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ENZYMES ACTIVITIES DURING VERMISTABILIZATION OF TOTAL PETROLEUM HYDROCARBON CONTAMINATED SOIL WITH VEGETABLE WASTES USING EARTHWORM, EUDRILUS EUGENIAE

Zoology

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ABSTRACT

Industrial sludge is hazardous and annoyance and inevitable end product of effluent treatment. Disposal of sludge without proper treatment cause severe pollution receiving aquatic system and disrupts agricultural environment. The inappropriate treatment methods lead more toxic to macro and micro fauna of soil ecosystem. From the above facts, the sludge must be adapted into balanced stabilization with an innovative, non-polluting, organic and cost effective biological remediation. The enzyme activities such as amylase, protease, cellulase and alkaline phosphatase of different vermicomposts were studied during vermistabilization of petroleum hydrocarbon contaminated soil with vegetable wastes and effective microorganisms. Standard techniques were used to analyze the enzyme activities in vermicomposts, and the results suggested that the enhanced enzymatic activities were seen over the experimental days. In 45% concentrations of total hydrocarbon petroleum contaminated soil, the activity levels of amylase and protease of vermicomposts reached their highest levels by the 60th day, which were 7.58±0.02 µg reducing sugar g-1 hr-1; 5.89±0.03 µ moles of amino acid g-1 hr-1; 864±60 µg reducing sugar g-1 hr-1; and 55±3.2 g PNG g−1 dwt h−1, respectively. From the results increasing level of enzyme activities showed when increasing experimental days. The enhanced effective microorganisms enrich the degradation of wastes as well as enrich nutrients availabilities in vermicomposts.

KEYWORDS

Total petroleum hydrocarbon, vegetable wastes, effective microorganisms, vermicomposting, enzyme activities

INTRODUCTION

Earthworms are essential to the physical and biological processes involved in the vermicomposting of waste materials. While the physical processes include air circulation, mixing, and mechanical grinding of the substrate, the biochemical technology is affect by the bacterial degradation of the materials in the gut of earthworms. Earthworms increase the surface area accessible for enzymatic activity by manually breaking down waste materials into smaller pieces. They also add mucus and turn it into liquid state. The biochemical breakdown of the material consumed is carried out by the endogenous enzymes produced in the earthworm stomach, or partly by external enzymes produced by intestinal biota, as well as by cast-dwelling flora and fauna after their excretions (Suthar, 2007). Although the biochemical breakdown of organic matter is mostly the responsibility of microorganisms, earthworms contribute significantly to this process by changing the materials with their digestive enzymes, enhancing the surface area available to bacterial activities (Dominguez, 2004).

Enzymes which break down complex chemical substances are secreted by bacteria and fungi, which then take up the simpler compounds and incorporate them into their cells. The earthworms and microorganisms which make up the vermicompost food chain release and recycle nutrients like potassium, phosphate, and nitrogen when organic matter breaks down (Aira et al., 2007). Actinomycetes are crucial to the breakdown of complex organic compounds including cellulose, lignin, chitin, and proteins during the vermicomposting process. According to Aira et al. (2007), microorganisms are not competing effectively for simple carbohydrate sources which are abundant during the early stages of composting. Because distinct extracellular enzymes produced by microbial decomposers break down substances containing C, N, or P, the relative allocation of resources to various enzymes may be tuned so that it targets the resource in the shortest amount of time.

Since enzymes are engaged in the biochemical transformation of both native and foreign substances in soils, they have been widely employed as an indication of the fertility of the soil and ecosystem site (Tate, 2000). Living or decaying cells, cell detritus, free enzymes, clay-adsorbed enzymes, and immobilized enzymes in humic complexes can all contain enzymes. These enzymes are responsible for the actions of enzymes (Nannipieri et al., 2002). Enzyme activities are correlated with the rates of breakdown of leaf litter, and the extracellular enzymes are the primary agents of plant tissue degradation (Benıtez et al., 2005). The main aim of the current study to use E. eugeniae to analyze the activity of enzymes including amylase, protease, and phosphatase in control and treated vermicomposts made from different combinations of soil polluted with petroleum hydrocarbons and vegetable wastes.

MATERIALS AND METHODS

Collection and Evaluation of Soil Contaminated with TPH

TPH leakage from moving cars and heating fuels near an oil refinery in Erode District, Tamil Nadu, India, polluted soil that was used for this investigation. The recovered contaminated soil was thoroughly mixed, dried for two hours at 150°C, then ground. According to accepted procedures, the samples were described in the lab (APHA, 2005).

Collection and Identification of Earthworm E. Eugeniae

The vermicomposting unit at Nasiyanur, Erode District, Tamil Nadu, India, is where earthworm E. eugeniae which was collected and further identified using Manual of earthworm identification (Julka, 2001). The earthworms were housed in clay pots with a capacity of 5 liters in the lab, which were filled with various mixtures of soil that had been polluted with total petroleum hydrocarbons and vegetable waste. Cow dung was moistened at a rate of 60 to 70% and provided as an extra carbon source. Similar-sized E. eugeniae were carefully chosen from the clay pots for further investigation.

Experimental Procedure

In a laboratory environment, the samples of polluted soil were allowed to air dried before being sieved through 2 mm screens. Subsequently, a cylindrical plastic container containing 500g of dirt was filled. The soil that had been polluted with TPH was fully mixed in at percentages of 15, 30, 45, 60 and 75%. There was also a control treatment (0%) that did not include TPH. Each pot in the incubation experiment was duplicated twice, for a total of 12 pots, in accordance with the fully randomized design of the study. Each container contained twenty-five fully grown earthworms. The pots were kept at 28±0.5°C in the dark for ninety days. The soil's moisture content was kept at 60% of its area of expertise capacity throughout the duration of the incubation phase. During the incubation phase, soil samples were taken and examined every month. The levels of amylase, protease, cellulose and alkaline phosphatase were measured.

Amylase Activity
1.0 g of regular compost/vermicompost was combined with 4 ml of starch dissolved in a 0.1 M pH 4.7 acetate buffer (1%) and allowed into incubate for 60 minutes at 27 °C. The solution was kept under the centrifuge at 4000xg for 15 min and filtered using filter paper (Whatman No. 1), with resulting filtrate tested to produce the total amount of reducing sugar through amylolytic activities (Miller, 1959). After the incubation period, starch solution was added to prepare control samples.

Protease Activity
The proteinase activity was determined as per the techniques given by McDonald and Chen (1965). The enzyme extract is prepared just as like as the extract preparation method for amylase activity, where in 20ml of sodium phosphate (0.05M) buffer of pH 6.0 is added instead of buffer pH 5.0.

Cellulase Activity
Cellulase activity is ascertained by adapting the method of Nokrans (1957). 1gm of intestinal tissue was used to make the enzyme extract and ground well in a frozen state, in a mortar along with 20ml dibasic sodium phosphate (1/15M). Throughout the process the constant temperature of 5°C is maintained using smashed icebergs.

Alkaline Phosphatase Activity
Alkaline phosphatase activity was assessed using the methodology outlined by Walter and Schutt (1974). Five milliliters of normal saline were used to homogenize 250 milligrams of fresh earthworm tissue that had been placed in a homogenizing tube. Following homogenization, the materials were centrifuged for 10 minutes at 28±2.0 rpm. A test tube containing 0.05 ml of supernatant was properly combined with 2 ml of alkaline buffer. Two milliliters of alkaline buffer were mixed with 0.05 milliliters of 0.7% saline to create a blank. For thirty minutes, the test tubes were maintained at 37°C in the incubator. Ten milliliters of 0.05 N NaOH solutions were added to the test tubes and thoroughly mixed following the incubation period.

The amount of liberated p-nitrophenol in tissue sample mixture gives an intense yellow colour that was measured spectrophotometrically at 405 nm wave length after adjusting the absorbance of the blank. A standard curve was drawn with the known quantity of paranitrophenyl phosphate in the same procedure and the values of liberated p-nitrophenol were determined from the standard curve. Protein concentration of each sample was quantified by the Lowry's method. A standard curve was drawn using BSA and the amount of protein in our earthworm tissue was calculated from the linear regression equation based on the standard curve (Lowry et al., 1951). The alkaline phosphatase activity was finally expressed in µg pnp/mg of protein/30 min.

Statistical Analysis
Two-way analyses of variance (ANOVA) were conducted by SPSS (version No.10) to evaluate the significance of differences among various soil samples in terms of their enzymatic properties.

RESULTS
Enzyme activity in general, is higher in vermicompost of E.eugeniae. The presence of earthworms significantly increased enzyme activity in PHC contaminated soil and vegetable wastes mixtures. The enzyme activity especially amylase, protease and alkaline phosphatase were significantly higher than the initial vermicompost. The changes observed in enzyme activities are presented in Tables 1 to 4 and Fig 1 to 4.

Amylase Activity
Table 1 and Fig.1 represent amylase activity in the vermicompost of E. eugeniae during the period of 60 days. The amylase activity was increased in all treatments and control from PHC contaminated soil and vegetable wastes mixtures. In E. eugeniae the amylase activity (µg reducing sugar g^{-1} hr^{-1}) increased from 0day to 60th day of experiments. In 45% and 60% concentrations of PTH the amylase activity was got high 7.02±0.04 µg reducing sugar g^{-1} hr^{-1} and 6.90±0.03µg reducing sugar g^{-1} hr^{-1} respectively). Lowest values were recorded in 75% followed by 30%, 15% and 0%.

The main function of amylases is to degrade the glycosidic bonds present in starch, converting complex carbohydrates into simpler sugars. Amylases degrade starch into minute components, finally producing maltose, and then break down into two molecules of glucose by maltase. Amylase (starch hydrolysis) activity in soil have been extensively examined because of their wide spread distribution in plants and soil and their role in the hydrolysis of carbohydrates. Amylase activity may also be increased by the addition of organic materials (Nannipieri et al., 1983). The significant increase in the amylase activity in all treatments and control was due to presence of higher organic content including sugar and higher amylolytic enzyme producing fungi and bacteria. These findings are supported by the findings of Vinotha et al. (2000) who has indicated that higher amylase activity in the fresh casts, particularly from pressmud, in both worms P.excavatus and E.eugeniae. In the present study the combinations of TPH and vegetable wastes were found highest enzyme activity was supported by Manimegala (2011) and Sarojini and Vrinda (2011) reported the vermicomposting of fly ash with various bedding material, the lower percentage of fly ash illustrate the maximum amylase activity.

Protease Activity
Table 2 and Fig. 2 show the results of protease activities during the vermicomposting of TPH contaminated soil with vegetable wastes in using earthworm species E. eugeniae. Maximum activities were found in 45% concentrations of TPH got 5.89±0.03 µ moles of amino acid g^{-1} hr^{-1}. Other concentrations showed the decreasing activities when increasing experimental days. The protease activity increased in all treatments, it may be due to substrate availability and microbial population and activity. These findings are supported by Brintha et al. (2015). In L. mauritii and P. excavatus are the potential species for rearing and bio-stabilization of poultry waste.

Cellulase Activity
Enzymes cellulases that decompose cellulose present in plant cell walls into simple sugars, which can be utilized as the basic ingredients for biofuels and several biobased chemicals, plastics, and other materials mentioned previously. During decomposition the complex form of cellulose is broken down to glucose and cellobiose by action of the enzyme cellulase. As a biological technique, composting includes many microorganisms and their composition and magnitude, are vital components of the composting techniques (Tiquia and Michel 2002). Devi et al. (2009) working on vegetable waste and cow dung reported similar trend in cellulase activity during vermicomposting. They further reported that after initial spurt for 30 days the enzyme activity significantly increased which was attributed to the increase in microbial population. In the present investigation, similar observation was recorded as the cellulase activity increased up to 60 days (Table 3 and Fig.3).

The enhanced level of cellulase and protease activities has been detected throughout the active phase of vermicomposting (Mondini et al., 2004). The microbial biomass is responsible for the biodegradation of organic materials, worm's fragmentation and conditions the substrates by rising surface area for microbial activity.

Phosphatase Activity
Table 4 and Fig 4 represent phosphatase activity in the vermicompost of E. eugeniae during the period of 60 days. The phosphatase activity was increased in all treatments and control from different PHC contaminated soil and vegetable wastes mixtures. Vermicompost of E. eugeniae shows the enrichment of phosphatase activity. Initially, the alkaline phosphatase activity was low (22±2.2μg PNG g^{-1} dwt h^{-1}). The alkaline phosphatase activity on 60th day increased to 55±3.2 μg PNG g^{-1} dwt h^{-1} in 45% concentration of TPH followed by 60%, 30%, 15% and 0%. Phosphatase acts as intermediary enzyme in the transformation of organic phosphorus into inorganic forms (Mansell et al., 1981). The phosphatase activity was high in all concentrations, but the 45 and 60% concentrations show highest activity, which could be due to the additional source of organic matter and nutrients supplied. Phosphatases are considered the main enzymes in phosphorus cycling in soil and indicate phosphorus mineralization power by the effective microorganisms.

The activity of alkaline phosphatase was determined to be significant during the trial, despite the fact that all treatments displayed a rising tendency. High enzyme activity is indicated by higher initial alkaline phosphatase activity in TPH-treated soils just after vegetable wastes containing beneficial bacteria are added. This finding is consistent with other research showing that vermicompost has a high alkaline phosphatase activity and that applying it increases the amount of phosphorus accessible in soils as well as the activity of this enzyme (Aira et al., 2008; Pramanik et al., 2007; Doan et al., 2013).

Additionally, a prior study (Ilker and Ismail, 2014) supports our data indicating a substantial negative connection between EC and alkaline phosphatase activities in soil containing vermicompost.

CONCLUSION

Because of the use of efficient microorganisms, the enzyme values demonstrated that the vegetable wastes were the most active vermicomposted material. Therefore, this waste considered to be ideal for soil application. Because of its high enzymatic activity, vermicompost made from household biowaste should be the most effective at accelerating the breakdown of petroleum hydrocarbons in the soil into a form that plants can use. The soil with the highest concentration of TPH contamination was the least active. Nevertheless, it can be said that the process of vermicomposting went smoothly in all variations and that the enzyme activity was enough throughout. Certain enzymes are positively impacted by the quantity of earthworms and beneficial microbes, according to the dependent courses that were discovered. Vermicomposting of vegetable waste with soil tainted by petroleum hydrocarbons and broken down by beneficial microorganisms is a low-maintenance, environmentally friendly, and nearly maintenance-free technology that helps produce cleaner products.

Table 1. Amylase (µg reducing sugar per gram) and Protease (µ moles of amino acid per gram) of different concentrations of total petroleum hydrocarbon contaminated soil (0, 15, 30, 45, 60 and 75%) treated with vegetable wastes (25%) and effective microorganisms using in E. eugeniae

TPH	Amylase			Protease		
	0 day	30 days	60 days	0 day	30 days	60 days
0%	6.02± 0.52	6.12± 0.03	6.35± 0.05	4.15± 0.02	4.29± 0.03	4.46± 0.02
15%	6.02± 0.52	6.46± 0.02	7.28± 0.03	4.15± 0.02	4.58± 0.03	4.98± 0.04
30%	6.02± 0.52	6.81± 0.06	7.45± 0.04	4.15± 0.02	4.65± 0.03	5.15± 0.02
45%	6.02± 0.52	7.02± 0.04	7.58± 0.02	4.15± 0.02	5.10± 0.02	5.89± 0.03
60%	6.02± 0.52	6.90± 0.03	7.52± 0.05	4.15± 0.02	5.02± 0.04	5.64± 0.03
75%	6.02± 0.52	6.85± 0.02	7.48± 0.03	4.15± 0.02	5.08± 0.02	5.35± 0.02
P Value	0.001987*			0.00000*		

Two-way ANOVA of amylase and protease activity
* Significance (P< 0.05)
** Insignificance (P>0.05)

Table 2. Cellulase (µg reducing sugar per gram) and alkaline phosphatase (μg PNG g^{-1} dwt h^{-1}) of different concentrations of total petroleum hydrocarbon contaminated soil (0, 15, 30, 45, 60 and 75%) treated with vegetable wastes (25%) and effective microorganisms using in E. eugeniae

TPH	Cellulase			Alkaline phosphatase		
	0 day	30 days	60 days	0 day	30 days	60 days
0%	315±20	429±33	446±37	22±2.2	32±2.6	35±3.5
15%	315±20	458±44	558±45	22±2.2	35±2.5	42±2.3
30%	315±20	465±46	615±40	22±2.2	32±2.2	46±3.4
45%	315±20	510±45	789±52	22±2.2	35±2.4	51±3.2
60%	315±20	502±54	864±60	22±2.2	38±2.3	54±3.5
75%	315±20	508±47	735±55	22±2.2	32±2.2	40±4.3
P Value	0.001987*			0.00000*		

Two-way ANOVA of cellulase and alkaline phosphatase activity
* Significance (P< 0.05)
** Insignificance (P>0.05)

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