Iznaga, Y., Lemus, M., González, L., Garmendía, L., Nadal, L. and Vallín, C. (2004). Antifungal activity of actinomycetes from cuban soils. Phytother. Res., **18**, 494-496.

Johnson, A. J., Kline, D. L. and Alkjaersig, N. (1969). Assay methods and standard preparations for plasmin, plasminogen and urokinase in purified systems. Thromb. Diath. Haemorrh., **21**, 259-272.

Lim, S. W., Kim, J. D., Kim, B. S. and Hwang, B. K. (2000). Isolation and numerical identification of Streptomyces humidus strain S5-55 antagonistic to plant pathogenic fungi. Plant Pathol. J., **16**, 189-199.

Moritz, J. S. and Latshaw, J. D. (2001). Indicators of nutritional value of hydrolyzed feather meal. Poultry Sci., **80**, 79-86.

Nustorova, M., Braikova, D., Gousterova, A., Vasileva-Tonkova, E. and Nedkov, P. (2006). Chemical, microbiological and plant analysis of soil fertilized with alkaline hydrolysate of sheep's wool waste. World J. Microbiol. Biotechnol., **22**, 383-390.

O'Brien, T. A., Herbert, S. J. and Barker, A. V. (2002). Growth of corn in varying mixtures of paper mill sludge and soil. *Commun. Soil Sci. Plant Anal.*, **33**, 635-646.

Onifade, A. A., Al-Sane, N. A., Al-Musallam, A. A. and Al-Zarban, S. (1998). A review: potentials for biotechnological applications of keratin-degrading microorganisms and their enzymes for nutritional improvement of feathers and other keratins as livestock feed resources. Bioresour. *Technol.*, **66**, 1-11.

Paluszak, Z. and Olszewska, H. (2000). Microbiological analysis of lessive soil fertilised with animal waste. Electronic J. *Polish Agric.* Univ., **3**, 1-9.

Pasupuleti, V. K., Holmes, C. and Demain, A. L. (2010). Applications of protein hydrolysates in biotechnology. (In V.K. Pasupuleti & A.L. Demain (Eds.). Protein hydrolysates in biotechnology Springer Netherlands (pp. 1–9). ISBN: 978-1-4020-6673-3 (Print)

Ros, M., Hernandez, M. T. and Garcia, C. (2003). Soil microbial activity after restoration of a semiarid soil by organic amendments. Soil Biol. Biochem., **35**, 463-469.

Speir, T. W., Pansier, E. A. and Cairns, A. (1980). A comparison of sulphatase, urease and protease activities in planted and fallow soils. Soil Biol. Biochem., **12**, 281-291.

Suzuki, Y., Tsujimoto, Y., Matsui, H. and Watanabe, K. (2006). Decomposition of extremely hard-to-degrade animal proteins by thermophilic bacteria. J. *Biosci.* Bioeng., **102**, 73-81.

Tejada, M., Hernandez, M. T. and Garcia, C. (2006). Application of two organic Aamendments on soil restoration: effects on the soil biological properties. J. Environ. Qual., **35**, 1010-1017.

Vasileva-Tonkova, E., Gousterova, A. and Neshev, G. (2009). Ecologically safe method for improved feather wastes biodegradation. Intern. Biodeterior. Biodegrad., **63**, 1008-1012.

Wang, X. and Parsons, C. M. (1997). Effect of processing systems on protein quality of feather meal and hog hair meals. *Poultry Sci.*, **76**, 491-496.

Zantua, M. J. and Bremner, J. M. (1975). Comparison of methods of assaying urease activity in soils. Soil Biol. Biochem., **7**, 291-295.

Zheljazkov, V. D. (2005). Assessment of Wool Waste and Hair Waste as Soil Amendment and Nutrient Source. J. Environ. Qual., **34**, 2310-2317.