World Journal of Zoology 6 (2): 142-148, 2011
ISSN 1817-3098

Screening Cellulolytic Bacteria from the Mid-Gut of the Popular Composting Earthworm, *Eudrilus eugeniae* (Kinberg)

[1]T. Shankar, [2]V. Mariappan and [2]L. Isaiarasu

[1]Department of Microbiology,
Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi-626124, India
[2]Department of Zoology,
Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi-626124, India

Abstract: Cellulose is the most abundant biopolymer in nature and constitutes a large pool of carbon source for the microorganisms responsible for the decomposition of organic matter in soil. Earthworms influence this decomposition by enhancing the structure and dynamics of the microbial population inside their gut as an efficient bioreactor. The assessment of cellulolytic activity in the microbes isolated from the mid-gut of the popular composting earthworm, *Eudrilus eugeniae* (Kinberg), revealed the bacterial community is responsible for the breakdown of cellulose and thereby decomposition of organic matter by this earthworm. The bacterial counts for viable microorganisms were made in the mid-gut section of *E. eugeniae* collected from vermicomposting unit in our college. The number of bacteria present in the mid gut identified by standard plate count technique was 5.2 x 10^7CFU/g. Based on the morphological characteristics of colony, 15 cultures (EWBC-A to EWBC-O) were raised. They were further screened for their cellulolytic activity in modified czapex-dox agar (sucrose replaced by carboxy methyl cellulose). Out of 15 cultures, only 9 cultures proved to be cellulolytic. Carboxy methyl cellulase assay was then performed to find out the most efficient cellulase producers. Accordingly, the culture designated as EWBC-M produced 0.1271 ± 0.002 IU/ml and the culture designated as EWBC-I produced 0.0345 ± 0.002 IU/ml of CMCase/ 0.3% substrate in 72hours at 37°C and at pH 6.

Key words: Bacterial enzyme · Cellulose · Carboxy methyl cellulase · Earthworm · *Eudrilus eugeniae* (Kinberg)

INTRODUCTION

Earthworms are one of the most important organisms among soil invertebrates owing to their beneficial effects on soil environment such as modification of soil physical properties and impact on decomposition of soil organic matter [1]. Major part of their beneficial effects on soil properties is attributed to their feeding activities and interactions with soil microorganisms [2]. There is increasing interest in how soil fauna shapes the composition of microbial communities by microbial grazing, disturbance and dispersal, thereby affecting decomposition and nutrient cycling. Earthworms thus represent an important portion of soil invertebrate biomass and in many ecosystem, earthworms are undoubtedly the key organisms in organic matter decomposition by modifying soil nutrient and microbial dynamics [3]. However, little is understood on the interactions between earthworm and soil microorganisms, including the gut microbial community. The present paper reported the carboxy methyl cellulase producing potential of the earthworm gut bacterial isolates which can be further optimized for the maximum cellulase production and it can be used for further industrial applications.

MATERIALS AND METHODS

Collection of Earthworm and Sampling Procedure: Earthworms (*Eudrilus eugeniae*) collected from the vermicomposting unit of our college, were washed with sterilized tap water and placed on sterile petriplate with moistened filter paper for 24 hours. They were then cleaned externally with 70% ethanol and dissected, weighed and homogenized for 5 minutes with a vortex mixture in sterile 0.85% NaCl solution.

Corresponding Author: T. Shankar, Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi-626124, India.
Mobile: +9952761350, E-mail: ewcellulase@gmail.com.

Isolation of Bacteria from Earthworm Gut: Isolation of bacteria was done by dilution plate method. For this, the mid gut of the earthworm was excised and the gut content (1g) was suspended in 10ml of sterile 0.85% NaCl solution, serially diluted (10^{-1} to10^{-7}). After serial dilution 0.1ml of solution was taken using sterile micropipette and plated on nutrient agar medium. The plates were then incubated at 28°C for 24 hours. Three replicates were maintained from each dilution.

Isolation of Cellulose Degrading Bacteria: The colonies were then isolated and streaked on Carboxy Methyl Cellulose (CMC) agar plates ($NaNo_3$-3.0g/L, K_2HPo_4-1.0g/L, $MgSo_4.7H_2O$-0.5g/L, KCl-0.5g/L, $FeSo_4.7H_2O$-0.01g/L, CMC-1.0g/L, Agar-2%, pH 7.0) and incubated for 24 hours at 30°C. The isolated colonies on these plates were maintained on CMC agar slants at 4°C for further analysis.

Screening of Cellulase Activity: The purified colonies were further screened for their cellulase activity. Pure cultures of bacterial colonies were transformed individually on CMC agar plates. After 72 hours of incubation, the plates were flooded with 1% congo red and the plates were allowed to stand for 20 minutes at room temperature. Then the plates were thoroughly washed with 1M NaCl solution. A clear zone formed around the growing colonies of cellulase positive cultures against the dark red background was taken as the indication of cellulase activity. The contrast was further enhanced by treating the plates with 5% acetic acid for 1 to 2 minutes and then washed off the excess acid with distilled water. The bacteria that showed good clearance beyond the area of growth were then selected for further studies as potential cellulase secretors. The isolated bacterial cultures were further screened for their extracellular cellulase (endoglucanase) enzyme production. The isolated bacterial colonies were further characterized for their morphological and biochemical characters by following standard keys of Bergey's Manual of Determinative Bacteriology.

Determination of Endoglucanase Activity: The supernatant of the culture broth centrifuged at 5000 rpm for 20 minutes at 4°C served as the enzyme source. This enzyme solution 0.5 ml was added to 0.5 ml of 1% substrate (CMC) taken in 0.2 M Citrate Phosphate buffer (pH-7) and incubated at 45°C for 30 minutes. The reaction was stopped by the addition of 2 ml dinitrosalicylicacid reagent by keeping for 5 minutes in boiling water bath and quick cooling to room temperature. The degree of enzymatic hydrolysis of the cellulase was determined spectrophotometrically (UV-Vis Spectrophotometer, Systronics, India) by measuring the absorbance at 540 nm. Endoglucanase was assayed as described by Ghose [4]. The enzyme activity was expressed in units as the amount of enzyme required to release 1µ mol of reducing sugar as glucose equivalent min/g of the enzyme sample.

$IU\ ml^{-2}$ = Concentration of the glucose / 0.18 x 0.5 x 30

Genomic DNA Extraction, Cloning and Sequencing of 16s rRNA Gene: The isolated bacterial strain was grown in 2ml nutrient broth overnight at 37°C. The culture was spun at 7000 rpm for 3 min. The pellet was resuspended in 400 µl of sucrose TE. Lysozyme was added to a final concentration of 8 mg/ml and incubated for 1h at 37°C. To this tube, 100 µl of 0.5M EDTA (pH 8.0), 60 µl of 10% SDS and 3 µl of proteinase K from 20 mg/ml were added and incubated at 55°C overnight. The supernatant was extracted twice with phenol: chloroform (1:1) and once with chloroform: isoamylalcohol (24:1) and ethanol precipitated. The DNA pellet was resuspended in sterile distilled water. The amplified product (1,500-bp) was purified using GFX ™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences) according to manufacturer's instruction. The 16S rDNA amplicon was cloned in pTZ57R/T vector according to the manufacturer's instruction (InsT/Aclone™ PCR Product Cloning Kit #K1214, MBI Fermentas). Full length sequencing of the rRNA gene (about 1500 bp) for the isolated bacteria was carried out in Royal life science (Hyderabad). The full-length sequences obtained were matched with previously published sequences available in NCBI using BLAST.

RESULT AND DISCUSSION

Bacteria Enumerated from Earthworm Gut: The number of cultivable aerobic bacteria present in the mid gut of earthworm, *Eudrilus eugeniae* (Kinberg), as identified by standard plate count technique was 5.2 x 10^7CFU/g (Fig. 1).

Cellulase Producing Bacterial Strains: Over the last forty years, research on the degradation of cellulose and lignocellulosic biomass by microorganisms has received a considerable amount of attention. The potential use of cellulose as a renewable resource for the production of important industrial chemicals such as glucose, alcohol, solvents and goods such as paper, rayon and cellophane has increased attraction.

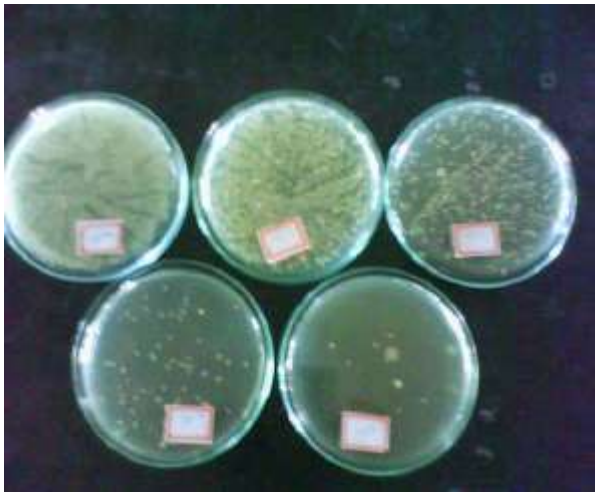

Fig. 1: Earthworm gut isolates in nutrient agar

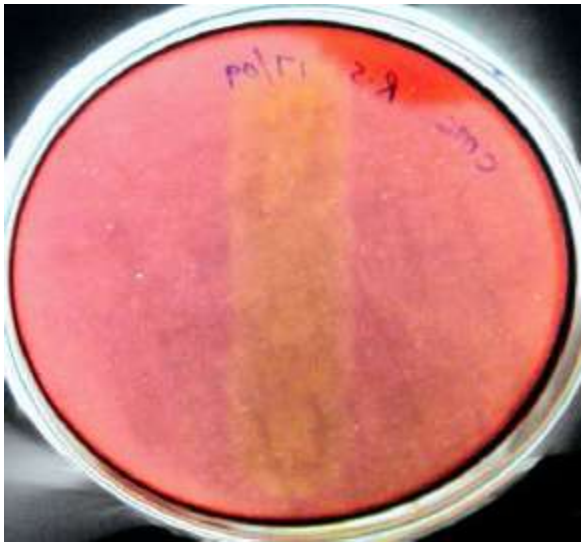

Fig. 2: Clearing of cellulose by EWBC-M strain in CMC agar plate

Cellulases have been utilized for the preparation of plant protoplast in genetic research and improvement of nutritional values of animal feed and as microbial proteins (SCP) remains a very attractive prospect in the long term [5]. In the present study, 15 different colonies were selected based on colony morphology and they were screened for their cellulase producing ability. Of these, 9 strains namely EWBC-C, EWBC-E, EWBC-F, EWBC-H, EWBC-I, EWBC-K, EWBC-M, EWBC-N and EWBC-O showed positive reactions indicated by a clearing zone of more than 10 mm in diameter (Fig. 2). Attempts to increase the production of enzyme cellulase from bacteria include several processes like mutation [6]; protoplast fission [7]; optimization of medium composition and environmental factors [8]. The bioconversion of various complex

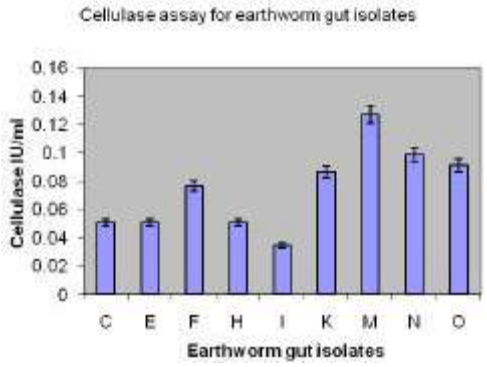

Fig. 3: Cellulase activity of earthworm gut isolates after 72 hours

Fig. 4: EWBC-M strainGram's reaction

cellulosic waste materials such as bagasse [9], saw dust [10], corncob [11] and coir retting effluents [12], have been reported.

Quantification of Cellulase Activity: The assay for endoglucanase activity is based on the ability of the cellulase enzyme produced by the strains to hydrolyze CMC to reducing sugars which could be measured using 3,5-dinitrosalicyclic acid (DNS) is shown in Fig. 3. The activities of the nine strains, screened as cellulase producers in the preliminary screening. At 72hours of incubation, EWBC-M exhibited the highest cellulase activity (0.1271 ± 0.002 IU/ml), which is significantly different from the rest of the strains. The next best cellulase activities were exhibited by EWBC-N and EWBC-O with 0.0987 ± 0.002 IU/ml and 0.0912 ± 0.002 IU/ml, respectively, which are significantly different. The minimum cellulase activity was exhibited by EWBC-I with 0.0345 ± 0.002 IU/ml. Maximum amount of enzyme production was observed in strain EWBC-M (Fig. 4). Hence it was used for further cellulase enzyme optimization, characterization and production. Cellulase yields appear to depend on a complex relationship involving a variety of factors like inoculum size (carbon source and cellulose quality), pH value, temperature,

Table 1: Growth and substrate utilization characteristics of B. pumilus.

Characteristic	B. pumilus phenotype	Characteristic	B. pumilus phenotype
Amylase	+	Substrate utilization	
Protease	+	D-Glucose	+
Lipase	+	L-Arabinose	+
Phosphatase	-	D-Xylose	+
DNase	-	D-Mannitol	+
Gelatinase	-	Galactose	+
Chitinase	-	Fructose	+
Growth temperature	5-50°C	Mannose	+
Growth ph	11-May	Nitrate	-
Nacl tolerance	10%	Adonitol	-
Oxidase	+	Dulcitol	-
Catalase	+	Sorbitol	-
Indole production	-	Inositol	-
Voges-Proskauer	+	Urea	-
Citrate utilization	-		

presence of inducers, medium additives, aeration and growth time etc. The particle size of cellulose can affect cellulase production by microorganisms [13].

Characterization of the Cellulase Producing Bacteria: In the present study, based on the morphological, physiological and biochemical characteristics of the suspected colony was identified as *Bacillus pumilus* EWBCM1 by the following standard keys of Bergey's Manual of Determinative Bacteriology (Table 1) and phylogenetic studies revealed that the 16S rRNA gene sequence of the strain *Bacillus pumilus* EWBCM1has 100% similarity with the nearest match in the Genbank.

Cellulose is probably the most abundant biological compound on terrestrial and aquatic ecosystem. It is the dominant waste material from agricultural industry in the form of stalks, stems and husk and is one of the most abundant renewable sources. By means of chemical or bioconversion methods, it is possible to transform this insoluble polymer into glucose, an excellent substrate for industrial fermentation. Mainly bacteria, fungi and actinomycetes achieve bioconversion of these materials. Several studies were carried out to produce cellulolytic enzymes in biowaste degradation process by several microorganisms including fungi such as *Trichoderma sp*. *Penicillium sp*. *Aspergillus sp*. respectively by Lakshmikant and Mathur [14]. Similarly cellulolytic property of bacterial species like *Pseudomonas*, *Cellulomonas*, *Cellulovibrio* and *Sporocytophaga sp*. was also reported Nakamura and Kappamura [15]. The specific cellulolytic activity shown by the bacterial species was reported to depend on the source of occurrence. The cellulase system of the mesophilic cellulolytic aerobe, *Cellulomonas fimi* is one of the first studied and has since been one of the most studied bacterial cellulase systems. Many spore forming bacteria have been isolated from factors that have a feed stock from cattle waste [16], cow manure [17], woody biomass [18] and municipal solid waste. Similarly many strains of cellulolytic anaerobic bacteria have been reported from various sources as human colon [19], estuarine sediments [20], fresh water sediments [21] and decomposing vegetation [22]. Because of the common occurrence of these bacteria in various natural environments, they are responsible for vast amount of cellulose degradation.

Reports of cellulolytic activity in the gut of some species of earthworms [23-26], especially in epigeic earthworms such as *Eisenia fetida* [27], indicate their ability to digest cellulose, although the effects exerted by earthworms on cellulolysis lie fundamentally in their interactions with microorganisms. These interactions are the subject of a certain amount of controversy, mainly because of the variety of species, substrate and experimental conditions assayed. It is generally agreed that microorganisms, especially fungi, are part of the diet of earthworms [3]; moreover, earthworms have been shown to graze selectively on fungal species [28]. Although earthworms can digest fungi and bacteria, an increase in the number of microorganisms during gut transit has also been reported [29].

Vermicomposting involves the biooxidation and stabilization of organic matter through the joint action of earthworms and microorganisms. The transformations in physicochemical and biochemical properties [3] and the short time in which they occur make them a suitable system for studying microbe-earthworm interactions.

The actions of earthworms during vermicomposting include not only digestion and release of easily assimilable substances, such as mucus for microbiota [30], but also the transport and dispersal of microorganisms through casting. Earthworm casts play an important role in decomposition because they have a different nutrient and microbiota composition to the material prior to ingestion, which makes possible a better exploitation of resources because of either the appearance of microbial species in fresh substrate or the pool of easily assimilable compounds of casts [3].

Degradation of cellulose in soils is a slow process that is limited by several factors involving cellulases, such as concentration, location and mobility of the enzymes [31]. Moreover, production of cellulases is regulated by the speed of accumulation of products [32]. Hemicelluloses and lignin content and the degree of crystallinity of cellulose itself also determine the rate at which cellulose is metabolized [33]. Decomposition of lignocellulosic residues is directly mediated by extracellular enzymes; therefore, analysis of the dynamic involved may clarify the mechanisms relating the rate of decomposition with substrate quality and nutrient availability [34].

Cellulose is the largest component of plant residues that enters terrestrial ecosystems [35] and therefore represents a huge source of energy for microorganisms, the main agents responsible for soil organic matter decomposition [36]. In nature, cellulolysis occurs as a result of the combined action of fungi and bacteria with different substrate requirements that shift their biomasses depending on what substrate is being metabolized [37]. The type of microorganisms involved depends on the environmental conditions; under aerobic conditions, they are mainly fungi, actinomycetes and bacteria and under anaerobic conditions, they are almost exclusively bacteria [33].

CONCLUSION

The data gathered in this study provides evidence for cellulase producing ability of the earthworm gut bacterial isolates. The production of cellulase and cellulase-lignocellulosic substrate interactions of bacterial strains in the earthworm gut was also evident in this study. This study gives us a hint that the microbial wealth of cellulase producing bacteria isolated from the earthworm gut can be harnessed for biotechnological processes.

ACKNOWLEDGEMENT

The facilities provided to us in the Department of Microbiology and Zoology in our college to carry out this study is gratefully acknowledged.

REFERENCES

1. Lee, K.E., 1992. Some trends and opportunities in earthworm research or: Darwins children- the future of our discipline. Soil. Biol. Biochem. 24: 1765-1771.
2. Edwards, C.A. and K.E. Fletcher, 1988. Interactions between earthworms and microorganisms in organic-matter breakdown. Agric. Ecosyst. Environ. 24: 235-247.
3. Dominguez, J., 2004. State of the art and new perspectives in vermicomposting research. In: Edwards, CA (Ed.), Earthworm Ecology. 2nd ed. CRC Press, Boca Raton. pp: 401-425.
4. Ghose, T.K., 1987. Measurement of cellulose activities. Pure &nAppl. Chem., 59: 257-268.
5. Kluepfel, K., F. Shareck, F. Mondou and R. Morosoli, 1986. Characterization of cellulase and xylanase activities of *Streptomyces lividans.* Appl Microbiol Biotechnol., 24: 230-234.
6. Haggett, K.D., W.Y. Choi and N.W. Dunn, 1978. Mutants of *Cellulomonas*, which produce increased levels of β-glucosidase. Europ. J. Appl. Microbiol. Biotechnol., 6: 189-191.
7. Gokhale, D.V., E. Soo-Han, V.R. Srinivasan and D.N. Deobagkar, 1984. Transfer of DNA coding for cellulases from *Cellulomonas sp*. To *Bacillus subtilis* by protoplast fusion. Biotechnol. Lett., 6: 627-632.
8. Prasetsan, P. and H.W. Doelle, 1987. Nutrient optimization for cellulase biosynthesis by a newly isolated *Cellulomonas sp*. Mircen. J., 3: 33-44.
9. Kansoh, A.L., S.A. Essam and A.N. Zeinat, 1999. Biodegradation and utilization of bagasse with *Trichoderma reesei*. Polym. Degrad. Stab., 62: 273-278.
10. Solomon, B.O., B. Amigun, E. Betiku, T. Ojumu and S.K. Layokun, 1999. Optimization of Cellulase production by *Aspergillus flavus* Linn isolate NSPR101grown on Bagasse. JNSChE. 16: 61-68.
11. Ojumu, T., V. Solomon, O. Bamidele, E. Betiku, S.K. Layokun and B. Amigun, 2003. Cellulase production by *Aspergillus flavus* Linn isolate NSPR101fermented in sawdust, bagasse and corncob. African J. Biotechnol., 2(6): 150-152.

12. Immanuel, G., R. Dhanusha, P. Prema and A. Palavesam, 2006. Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment. Int. J. Environ. Sci. and Technol., 3(1): 25-34.
13. Greaves, H., 1971. The effect of substrate availability on cellulolytic enzyme production by selected wood rotting microorganisms. Australian J. Bio. Sci., 24: 1167-1180.
14. Lakshmikant, K. and S.N. Mathur, 1990. Cellulolytic activities of *Cheatomium globosum* on different cellulosic substrate. W.J. Microbiol. Biotech., 11: 23-26.
15. Nakamura, K. and K. Kppamura, 1982. Isolation and identification of crystalline cellulose hydrolyzing bacterium and its enzymatic properties. J. Ferment. Technol., 60(4): 343-348.
16. Sharma, V.K. and P.N. Hobson, 1985. Isolation and cellulolytic activities of bacteria from a cattle waste anaerobic digester and the properties of some *Clostridium* species. Agri. Was., 14: 173-196.
17. Palop, M.L., S. Valles, F. Pinaga and A. Flors, 1989. Isolation and characterization of an anaerobic, cellulolytic bacterium, *Clostridium celerecrescens*. Int. J. Sys. Bacteriol., 39: 68-71.
18. Sleat, R. and R.A. Mah, 1984. *Clostridium populeti sp. Novo*. A cellulolytic species from a woody- biomass digester. J. Sys. Bacteriol., 35: 160-163.
19. Skinner, F.A., 1960. The isolation of anaerobic cellulose decomposing bacteria from soil. J. Gen. Microbiol., 22: 539-554.
20. Madden, R.H., M.J. Bryder and N.J. Poole, 1982. Isolation and characterization of an anaerobic, cellulolytic bacterium *Clostridium papoyrosolvens* sp. Nov. a cellulolytic thermophile. Int. J. Sys. Bacter., 32: 87-91.
21. Leschine, S.B. and E. Canale, 1983. Mesophilic cellulolytic Clostridia from fresh water environments. Appl. Environ. Microbiol., 46: 728-737.
22. Petidemange, E. F. Caillet, J. Giallo and C. Caudin, 1984. *Clostridium cellulolyticum* sp. Nova, a cellulolytic mesophilic species from decayed grass. Int. J. Sys. Bacteriol., 34: 155-159.
23. Urbasek, F. and V. Pizl, 1991. Activity of digestive enzymes in the gut of five earthworm species (Oligochaeta: Lumbricidae). Rev. Ecol. Biol. Sol., 28: 461-468.
24. Lattaud, C., S. Locati, P. Mora and C. Rouland, 1997a. Origin and activities of glycolytic enzymes in the gut of the tropical geophagous earthworm *Millsonia anomala* from Lamto (Cote d'lvoire). Pedobiologia. 41: 242-251.
25. Zhang, B.G., C. Rouland, C. Lattaud and P. Lavelle, 1993. Activity and origin of digestive enzymes in the gut of tropical earthworm *Pontoscolex corethurus*. Eur. J. Soil. Biol., 29: 7-11.
26. Lattaud, C., B.G. Zhang, S. Locati, C. Rouland and P. Lavelle, 1997b. Activities of the digestive enzymes in the gut and in tissue culture of a tropical geophagus earthworm, Polipheretima elongate (Megascolecidae). Soil. Biol. Biochem., 29: 335-339.
27. Zhang, B.G., G.T. Li, T.S. Shen, J.K. Wang, Z. Sun, 2000. Changes in microbial biomass C, N and P and enzymes activities in soil incubated with the earthworms *Metaphire guillelmi* or *Eisenia fetida*. Soil. Biol. Biochem., 32: 2055-2062.
28. Moody, S.A., M.J.I. Briones, T.G. Pierce and J. Dighton, 1995. Selective consumption of decomposing wheat straw by earthworms. Soil Biol Biochem., 27: 1209-1213.
29. Schonholzer, F., D. Hahn and J. Zeyer, 1999. Origin and fate of fungi and bacteria in the gut of *Lumbricus terrestris L.* studied by image analysis. FEMS Microbiol. Ecol., 28*:* 235-248.
30. Brown, G.G. and B.M. Doube, 2004. Functional interactions between earthworms, microorganisms, organic matter and plants. In: Edwards, C.A. (Ed.), *Earthworm Ecology*, 2nd ed. CRC Press, Boca Raton. pp: 213-224.
31. Sinsabaugh, R.L. and A.E. Linkins, 1988. Adsorption of cellulase components by leaf litter. Soil Biol Biochem., 20: 927-931.
32. Goyal, A. and B. Ghosh, D. Eveleigh, 1991. Characteristics of fungal cellulases. Bioresour. Technol., 36: 37-50.
33. Lynd, L.R., P.J. Weimer, W.H. Van Zyl. and I.S. Pretorius, 2002 Microbial cellulose utilization: fundamentals and biotechnology. Microbiol. Mol. Biol. Rev., 66: 506-577.

34. Sinsabaugh, R.L. and A.E. Linkins, 1993. Statistical modeling of litter decomposition from integrated cellulose activity. Ecol., 74: 1594-1597.
35. Richmond, P.A., 1991. Occurrence and functions of native cellulose. In: Haigler, CH, Weimer, JP (Eds.) Biosynthesis and Biodegradation of cellulose. Dekker, New York, pp: 5-23.
36. Lavelle, P. and A.V. Spain, 2001. Soil Ecology. Kluwer Academic Publishers, London. pp: 52-96.
37. Hu, S. and A.H.C. Van Bruggen, 1997. Microbial dynamics associated with multiphasic decomposition of 14C-labelled cellulose in soil. Microb. Ecol., 33: 134-143.