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Investigation of Some Biological Properties of Coelomic Fluid Originating from the Red Worm *Eisenia fetida*

Kırmızı Solucan Eisenia fetida Kaynaklı Sölomik Sıvının Bazı Biyolojik Özelliklerinin Araştırılması

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Abstract

Eisenia fetida is abundant and vital member of the soil environment. The coelomic fluid they secrete help their physiological activities. This liquid, which they release during stress, also has a significant effect on soil health and ecotoxicity. In this study, some biological properties of coelomic fluid were investigated. For this purpose, 3 bacteria were selected from coelomic fluid, they were identified based on the 16S rRNA sequence and 2 of these were similar to *Bacillus cereus* and 1 to *Bacillus thuringiensis*. When the enzymatic activities in the coelomic fluid were controlled, the presence of lipase, cellulase, amylase, protease, phytase and arylsulfatase activities were detected. The antibacterial effects of the coelomic fluid was investigated against *Enterococcus faecalis, Escherichia coli, Staphylococcus epidermidis, Staphylococcus aureus, Klebsiella pneumoniae, Salmonella typhimurium, Yersinia enterocolitica* and *Pseudomonas aeruginosa*. According to the results, it showed inhibitory effects on test bacteria. Additionally, the thin layer chromatography results showed the presence of glucose and maltose in the coelomic fluid. Protein pattern of fluid showed five clear protein bands in the range of 17–46 kDa. With this study, some biological properties of coelomic fluid have been revealed, and will shed light on future studies.

Keywords: Antimicrobial activity, Coelomic fluid, Earthworm, Eisenia fetida, Enzyme activity, Protein profile

Öz

Eisenia fetida, toprak ortamının bol bulunan hayati üyelerindendir. Salgıladıkları sölomik sıvı fizyolojik aktivitelerine yardımcı olur. Stres sırasında serbest bıraktıkları bu sıvı, toprak sağlığı ve ekotoksisite üzerinde de önemli bir etkiye sahiptir. Bu çalışmada, sölomik sıvının bazı biyolojik özellikleri araştırılmıştır. Bu amaçla sölomik sıvıdan 3 bakteri seçilmiş, 16S rRNA dizilimine göre tanımlanmış ve bunlardan 2 tanesi *Bacillus cereus* ve 1 tanesi *Bacillus thuringiensis* ile benzerlik göstermiştir. Sölomik sıvıdaki enzimatik aktiviteler kontrol edildiğinde lipaz, selülaz, amilaz, proteaz, fitaz ve arilsülfataz aktivitelerinin varlığı tespit edilmiştir. Sölomik sıvının antibakteriyel etkileri *Enterococcus faecalis, Escherichia coli, Staphylococcus epidermidis, Stapylococcus aureus, Klebsiella pneumoniae, Salmonella typhimurium, Yersinia enterocolitica* ve *Pseudomonas aeruginosa*'ya karşı araştırılmıştır. Sonuçlara göre sölom bakterileri üzerinde inhibitör etkiler göstermiştir. Buna ek olarak, ince tabaka kromatografisi sonuçları ise sölomik sıvıda glikoz ve maltoz varlığını göstermiştir. Sıvının protein paterni, 17-46 kDa aralığında beş açık protein bandı göstermiştir. Bu çalışma ile sölomik sıvının bazı biyolojik özellikleri ortaya konulmuş olup, bundan sonraki çalışmalara ışık tutacaktır.

Anahtar Kelimeler: Antimikrobiyal aktivite, Sölomik sıvı, Solucan, Eisenia fetida, Enzim aktivitesi, Protein profili

1. Introduction

Protection of soil life is the first thing against the sustainable management of its biological properties of soil life for long-term quality and productivity. It is explained as soil biota helps to soil fertility, but there is not so much informations about soil-dwelling organisms and the functioning of soil ecosystems. The role of earthworms in fertility of soil has been known since 1881 (Bhadauria and Saxena 2010). Earthworms are the *Annelidae*, *Lumbricidae* family, oligochaetes class and it has up 8000 species (Blouin et al. 2013). Soil fauna, like earthworms, indirectly change the soil and subsoil environment through their biological structures (dumps, galleries, pellets, etc.), thereby influencing soil plant growth, physical features and cycling of nutrient (Scheu

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2003). Earthworms are an important for soil fauna in many of the natural ecosystems and make up a big portion of the macrofauna biomass (Lavelle 1995).

Earthworms are metamerically segmented organisms (Gudeta et al. 2020). Although the worm has a primitive organization, it's circulatory, nervous, muscular, digestive, excretory and reproductive systems are developed well. It's one of important feature is its body segmentation. It has the same general functions like the division of the body to organs, different segments perform different functions (Aydilek 2005).

Earthworms secrete a pungent yellow liquid on stress (heat, cold, mild electric current, etc.) (Nadana et al. 2020). This fluid secreted from the coelom, which is the space between the body wall and the alimentary canal, is called coelomic fluid (CF). The yellow colour comes from riboflavin, which is found partly in the fluid. However, CF is a mixture contains aromatic metabolites are known as species-specific small molecules, but its true content is not fully known (Rochfort et al. 2017). However, researchers determined that this liquid contains some enzymes such as fibrinolytic enzymes, metalloenzymes, lysozymes, proteases, and antimicrobial proteins, polysaccharides, some nutrients and also defense substances that support the growth of plants beside inhibit plant from diseases (Wang et al. 2007, Gudeta et al. 2021). Bioactive components in the liquid have cytolytic, proteolytic, antimicrobial, anti-inflammatory, antioxidant and antitumor, hemolytic, hemagglutination, tumorolytic and mitogenic activities (Li et al. 2017). Antimicrobial substances like antigen binding proteins and lysozyme that are the significant elements for immune system have also found in the CF (Wang et al. 2006). Antimicrobial peptides are the most important defense components, are now considered as universal host defense tools against microbial infection (Valembois et al. 1992, Scherbert and Messner 1997).

Researchers have investigated the activities of several enzymes such as protease, amylase, and prophenoloxidase isolated and purified from CF (Kauschke et al. 2007, Ramian et al. 2018).

CF has also been reported to have anticancer activity. The high concentration of CF exerted a toxic effect on HeLa cells, causing cell lysis and fragmentation (Esaivani et al 2017).

Many techniques have been developed so as to obtain CF from earthworms for various biological activities etc. There are usually four methods are practicing nowadays for the collection of CF from earthworms namely; warm water method, electric shock method, cold shock method and heat shock method (Patil and Biradar 2017).

CF with complex matrix with many biological functions and its own immune defense and microbial fertilizer has long been of interest (Cooper et al. 2002, Shen 2010). With the development of biotechnology, the bioactive compounds in the worm began to attract more and more attention.

Eisenia fetida (older spelling: *foetida*), also known as the California red worm, is one of the richest terrestrial species. They have an important role to maintain the ecological function of the soil by restoring and converting waste products into high-value useful plant growth medium (vermicompost: biofertilizer) (Devi and Khwairakpam 2020).

The aim of our study is to reveal some important biological properties of CF from *E. fetida*. For this, we intend to contribute to the literature by isolating bacteria from CF, presence of enzymes, antibacterial effect, presence of sugar, protein content in the fluid, and also by detecting the protein profile of the fluid with SDS-PAGE.

2. Materials and Methods

2.1. Materials

The worm *Eisenia fetida*, which was used as a CF source in the study, was obtained from Solitera Compost Ecological Products Food, Agriculture and Livestock Company (Bursa), which produces chemical fertilizers and earthworms. For antibacterial activity tests, *Pseudomonas aeruginosa ATCC 35032 Yersinia enterocolitica ATCC 9610 Klebsiella pneumoniae ATCC 70603 Staphylococcus aureus ATCC 25923 Salmonella typhimurium ATCC 14028 Escherichia coli ATCC 25922 Enterococcus feacalis ATCC 29212 and Staphylococcus epidermidis ATCC 12228* were obtained from Department of Microbiology and Infection Diseases, Faculty of Medicine, Bursa Uludag University.

2.2. Methods

2.2.1. Extraction of coelomic fluid by salt shock method

The earthworms were washed twice with distilled water and 0.85% NaCl (physiological salt water) respectively, dried on a paper, and then put in a glass beaker. The 5 V stimulation for 30 s was applied to the earthworms. It was induced them to release CF from the epidermal dorsal pores. The collected CF was vortexed and filtered with 0.22 µm syringe filter

(Macherey-Nagel sterile filter) (Vasantha et al. 2019). Cell-free supernatant was used in the experiments.

2.2.2. Isolation and identification of bacterial strains

Nutrient agar medium was used to isolate bacteria. 1 mL of CF obtained from the worm was mixed by vortexing in 10 mL of 0.85% of NaCl solution A series of dilutions were made from the solution sample and inoculated into petri dishes. They were incubated overnight at 37 ˚C. Identification and phylogenetic analyzes of 3 selected bacteria were performed by Refgen Biotechnology company (Ankara, Turkey). The bacterial identification and phylogenetic analysis were done after extraction of genomic DNA of bacteria (Qbiogene, Montreal, PQ, Canada). The sequence was analyzed with ABI 3100 Genetic Analyzer (Applied Biosystems, USA) and sequences were checked and analyzed by a similarity search on the NCBI GenBank database using BLAST (Basic Local Alignment Search Tool) to compare with the related sequences (Altschul et al. 1990). The CLUSTAL W was used to obtain multiple alignments of nucleotides for 16S rRNA genes. The phylogenetic analysis was constructed by MEGA 6.0, the phylogenetic tree was created using the neighbor-joining method (Saitou and Nei 1987). Gram and spore staining were also performed. The sequences were submitted to NCBI GenBank database and accession numbers were obtained.

2.2.3. Protein concentration determination

The biomass of protein content in CF was determine by Lowry et al. (1951) method. The protein concentration of the test sample was performed from a standard curve.

2.2.4. Enzyme activities of coelomic fluid

The presence of important enzymes such as lipase, cellulase, amylase, protease, phytase and arylsulfatase activities in CF was investigated.

Lipase

Titrimetric analysis was used to determine the lipase activity (Sugihara et al. 1991). Reaction mixture (4.5 mL of 50 mM Tris-HCl (pH 7.0) and 0.1 M $CaCl_2$), 1 mL of olive oil and 1 mL of filtered CF were incubated at 30 ˚C for 30 minutes by shaking. The 20 mL of 99.8% ethanol was used to stop enzymatic reaction. The pH was titrated to 10.5 by using a burette containing 50 mM KOH. One unit enzyme activity was described as the amount of enzyme releasing 1 µmol of fatty acid.

Cellulase

The method by Miller (1959) was used for the determination of cellulase enzyme activity. 0.5 mL of 1% carboxyl methyl cellulose substrate solution was added to 0.5 mL of CF and solution was incubated at 37 ˚C for 30 minutes in a water bath. In order to stop the reaction, 1 mL of Dinitrosalicylic acid (DNS) reagent (Garriga et al. 2017) was put and tubes were incubated in boiling water for 5 min., the absorbance was measured at 540 nm after cooling. One unit (U) of enzyme activity was defined as the enzyme amount that releases 1 µmol glucose per minute.

Amylase

Amylase activity was assayed by the starch-iodine method (Yoo et al. 1987). 5 mL of substrate solution (0.1% w/v) was kept for 10 min at 37 ˚C in a water bath and, then CF (0.5 mL) was added and incubated under the test conditions. After that 5 mL stopping reagent (0.1 M HCI) was added to the reaction mixture. 0.5 mL of mixture was added to 5 mL iodine solution. The absorbance was measured at 620 nm. One unit of enzyme was defined as the amount of enzyme that hydrolyzed 1 mg of starch (0.1%) under experimental conditions.

Protease

Total protease activity was measured using a casein substrate Anson Method (Keay and Wildi 1970). One mililiter of the CF, 1 mL 0.05 M phosphate buffer-0.1 M NaOH (pH 7.0 adjusted with phosphoric acid) was taken in a test tube containing 2% casein. Then test tube was placed in a water bath at 37 ˚C for 10 min. After reaction, 2 mL 0.4 M Trichloroacetic acid (TCA) was added for stopping the reaction. After 20 min stand at 37 ˚C, the solution was centrifuged at 6000 rpm for 10 min. From 1 mL supernatant was treated with 5 mL 0.4 M $NaCO_3$. To this mixture 1 mL of 1:3 diluted Folin-Ciocalteu reagent was added, the contents were mixed properly and placed in dark for 20 min before the absorbance was measured at 660 nm. A standard curve was plotted from the concentrations of 0-60 µg/mL tyrosine and one unit of protease activity was defined as the amount of enzyme required to liberate 1 µg/mL tyrosine.

Phytase

Phytase activity was assayed according to the method described by Choi et al. (2001). 0.1 mL of CF and 0.9 mL of 2 mM sodium phytate was mixed in the test tubes containing 0.1 M Tris-HCl buffer (pH 7.0). The reaction mixture was incubated in a water bath at 37 ˚C for 10 min

and then the reaction was stopped by adding 0.75 mL of 5% (w/v) TCA. Freshly prepared 1,5 mL of color reagent [four volumes of 2.5% (w/v) ammonium molybdate in 5.5% (v/v) sulfuric acid and one volume of 2.5% ferrous sulfate (w/v)] was added to the test tube and the released phosphate was measured at 700 nm. One enzyme unit was defined as the amount of phytase liberating 1 µmol of inorganic phosphate in a minute under the assay conditions.

Arylsulfatase

Arylsulfatase activity was determined by using method of Ohresser et al. (1997). 0.4 M glycine-NaOH buffer pH 9.0, 10 mM imidazole and the enzyme substrate 0.3 mM 5-bromo-4 chloro-indolylsulphate (X-SO_4) were added to 400 µl CF in a total volume of 500 µl the reaction mixture. The sample was incubated at 37 ˚C for 1 h with X-SO_4, then 500 µl of 4% SDS in 0.2 M Na acetate buffer pH 4.8 and 100 µl of 10 mg/mL tetrazotized-o-dianisidine (Fast Blue B) (Sigma) was used to stop reaction. Fast Blue B coupled to the α-naphthol released in the reaction, a purple precipitate which is soluble in SDS was observed. The absorbance at 540 nm was measured within 2 min after the addition of Fast Blue B. Arylsulfatase activity was expressed as α-naphthol concentration formed in 1 hour at 37˚C according to the total amount of protein (µg α-naphtol/mg protein hour).

2.2.5. Agar well diffusion method for antibacterial activity

Agar well diffusion method was used to determine CF's antibacterial activity (Haque et al. 1995). The test strains were incubated at 37˚C for 24 h in nutrient broth. The inoculates with a density equivalent to1.5 x 10^8 CFU/mL were spread over the Nutrient Agar (NA) with a cotton swab dipped in. Then, wells (7 mm in diameter) were made in the inoculated agar under sterile conditions. Filtered CF (0.22 µm syringe filter, Macherey-Nagel) was collected in sterile 1.5 mL microcentrifuge tubes and applied in concentration of 1 mg protein/mL dH_2O. 100 µl of diluted solutions of bacitracin (Merck), ampicillin (Calbiochem), penicillin (Merck) (1mg/mL) and CF filtrate and distilled water as control were added to wells. The plates were left to dry for 1 h at room temperature. All the plates were incubated at 37 ˚C for 48 h and observed for zones of inhibition. Zones were recorded by measuring in millimeter(mm).

2.2.6. SDS-PAGE

Characterization of CF proteins was determined by sodium dodecyl sulphate / polyacrylamide gels (SDS–PAGE) according to Laemmli (1970). The protein separation was accomplished by using 10% SDS run at 100 V for 2 h.

2.2.7. Determination of carbohydrates by TLC method

Thin layer chromatography (TLC) method was used according to Stahl (1965). for the determination of carbohydrates in CF and in commercial coelomic fluid (CCF) procured from Solitera Compost Ecological Products Food, Agriculture and Livestock Company (Bursa) Silica gel plates with a size of 20×10 cm and a thickness of 0.2 mm were used (Merck, 100390). The CF was applied as 5 µl with a microsyringe (Hamilton), and the plates were allowed to dry at room temperature. Butanol, ethanol, distilled water (5:3:2) were used as solvents. The plate remained in the tank until the solvent removed. Then the plate was removed and kept in an oven at 110 ˚C for 10 minutes to dry out. A solution of sulfuric acid and methanol (1:3, v/v) was carefully sprayed onto the plate. The plate was then dried in an oven again at 110 ˚C for 10 minutes, to monitor carbohydrates as black or brown spots on the white plate (Robyt and White 1987).

3. Results and Discussion

Earthworms release liquid from the coelom to the external environment in case of rough use or stress. CF, which consists of the aqueous fluid released from the dorsal pores of the worms and composed of coelomocytes such as amoebocytes, mucocytes, circular cells and chloragogen cells, plays a very important role in the fight against pathogenic microorganisms in the immunity of the worms. There are many chemical components with medicinal value in the CF. With the development of biotechnology, the bioactive compounds in the worm began to attract the attention of more and more scientists (Cooper et al. 2002, Shen 2010).

In this study, some biological properties of CF obtained from *E. fetida* were revealed. Bacterial load of CF was determined as 10^3 CFU/mL in NA. There were over 100 bacterial colonies on petri dish, morphologically different three bacteria were selected. They were named as bacteria 5, 6, and 7. As can be seen on phylogenetic tree, bacteria 5, 6 showed 100% sequence identity with *Bacillus cereus* and bacteria 7 showed 100% similarity with *Bacillus thuringiensis* (Figure 1). The partial 16S rRNA of the isolated bacteria 5, 6, and 7 were deposited in GenBank (accession numbers OM457246, OM457247, and OM458830 respectively).

Yakkou et al. (2021) was analyzed the structure of the bacterial community of the earthworm's CF and it was obtained that 28 different bacterial isolates were found from total 70 colonies. Out of them 16 isolated bacteria were selected and they were found as *Aeromonas, Bacillus, Buttiauxella,*

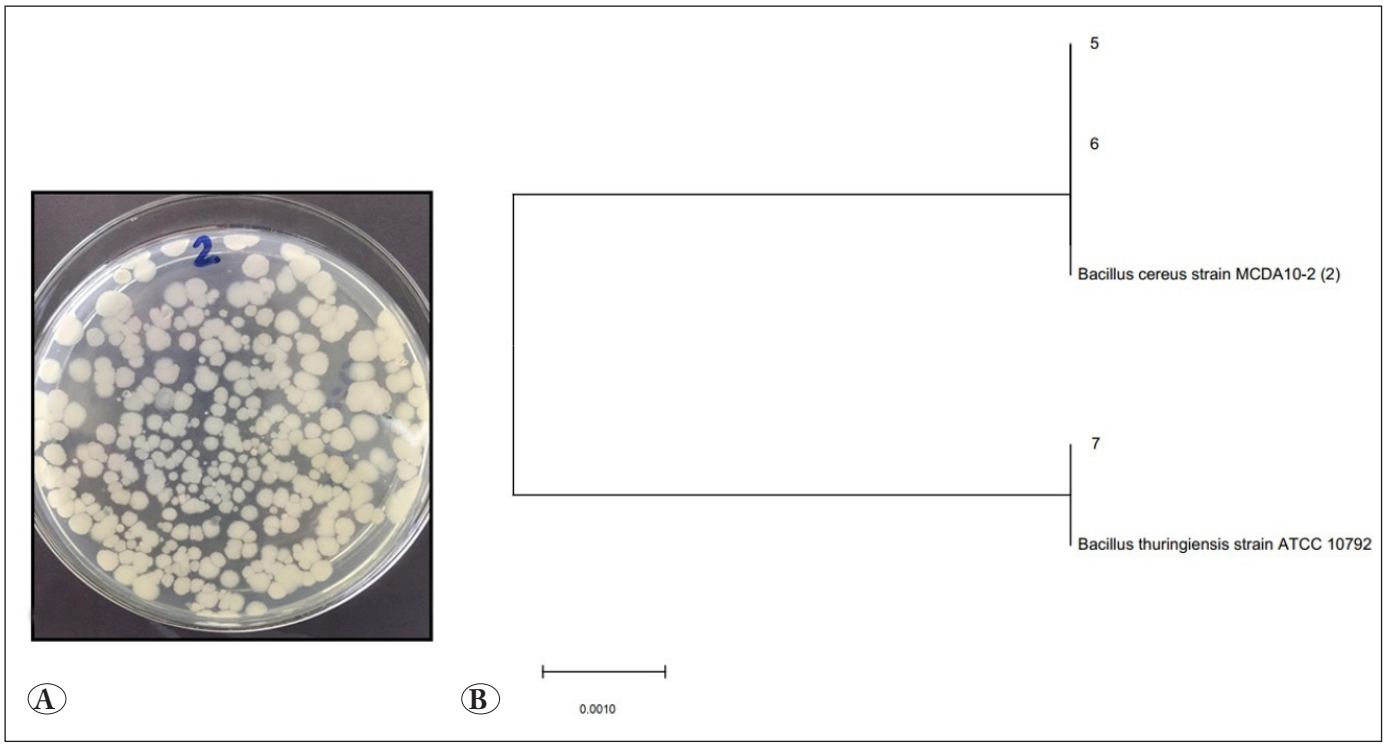

Figure 1. Bacterial load in CF **(A)**, Phylogenetic tree of four bacteria based on the neighbor-joining method **(B)**. Bar, 0.0010 substitutions per nucleotide position.

Enterobacter, Raoultella, *Pantoea*, and *Pseudomonas*. Among them there are species which *Bacillus thuringiensis, Bacillus altitudinis, Bacillus licheniformis, Pseudomonas aeruginosa, Pseudomonas azotoformans, Pseudomonas helmanticensis* have important role in the rhizosphere and beneficial bacteria for plant growth and also used in soil fertilization and biocontrol (Mishra et al. 2014, Yousra et al. 2018).

Earthworms are considered as major terrestrial ecosystem engineers for their ability to modify physical (aggregate structure, porosity), chemical (nutrient supply and cycling) and biological (soil fauna, microbial and enzymes activities) properties of the soil profile. The decomposition and mineralization of organic matter in nature is associated with the presence of extracellular and intracellular enzymes of both soil and worm gut bacteria (Jouni 2018).

They are also known to have many digestive enzymes such as amylase, cellulase, lipase, lumbrokinase, endoglucanase and β-glucosidase etc. These enzymes are also useful for biomass utilization (Akazawa et al. 2018). Earthworms contain abundant of enzymes useful for bioremediation of contaminated soils, in animal feeds for improving the digestion of proteins, lipids, and carbohydrates, and also in the saccharification of starch, detergents, animal and human therapeutics (Ramian et al. 2018).

In present study the enzyme activity of CF was demonstrated in Figure 2. The presence of lipase, cellulase, amylase, protease, phytase and arylsulfatase enzymes were detected in CF obtained from *E.fetida*. The activity values for lipase, cellulase, amylase, protease and phytase in the coelom fluid were compared with the enzyme activity results obtained from our previous studies with *Bacillus* sp. bacteria. While lipase was 9.5 U/mL in CF, it was 6.6 U/mL in *Bacillus cereus* ATA179 (Demirkan et al. 2020); while cellulase was 48 U/mL in CF, it was 68 U/mL in *Bacillus* sp. (Msakni 2020); while amylase was 496.2 U/mL in CF, it was 21.2 U/mL in *Bacillus* sp.M10 (Demirkan et al. 2016); while protease was 115.6 U/mL in CF, 224 U/mL in *Bacillus* sp. N-40 (Sevinç and Demirkan 2011); while phytase was 528 U/mL in CF, it was 210 U/mL in *Bacillus megaterium* EBD 9-1 (Demirkan et al. 2017). The arylsulfatase as value was compared with the data of the study conducted with *Chlamydomonas reinhardtii* (Sevgi and Demirkan 2021). The arylsulfatase activity was found to be quite low in CF. The presence of phytase enzyme in fluid was reported for the first time in this study. These enzymes in CF may be released by *Bacillus* sp. bacteria found in CF. According to these results, the fact that amylase and phytase activities were found high in the CF, which are useful for biomass utilization.

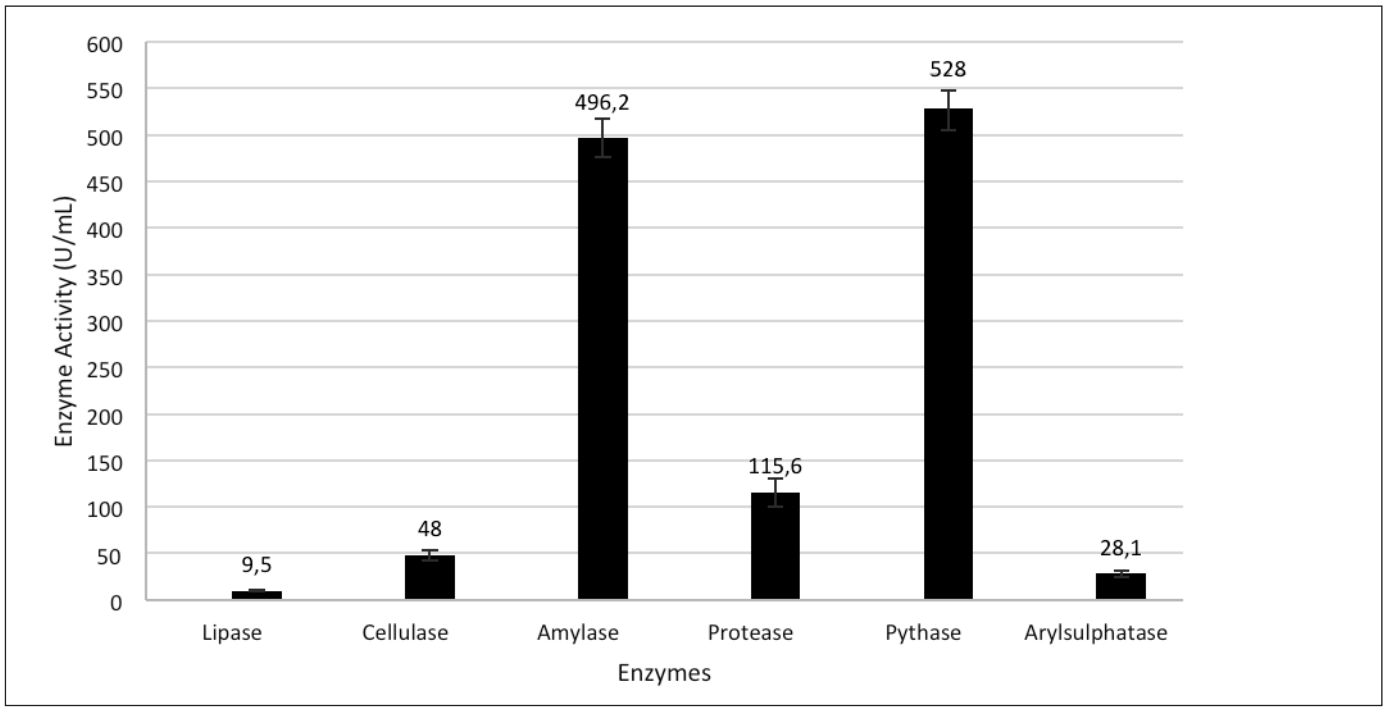

Figure 2. Enzyme activities in coelomic fluid from *E. fetida*.

Some researchers have investigated the enzymes in the *E. fetida* CF. Akazawa et al. (2018) reported the presence of lumbrokinase, amylase, endoglucanase, glucosidase and lipase in CF of *E. fetida*. Raw-starch-digesting (Amy I) and cold-adapted (Amy II) α-amylases from the earthworm *E. fetida* were purified and characterized by Tsukamoto et al. (2021). Ramian et al. (2018) reported the effects of pH, temperature and metals on amylase enzyme in CF obtained from *Allolobophora chlorotica* worm and Kauschke et al. (1997) reported the presence of proteolytic enzymes in CF from earthworms *Lumbricus terrestris*, identified eight proteases at different electrophoretic mobility and showed their trypsin and chymotrypsin and found relatively heat stable, are classified as serine proteases. Bilej et al. (1995) identified a cytolytic protein in the CF of *E. fetida* earthworms and is capable of lysing different mammalian tumor cell lines. Yamaji et al. (1998) identified a newly sphingomyelin-specific binding protein called Lysenin, purified from CF of the earthworm *E.fetida*, which induces erythrocyte lysis. Kizilkaya et al. (2010) reported the enzyme activities such as urease, phosphatase and arylsulphatase during vermicomposting by earthworm *E. fetida*. Eue et al. (1998) also reported hemolytic activity in CF of *E. fetida*.

Plants grown on vermicompost treated soils have been shown to be more resistant to diseases and pests. The antibacterial and antifungal effect of vermicompost on plants is due to the CF that worms secrete out of their bodies for various reasons. Enzymes and proteins such as agglutinin, fetidin, lumbricin and chitinase in the structure of CF mixed with vermicompost act against some fungi, bacteria and pests that contain chitin in their structure, thus weakening the effects of lots of diseases and pests. (Wang et al. 2006, Gudeta et al. 2021).

In this study, agar well diffusion assay was applied for the antibacterial activity of the CF extracted from *E. fetida* against pathogenic bacteria causing diseases in human such as *Enterococcus feacalis*, *Escherichia coli*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Yersinia enterocolitica* and *Pseudomonas aeruginosa* and the data were compared with standard antibiotics as controls (Table 1). As a result, CF obtained from *E. fetida* have shown zones of inhibition between 15-35 mm against of the pathogenic bacterial strains. It was observed that the effect of CF was significantly higher than standard antibiotics particularly bacitracin (BAC). The highest inhibitory effects of CF were identified on the growth of *K. pneumoniae* and *E. coli*. The antibacterial impact of CF was studied highest to lowest as follows *K. pneumonia*, *E. coli* = *Y. enterocolitica*, *S. typhimurium*, *P. aeruginosa*, *S. aureus*, *S. epidermidis* (Table 1). The findings showed that CF had a high potential to inhibit the bacterial growth used in the research. Due to

Table 1. Antibacterial activity of coelomic fluid (CF) against different pathogens.

Test bacteria	Diameters of inhibition zones (mm)			
	CF (1 mg/mL)	Antibiotics (1 mg/mL)		
		AMP	PEN	BAC
P. aeruginosa ATCC 35032	21	12	11	8
Y. enterocolitica ATCC 9610	25	21	12	15
K. pneumoniae ATCC 70603	35	17	14	12
S. aureus ATCC 25923	20	20	24	15
S. typhimurium ATCC 14028	22	16	15	9
E. coli ATCC 25922	35	22	18	19
E. faecalis ATCC 29212	25	17	26	19
S. epidermidis ATCC 12228	15	9	12	10

BAC: bacitracin, **AMP:** Ampicillin, **PEN:** Penicillin.

extensive antibacterial properties of CF, extracted CF may have potential applications for the pharmaceutical industry.

Earthworms always expose the invasion of pathogen microorganisms in their environments, so they live because of helping their defense system, since the beginning of evolution (Engelmann et al. 2004). Because of this, CF of the earthworms have more than 40 proteins and they exhibit some biological activities like antimicrobial, cytolytic, haemolytic, haemagglutinating, proteolytic, mitogenic and tumorolytic activities (Cooper and Roch, 2003, Gudeta et al. 2020).

There are so much studies about antibacterial components in CF of earthworms in literature. Valembois et al. (1992) reported that the CF from *E. fetida* has an antibacterial activity against Gram negative *Aeromonas hydrophila* and Gram positive *Bacillus megaterium* as pathogenic bacteria. Scherbert and Messner (1997) identified some lysozyme like molecules as bacterolytic molecules and found active only against gram positive bacteria. Murugan and Umamaheswari (2021) assayed CF of *Eudrilus eugeniae* on gram positive bacteria (*S. faecalis, B. subtilis, Micrococcus luteus*) and gram negative bacteria (*E. coli, Salmonella abony, P. aeruginosa*) and identified the best inhibitory effects on the growth of *P. aeruginosa* and *B. subtilis*. Milochau et al. (1997) stated that CF of *Eisenia fetida andrei* ndicates strong hemolytic activity against some mammalian erythrocytes and bacteria. Vasanthi et al. (2013) reported that the extract from *Eudrilus eugeniae* showed strong antimicrobial activity against *E. coli, B. subtilis, S. abony, S. aureus* and *K. pnuemoniae* bacteria and *Aspergillus niger, Aspergillus flavus, Candida albicans* and *Penicillum notatum* fungus. Tutar and Karaman (2017) also found the zone diameters of the CF obtained from *E. fetida* between 8-54 mm against the microorganisms *Erwinia chrysanthemi, Erwinia herbicola, Pseudomonas cepacia, Pseudomonas fluorescens*, *Pseudomonas syringae, Xanthomonas carotae* and *Xanthomonas campestris*

The presence of sugar in the CF extracted from *E. fetida* was determined by TLC. According to the TLC result, when glucose and maltose were used as standards, the presence of very little glucose and maltose was observed. However, the presence of sugar was not observed in commercial CF (Figure 3).

Konosu et al. (1966) showed glucose and maltose in TLC using several different solvent systems. The properties of the CF in starch column chromatography showed three peaks and the behaviors agreed well with glucose and maltose. Clark (1964) also reported the presence of glucose, maltose and trisaccharide in coelomic fluid of other species of marine worm, *Nephtys hombergi*. Yuk et al. (2012) reported that 1D NMR spectrum of the CF carbohydrates are present at trace levels of various sugars including maltose, glucose. Ramian et al. (2018) also reported the amylases in CF and body extract from the earthworm *Allolobophora chlorotic* and showed the enzyme hydrolyzed raw starch to main products.

In this study, when the protein profiles of the CF were analyzed by SDS-PAGE, several proteins, which were estimated as dark and light bands, were detected. The molecular weights of these proteins are estimated to be between 15-46 kDa. Estimated molecular weights among

3 of them were found between 46-32 kDa, while one of them was 32 kDa and another one as 15 kDa (Figure 4). The protein bands obtained in this study are predicted to be phytase, amylase, protease or antibacterial peptides. The protein that appears as a thick band is estimated to be a phytase enzyme. Because, the molecular weight of phytase enzymes were determined as approximately 45 kDa (Demirkan et al. 2017).

In the CF of *A. caliginosa* several proteolytic bands below 94 kDa were detected and the inhibition assays showed that these CF protease as serine proteases (Kauschke et al. 2007). Milochau et al. (1997) reported Fetidin proteins' molecular masses 40 kDa and 45 kDa and determined that they were stable under at 56°C. Bilej et al. (1995) depicted a protein in CF with western blot analysis with 42 kDa molecular weight. Molecular mass of lysenin protein from CF of *Eisenia fetida* had found as 41 kDa (Yamaji et al 1998). Three proteins Hemolytic protein (H) 1, H2, H3 had hemolytic activity had molecular weights of 46, 43 and 40 kD, respectively (Eue et al. 1998).

They also defend themselves against pathogenic microbes and pests through their humoral defense mechanism which includes bioactive molecules. Other metabolites such as hormones, enzymes, vitamins, proteins, different macro and micronutrients, and a massive amount of microbes facilitate a conducive environment for the growth of a plant by reducing stress conditions and create unfavorable condition for pathogenic soil microbes and pests (Gudeta et al. 2021).

4. Conclusion

Earthworm coelomic fluid contains important defense components such as bacteriostatic/bactericidal, hemolytic, cytolytic and proteolytic substances. Many researchers have focused on the study of bioactive proteins in worm coelomic fluid. In this study, some biological properties of *E. fetida* coelomic fluid were revealed. Especially, due to the high antibacterial properties of coelomic fluid, it has a high potential for use in the pharmaceutical industry. It has the potential to be used in enzyme production with the isolation of bacteria in coelomic fluid that secrete enzymes of industrial importance. The data obtained are of a nature to shed light on other studies.

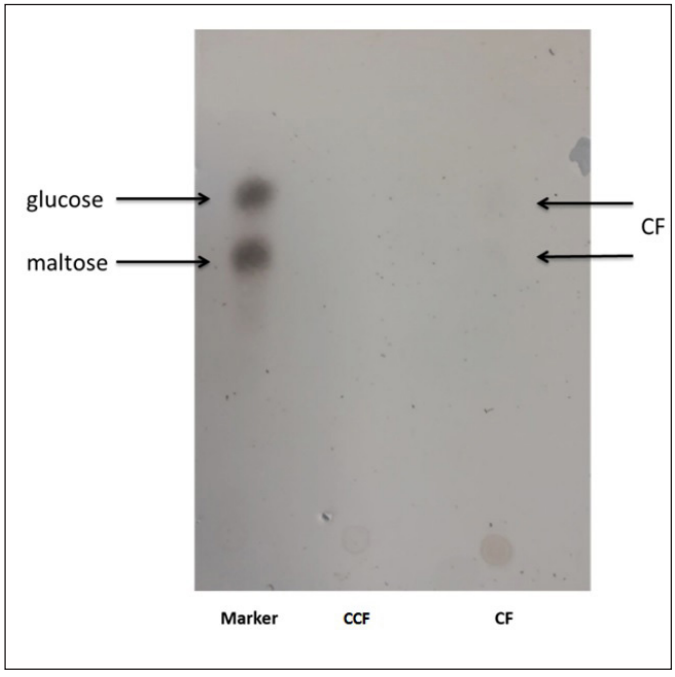

Figure 3. Determination of the presence of glucose and maltose by thin layer chromatography. **CF:** coelomic fluid from *E.fetida,* **CCF:** commercial coelomic fluid product.

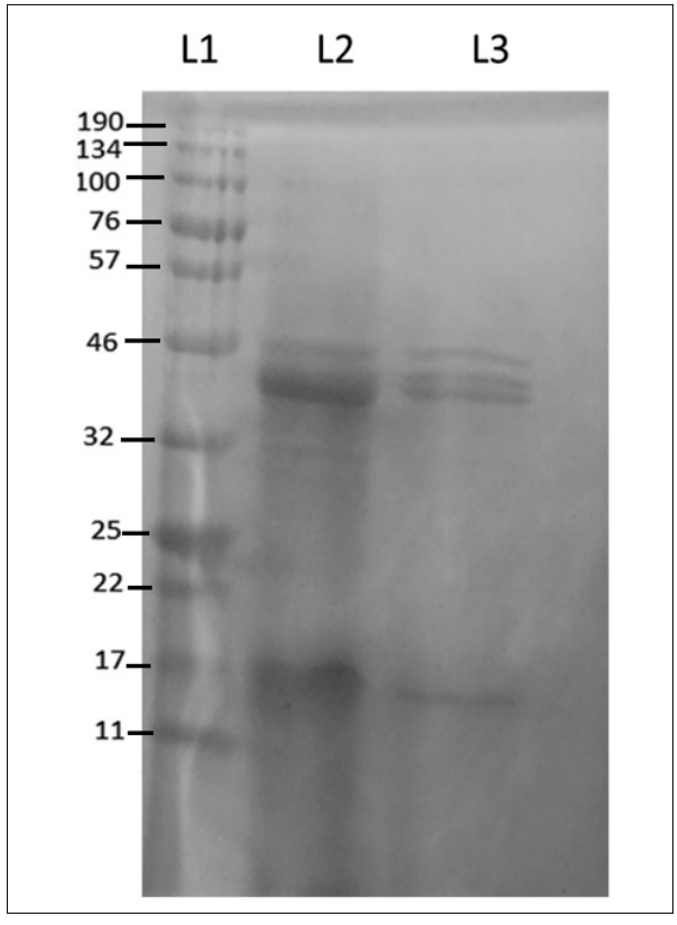

Figure 4. SDS-PAGE showing protein profile standard protein markers (L1), coelomic fluid (L2), 1:10 diluted coelomic fluid (L3).

5. References

Akazawa, S.I., Tokuyama, H., Sato, S., Watanabe, T., Shida, Y., Ogasawara, W. 2018. High-pressure tolerance of earthworm fibrinolytic and digestive enzymes. J. Biosci. Bioeng., 125(2): 155-159. https://doi.org/10.1016/j.jbiosc.2017.08.011

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. 1990. Basic local alignment search tool. J. Mol. Bio. 215(3): 403-410. https://doi.org/10.1016/S0022-2836(05)80360-2

Aydilek, S. 2005. The effect of coelomic fluid of earthworms against various cells. MSc, İstanbul University, İstanbul, Turkey.

Bhadauria, T., Saxena, K.G. 2010. Role of earthworms in soil fertility maintenance through the production of biogenic structures. Appl. Environ. Soil Sci. https://doi.org/10.1155/2010/816073

Bilej, M., Brys, L., Beschin, A., Lucas, R., Vercauteren, E., et al. 1995. Identification of a cytolytic protein in the coelomic fluid of *Eisenia foetida* earthworms. Immun. Lett. 45: 123-128. https://doi.org/10.1016/0165-2478(94)00248-P

Blouin, M., Hodson, M.E., Delgado, E.A., Baker, G., Brussaard, L. et al. 2013. A review of earthworm impact on soil function and ecosystem services. Eur. J. Soil Sci. 64: 161-182. https://doi.org/10.1111/ejss.12025

Choi, Y.M., Suh, H.J., Kim, J.M. 2001. Purification and properties of extracellular phytase from *Bacillus* sp. KHU-10. J. Protein Chem. 20: 287-292. https://doi.org/10.1023/a:1010945416862

Clark, M.E. 1964. Biochemical studies on the coelomic fluid of Nephtys hombergi (Polychaeta: Nephtyidae), with observations on changes during different physiological states. Biol. Bull. 127(1): 63-84. https://doi.org/10.2307/1539345

Cooper, E.L., Ellen, K., Andrea, C. 2002. Digging forinnate immunity since Darwin and Metchnikoff. Bio Essay, 24: 319-333. https://doi.org/10.1002/bies.10077

Cooper, E.L., Roch, P. 2003. Earthworm immunity: a model of immune competence. Pedobiologia, 47: 676-688. https://doi.org/10.1078/0031-4056-00245

Demirkan, E., Aybey Çetinkaya, A., Abdou, M. 2021. Lipase from new isolate *Bacillus cereus* ATA179: optimization of production conditions, partial purification, characterization and its potential in the detergent industry. Turk. J. Biol. 45(3): 287-300. https://doi.org/10.3906/biy-2101-22

Demirkan, E., Sevgi, T., Akcakoca, D., Ersoy, F. 2017. Partial purification, characterization and wheat bran degradation studies of a new phytase from the *Bacillus megaterium* EBD 9-1 strain. Turk. J. Biochem. 4(3): 329-337. https://doi.org/10.1515/tjb-2016-0265

Demirkan, E., Sevgi, T., Baskurt, M. 2016. Optimization of Physical factors affecting the production of the α-Amylase from a newly Isolated *Bacillus* sp. M10 strain. Karaelmas Sci. Eng. J. 6(2): 1-8.

Devi, C., Khwairakpam, M. 2020. Feasibility of vermicomposting for the management of terrestrial weed Ageratum conyzoides using earthworm species *Eisenia fetida*. Environ. Technol. Innov. 18: 100696. https://doi.org/10.1016/j.eti.2020.100696

Engelmann, P., Kiss, J., Cooper, E.L., Nemeth, P. 2004. Earthworm leukocytes kill Hela, Hep-2, PC -12 and PA 317 cells invitro. J. Biochem. Biophys. Meth. 61: 215-228. https://doi.org/10.1016/j.jbbm.2004.04.004

Esaivani, C., Vasanthi, K., Singh, A.J. 2017. An Investigation on antimicrobial potency of coelomic fluid of earthworm *Eudrilus eugeniae.* British J. Med. Health Res. 4(4): 2394-2967.

Eue, I., Kauschke, E., Mohrig, W., Cooper, E.L. 1998. Isolation and characterization of earthworm hemolysins and agglutinins. Dev. Comp. Immunol. 22: 13-25. https://doi.org/10.1016/s0145-305x(97)00049-9

Garriga, M., Almaraz, M., Marchiaro, A. 2017. Determination of reducing sugars in extracts of *Undaria pinnatifida* (harvey) algae by UV-visible spectrophotometry (DNS method). Actas de Ingeniería 3: 173-179.

Gudeta, K., Kumari, S., Bhagat, A., Julka, J.M., Goyal, R. 2020. Mitogenic, Antioxidative and Antimicrobial Activities of G-90 and Coelomic Fluid From Earthworm As A Therapeutic Agent. Inter. J. Pharmaceut. Sci. Res. 11(11): 5298-5307. https://doi.org/ 10.13040/IJPSR.0975-8232.11(11).5298-07.

Gudeta, K., Julka, J.M., Kumar, A., Bhagat, A., Kumari, A. 2021. Vermiwash: An agent of disease and pest control in soil, a review. Heliyon, 7(3). https://doi.org/10.1016/j.heliyon.2021.e06434.

Haque, S.F., Sen, S.K., Pal, S.C. 1995. Nutrient optimization for production of broad spectrum antibiotic by Streptomyces antibioticus SR15. 4. Acta Microbiol. Immun. Hungar. 42 (2): 155-162.

Jouni, F. 2018. Synergistic interaction earthworm-microbiota : a role in the tolerance and detoxification of pesticides?. Présentée pour obtenir le grade de docteur de L'Université d'Avignon.

Kauschke, E., Pagliara, P., Stabili, L., Cooper, E.L. 1997. Characterization of Proteolytic Activity in Coelomic Fluid of *Lumbricus terrestris L.* (Annelida, Lumbricidae) Comp. Biochem. Physiol. Part B: Biochem. Mol. Biol. 116:235-242. https://doi.org/10.1016/S0305-0491(96)00248-9

Kauschke, E., Mohrig, W., Cooper, E.L. 2007. Coelomic fluid proteins as basic components of innate immunity in earthworms. Eur. J. Soil Biol. 43: 110-115. https://doi.org/10.1016/j.ejsobi.2007.08.043

Keay, L., Wildi, B.S. 1970. Proteinases of the genus Bacillus. I. Neutral proteinases. Biotech. Bioeng. 12: 179-212. https://doi.org/10.1002/bit.260120205.

Kizilkaya, R., Dengiz, O. 2010. Variation of land use and land cover effects on some soil physico-chemical characteristics and soil enzyme activity. Zemdirbyste. 97(2): 15-24.

Konosu, S., Chen, Y.N., Hashimoto, Y. 1966. Constituents of the extracts of a marine worm, *Perinereis brevicirrus*. Bull. Jpn. Soc. Sci. Fish. 32(10): 881-886.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227(5259): 680-685.

Lavelle, P., Chauvel, A., Fragoso, C. 1995. Faunal activity in acid soils. In: Date R.A., Grundon N.J., Rayment G.E., Probert M.E. (editors). Plant-Soil Interactions at Low pH: Principles and Management. Developments in Plant and Soil Sciences, Springer, Dordrecht, pp. 201-211. https://doi.org/10.1007/978-94-011-0221-6_29

Li, C., Chen, M., Li, X., Yang, M., Wang, Y., Yang, X. 2017. Purification and function of two analgesic and anti-inflammatory peptides from coelomic fluid of the earthworm, Eisenia foetida. Peptides. 89: 71-81. https://doi.org/10.1016/j.peptides.2017.01.016.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

Miller, G. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31: 426-428. https://doi.org/10.1021/ac60147a030

Milochau, A., Lassègues, M., Valembois, P. 1997. Purification, characterization and activities of two hemolytic and antibacterial proteins from coelomic fluid of the annelid *Eisenia fetida andrei*. Biochim. Biophys. Acta (BBA)-Protein Struc. Mol. Enzym. 1337(1): 123-132. https://doi.org/10.1016/S0167-4838(96)00160-4

Mishra, P.K., Bisht, S.C., Jeevanandan, K., Kumar, S., Bisht, J.K., Bhatt, J.C. 2014. Synergistic effect of inoculating plant growth- promoting *Pseudomonas* spp. and *Rhizobium leguminosarum*-FB1 on growth and nutrient uptake of rajmash (*Phaseolus vulgaris* L.). Arch. Agron. Soil Sci. 60: 799-815. https://doi.org/10.1080/03650340.2013.843773

Msakni, S. 2020. Screening of cellulase enzyme production by *Bacillus* sp. isolated from Turkish soils, nutritional optimization, partial purification of enzyme, characterization and effectiveness of plant waste removal in wool fabric. MSc, Bursa Uludağ University, Bursa, Turkey.

Murugan, S., Umamaheswari, S. 2021. Identification of lysenin protein function in coelomic fluid of *Eudrilus Eugeniae*. Eur. J. Mol. Clin. Med. 8(3): 2021.

Nadana, G.R.V., Selvaraj, K., Sivakumar, P., Palanichelvam, K. 2020. Coelomic fluid of earthworms extruded by cold stress method has commercially significant compounds and trigger seed germination in *Vigna radiata* L. Environ. Technol. Innov. 19: 100814. https://doi.org/ 10.1016/j.eti.2020.100814

Ohresser, M., Matagne, R.F., Loppes, R. 1997. Expression of the arylsulfatase reporter gene under the control of the nit 1 promoter in *Chlamydomonas reinhardtii*, Curr. Gen. 31: 264-271. https://doi.org/10.1007/s002940050204

Patil, S.R., Biradar, P.M. 2017. Earthworm's coelomic fluid: extraction and importance. Int. J. Adv. Sci. Res. 2(2): 1-4.

Ramian, P., Arabi, M., Hemmati, R. 2018. Novel Amylase in Coelomic Fluid and Body Extract from the Earthworm Allolobophora Chlorotica. Biomacromol. J. 4(1): 35-45.

Robyt, J.F., White, B.J. 1987. Biochemical techniques, Theory and practice. Wadsworth. Inc., Belmont, California, USA.

Rochfort, S., Wyatt, M.A., Liebeke, M., Southam, A.D., Viant, M.R. et al. 2017. Aromatic metabolites from the coelomic fluid of Eisenia earthworm Species. Eur. J. Soil Biol. 78: 17-19. https://doi.org/10.1016/j.ejsobi.2016.11.008

Saitou, N., Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evo. 4(4): 406-425. https://doi.org/10.1093/oxfordjournals.molbev.a040454

Scherbert, I., Messner, B. 1997. Untersuchugen uber das vor kommenvon lysozyme bei anneliden. Zool. Jahr. Physiol. Biol. 76: 36-50.

Scheu, S. 2003. Effects of earthworms on plant growth: patterns and perspectives. Pedobiologia, 47(5): 846–856.

Sevgi, T., Demirkan, E. 2021. Evaluation of the effects of temperature, light, and UV-C radiation on HSP70A expression in *Chlamydomonas reinhardtii*. Turk. J. Bot. 45: 671-680. https://doi.org/10.3906/bot-2012-43

Sevinc, N., Demirkan, E. 2011. Production of protease by *Bacillus* sp. N-40 isolated from soil and its enzymatic properties. J. Biol. Environ. Scie. 5(14): 95-103.

Shen, Y. 2010. Earthworms in Traditional Chinese Medicine. Zoology in the Middle East, 51 (sup2): 171-173. https://doi.org/10.1080/09397140.2010.10638470.

Stahl, E. (Ed.). 1965. Thin-layer chromatography: a laboratory handbook. Springer Science and Business Media.

Sugihara, A., Tani, T., Tominaga, Y. 1991. Purification and characterization of a novel thermostable lipase from *Bacillus sp*. J. Biochem.109(2): 211-216.

Tsukamoto, K., Ariki, S., Nakazawa, M., Sakamoto, T., Ueda, M. 2021. Novel cold-adapted raw-starch digesting α-amylases from *Eisenia fetida*: Gene cloning, expression, and characterization. Biotechol. Rep. 31: e00662. https://doi.org/10.1016/j.btre.2021.e00662

Tutar, U., Karaman, İ. 2017. Investigation of antibacterial properties of mucus and coelomic fluid obtained from *Eisenia fetida*. Cumhuriyet Sci. J. 38(3):427-434. https://doi.org/10.17776/csj.340474

Valembois, P., Lassegues, M., Roch, P. 1992. Formation of brown bodies in the coelomic cavity of the earthworm *Eisenia foetida Andrei* and attendant changes in shape and adhesive capacity of constitutive cells. Develop. Comp. Immun. 16: 95-101. https://doi.org/10.1016/0145-305x(92)90010-a

Vasantha, N.C., Rajagopalan, K., Selvan Christyraj, J.D., Subbiahanadar Chelladurai, K., Ganesan, M. et al. 2019. Heat-inactivated coelomic fluid of the earthworm Perionyx excavatus is a possible alternative source for fetal bovine serum in animal cell culture. Biotechnol. Prog. 35(4): e2817. https://doi.org/10.1002/btpr.2817

Vasanthi, K., Chairman, K., Ranjit Singh, A.J.A. 2013. Antimicrobial activity of earthworm (*Eudrilus eugeniae*) paste. Afr. J. Environ. Sci. Technol. 7(8): 789-793. https://doi.org/10.5897/AJEST2013.1455

Wang, C., Sun, Z., Zheng, D. 2006. Research advance in antibacterial immunity ecology. J. Appl. Ecol. 17(3): 525-529.

Wang, C., Sun, Z.J., Liu, Y.Q., Zheng, D.M., Liu, X.L. et al. 2007. Earthworm polysaccharide and its antibacterial function on plant-pathogen microbes in vitro. Eur. J. Soil Biol. 43: 135-142. https://doi.org/10.1016/j.ejsobi.2007.08.035.

Yakkou, L., Houida, S., Dominguez, J., Raouane, M., Amghar, S. et al. 2021. Identification and characterization of microbial community in the coelomic fluid of earthworm (*Aporrectodea molleri*). Microbiol. Biotechno. Lett. 49(3): 391-402. https://doi.org/10.48022/mbl.2104.04013

Yamaji, A., Sekizawa, Y., Emoto, K., Sakuraba, H., Inoue, K., et al. 1998. Lysenin, a novel sphingomyelin-specific binding protein. J. Biol. Chem. 273: 5300-5306. https://doi.org/10.1074/jbc.273.9.5300

Yoo, Y.J., Hong, J., Hatch, R.T. 1987. Comparison of a-amylase activities from different assay methods. Biotechnol. Bioeng. 147-51. https://doi.org/10.1002/bit.260300120

Yousra, B. 2018. Diversity in the susceptibility of *Botrytis cinerea* strains to the biological control agent *Pseudomonas helmanticensis*. IOBC/WPRS Bull. 133:100-104.

Yuk, J., Simpson, M.J., Simpson, A.J. 2012. Coelomic fluid: a complimentary biological medium to assess sub-lethal endosulfan exposure using 1 H NMR-based earthworm metabolomics. Ecotoxicol. 21(5): 1301-1313. https://doi.org/10.1007/s10646-012-0884-5