International Journal of Sciences & Applied Research

www.ijsar.in

Study of Cellulase activity in *Eisenia foetida* used in the degradation of paper waste

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Abstract

Introduction: Paper is one of the organic waste posing major environmental and disposal problems. Cellulose is the major constituent of paper and cellulase being the key enzyme involved in the degradation of paper is of due importance.

Aim: In this view, the effect of vermicomposting paper waste is dealt in the present study with cellulase activity and its quest of origin being observed with prime significance.

Materials and Methods: The experiments were conducted in triplicates for each treatment taken, (T1) – newspaper waste and cow dung, (T2) – written paper and cow dung. The epigeic earthworm species, *Eisenia foetida* is used in the study. The earthworms were bred in both the treatments and their controls were devoid of worms. This setup was monitored over a period of 60 days.

Results and Conclusion: The study reveals a peak increase in the cellulase activity on the 45^{th} day of the experimental period in T1. Further, the *Eisenia foetida* gut isolate on the 60^{th} day was found to be of the *Bacillus* genera in both the treatments. The phylogenetic analysis of the retrieved cellulase gene of *Eisenia foetida*, *Bacillus subtilis* and *Bacillus pumilus* disclosed a close relation of *Bacillus pumilus* to *Eisenia foetida* for cellulase gene leading to an inference of horizontal gene transfer (HGT) that would have occurred between the prokaryote's (Bacteria) and eukaryote's (Earthworm) genome during the course of evolution. Thus, vermicomposting using *Eisenia foetida* can be regarded as an alternative method of degrading the cellulosic paper waste.

Keywords: Vermicompost, *Eisenia foetida*, Paper waste, Cellulase, *Bacillus pumilus*, *Bacillus subtilis*, Horizontal Gene Transfer, Phylogenetic analysis

Introduction

Paper pervades all sectors of our activity from book to bullets and from morning newspaper to nuclear technology. From time immemorial, paper has played a key role in the evolution of our civilization. Moreover, the need for documentation of knowledge and record keeping has long been perceived to be linked to the intellectual prowess of a nation. But this paper is also a cause for concern when it reaches the land-fills, incinerators, and sometimes the ocean after use, leading to the pollution of the environment.

Paper is made up of cellulosic fibre sourced from plants. Cellulose is the most abundantly produced homopolymer in terrestrial environments, consisting of glucose units joined by β– 1, 4 bonds. Due to this molecular complexity, the degradation of cellulose is a slow process that is limited by several factors involving cellulases, such as concentration, location and mobility of the enzymes (Sinsabaugh and Linkins, 1988). Cellulolysis occurs as a result of the combined action of fungi and bacteria with different substrate requirement that shift their biomass depending on what substrate is being metabolized (Aira *et al*., 2007). Under aerobic conditions they are mainly fungi, bacteria and actinomycetes; and under anaerobic conditions, they are mostly bacteria (Richmond, 1991).

Earthworms play an important role in the degradation of substrate indirectly by affecting microbial population structure and dynamics and also directly, simply because their gut is capable of undertaking cellulolytic activity. Thus, products of cellulose hydrolysis are available as carbon and energy sources for other microbes that inhabit the environment in which cellulose is biodegraded, and this availability forms the basis of many biological interactions which involves stimulation or deactivation of the microbial populations. Not surprisingly, over the years a wide range of equally varied cellulose degrading microbial communities have evolved.

Henceforth, vermicomposting is one of the many ways to reduce this organic waste (paper) of cellulosic origin. *Vermes* is Latin word for worms and vermicomposting is essentially composting with worms (Ghatnekar *et al*., 1998). Elucidations as to whether the cellulose activity exhibited by the earthworm itself was partly because of some genes being transferred and incorporated in the earthworm's genome are from microorganisms' during the course of evolution needs to be answered. This transfer of genome is referred to as the horizontal gene transfer (HGT).

A significant correlation between cellulase and microflora of earthworm gut were also recorded by Kumar *et al*., (2010). Several approaches to validating the HGT (horizontal gene transfer) interferences and benchmarking methods have been adopted, in which the phylogenetic method appears to be the preferred standard of proof for HGT (Mark *et al*., 2006; Kechris *et al*., 2006). Phylogenetic method lends them the ability to better characterize the HGT events inferred, notably by designating the donor species and the time of transfer (Ravenhall *et al*., 2015). A cladogram or a phylogram are the representative forms of the phylogenetic tree reconstruction. Biased biological functions of horizontally transferred genes in prokaryotic genomes were tested by Yoji *et al*., (2004). The reports on plant-parasitic nematodes and the higher termite *Nasutitermestakasa goensis* have cohered entire genomic structure of their cellulase gene (Aira *et al*., 2006; Giritch *et al*., 2006).

Therefore, a study providing information on the subject covering various aspects viz., cellulosic waste degradation solely by earthworm gut activity, or by interaction among the members of cellulose decomposing microbial communities with earthworm individual as well as in combination and the origin of cellulase, is focused.

Materials and methods

Collection of earthworm and organic wastes

The earthworm species, *Eisenia foetida* was obtained from the vermicompost pit of Nirmala College for Women (Autonomous), Coimbatore. The species was identified in the college by the Department of Zoology. The two different types of paper waste generated from the college campus were collected and segregated as newspaper waste and written paper waste. These papers were shredded manually. The cow dung was obtained from a local cowshed and was sun dried and flaked.

Experimental design

The experiment was conducted in square plastic pots measuring 17 x 17 x 17 cm of length, breadth and height respectively. Holes were drilled at the bottom of the pots so as to drain excess water. The pots were filled bottom up with successive layers of pebbles, coconut husk, cow dung flakes and shredded papers respectively. The paper waste was mixed with cow dung flakes in the ratio of 1:1. All pots were maintained in triplicates. Water was sprinkled daily on all pots to maintain the moisture content and turned at regular intervals for proper mixing and aeration. The experimental pots were kept under shade and covered with gunny bags to prevent moisture loss. This setup was maintained for 15 days for partial degradation and stabilization. After 15 days, 20 non-clitellated earthworms were introduced into each treatment pots containing newspaper waste (T1) and written paper waste (T2). The control pots of newspaper waste (C1) and written paper waste (C2) were devoid of earthworms. This setup was also sprinkled with water daily and was monitored for a period of 60 days.On the 15^{th}, 30^{th}, 45^{th} and 60^{th} day of the experimental period, the earthworms were carefully removed and the samples of compost and vermicompost from all experimental units were collected and used to determine the cellulase activity. On the 60^{th} day of the experimental period, the earthworms from the treatment pots were carefully removed to examine its gut isolate bacteria and the information is used further for phylogenetic analysis.

Cellulase activity

Cellulase activity in all compost and vermicompost samples was determined by the dinitrosalicylic (DNS) acid method (Miller, 1959).

Statistical analysis

Using SPSS 16.0 package, Two way ANOVA was determined to analyze significant differences ($P < 0.05$) between the composted and vermicomposted samples for Cellulase activity.

Isolation and Identification of Bacteria from Earthworm Gut

Isolation of bacteria was done by dilution plate method (Shankar *et al*., 2011) and identified using its macroscopic and cultural characteristics.

Phylogenetic analysis

The cellulase gene for *Eisenia foetida* and the identified bacterial genera (*Bacillus*) was retrieved from GenBank. The *Bacillus spp*., for which complete CDS (cDNA Sequence) for cellulase were available in GenBank, alone were retrieved for further analysis (Benson *et al.,* 2005). To infer horizontal gene transfer event, phylogenetic tree was constructed using phylogeny.fr web interface (Dereeper *et al.*, 2008). "One click mode" was selected to perform the phylogenetic analysis using the following procedure:

- Multiple Sequence Alignment using 'MUSCLE' module
- Curation of the Alignment using 'Gblocks' module
- Phylogenetic tree construction using 'PhyML' module
- Tree rendering using 'TreeDyn' module

Results and discussion

Cellulase Activity

The highest activity was recorded on the 45^{th} day of the experimental period for T1 (993.33 ± 11.55) followed by T2 (932.00 ± 34.70) which were significantly different ($P<0.05$) from their control C1 (829.33 ± 11.37) and C2 (788.67 ± 25.01) respectively (Table 1). The 60^{th} day samples (C1 – 900.00 ± 10.00, T1 – 900.00 ± 6.00, C2 – 859.33 ± 9.87 and T2 – 861.33 ± 18.15) exhibited the second best activity. A markedly significant difference between the treatment pots (T1 – 573.33 ± 7.57 and T2 – 505.33 ± 5.03) was observed on the 30^{th} day of analysis.

Table 1: Cellulase activity of the composted and vermicomposted samples of different paper waste (µg reducing sugar/ $g^{-1}hr^{-1}$).

Sample	Days			
	15	30	45	60
C1	245.33 ± 5.03*	470.00 ± 11.14*	829.33 ± 11.37*	900.00 ± 10.00*
T1	308.00 ± 8.00*	573.33 ± 7.57*	993.33 ± 11.55*	900.00 ± 6.00*
C2	239.33 ± 13.32*	416.67 ± 3.06*	788.67 ± 25.01*	859.33 ± 9.87*
T2	286.00 ± 8.00*	505.33 ± 5.03*	932.00 ± 34.70*	861.33 ± 18.15*
SEd	31.94374			
CD(P<0.05)	65.06900			

Values are Mean ± Standard Deviation of three samples in each group; SEd– Standard Error of the Difference; CD – Critical Difference;* - Significant at P < 0.05 level;C1 – Newspaper waste, T1 – Newspaper waste + Earthworm, C2 – Written paper waste, T2 – Written paper waste + Earthworm.

Production of cellulases is regulated by the speed of accumulation of products (Goyal *et al*., 1991). Hemicelluloses and lignin content and the degree of crystallinity of cellulose itself also determine the rate at which cellulose is metabolized (Lynd *et al*., 2002). Earthworms voraciously feed on organic wastes utilizing only a small portion for their body metabolic activities and excrete a large part of the consumed materials in a half digested form (Edward and Lofty, 1977; Kale and Bano, 1986). The half-digested material decompose rapidly and is transformed into vermicompost within a short time since the intestines of earthworms harbor wide range of microorganisms, enzymes, hormones etc. The feeding activity of the earthworms and their voiding of microbial and enzymatically enriched casts could have also contributed to higher enzyme activities of vermicompost (Devi *et al*., 2009).

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 (Hu and Bruggen, 1997). 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 (Lynd *et al*., 2002). Reports of cellulolytic activity in the gut of some species of earthworms (Urbasek *et al*., 1991; Lattaud *et al*., 1997; Zhang *et al*., 1993), especially in epigeic earthworms such as *Eisenia foetida* (Zhang *et al*., 2000), 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 (Shankar *et al*., 2011).

Isolation and Identification of Earthworm Gut Bacteria

After subjecting the earthworm to vermicomposting on different paper waste, the bacteria present in the gut of *Eisenia foetida* was isolated and identified on the 60^{th} day, predominantly belongs to the *Bacillus* genera for both the treatments T1 and T2.

There are similar studies where *Bacillus* has been reported to be isolated from the gut of *Eisenia foetida* (Jyotsana *et al*., 2010) and had also emphasized that these gut associated microflora assists the earthworms significantly to hasten the decomposition of organic matter by producing certain enzymes namely cellulase, amylase, protease etc. Although dependent upon earthworm species, it is known that earthworms interact with microorganisms (fungi, bacteria and actinomycetes) on three broad spatial scales - burrow linings, casts and earthworm gut or intestine (Brown and Doube, 2004). Higher

microbial numbers, diversity and activity are also known to be related to passage of microorganisms through the earthworm gut, as well as the promotion therein, and the 'awakening' of dormant gut flora (Brown *et al*., 2000); this is, however, very much related to gut passage time (Brown and Doube, 2004). Importantly, the increased gut associated microflora are then excreted throughout the media within earthworm casts and via microbial adherence to earthworm skin whilst the transit and dispersal mechanisms associated with the water flow also help to further dissipate microorganisms (Edwards and Bohlen, 1996). This is not only due to the aforementioned intestinal promotion of microorganisms but also due to the inherently high organic matter levels, resulting in further microbial activity and proliferation (Brown and Doube, 2004).

Dynamics and succession of microorganisms within earthworm casts is complex, and relies heavily upon many factors such as type of food ingested, gut passage time and ingested and inherent gut microorganisms. It has also been noted that water soluble (Edwards and Bohlen, 1996) low molecular weight (Barrois and Lavelle, 1986; Lavelle *et al*., 1993) organic compounds are added to earthworm gut contents during digestion, such as enzymatic fluid and mucus based solutions (Brown and Doube, 2004), which also subsequently stimulate microbial activity both in the gut and in the egested casts (Edwards and Bohlen, 1996). Also, the increase of microbial population may be due to the congenial condition for the growth of microbes within the digestive tract of earthworm and by ingestion of nutrient rich organic wastes which provide energy and also act as a substrate for the growth of the microorganisms (Tiwari *et al*., 1989).

Phylogenetic analysis

The annotation for the retrieved cellulase gene in *Eisenia foetida* and the different strains of two *Bacilli* species namely *Bacillus subtilis* and *Bacillus pumilus* is presented in Table 2. These sequences were subjected for phylogenetic construction of gene tree to infer HGT events likely to have resulted between the prokaryote's (Bacteria) and eukaryote's (Earthworm) genome.

Table 2: Annotation of the cellulase gene sequences.

S. No.	Accession/GI: Region	Organism	Name of the gene	Length in bp
1	923995665: 365132-366265	*Bacillus pumilus* JRS3	Cellulase	1133
2	D01057	*Bacillus subtilis*	Cellulase	1928
3	M16185.1	*Bacillus subtilis* DLG	endo-beta-1,4-glucanase	1920
4	JXBC01000002.1|: 934156-935316	*Bacillus subtilis* strain HM-66	Cellulase	1161
5	M38634.1	*Bacillus subtilis*	Cellulase	770
6	KJ130415.1_a	*Bacillus subtilis* strain N22	Cellulase	729
7	KJ130415.1_b	*Bacillus subtilis* strain N22	endo-beta-1,4-glucanase	729
8	LYUI01000018.1|: 222760-223845	*Bacillus subtilis* strain SRCM101280	endoglucanase	1086
9	AB679653	*Eiseniafoetida*	endo-beta-1,4-glucanase	1371

GI – Gene Identification number; bp – base pair

It was interesting to note the revelation of phylogenetic analysis using cladogram representation (Figure 1) and phylogram representation (Figure 2) that, *Bacillus pumilus* showed closest relation to *Eisenia foetida* for cellulase gene. Contrastingly, *Bacillus pumilus* belonging to the same genera of *Bacillus subtilis* were not closely related as they branch out with different nodal points showing least evolutionary significance of difference for cellulase genome. This leads to a clear inference for the occurrence of horizontal gene transfer for cellulase activity had been obtained by *Eisenia foetida* from its symbiotically ingested microbe *Bacillus pumilus* during the course of evolution.

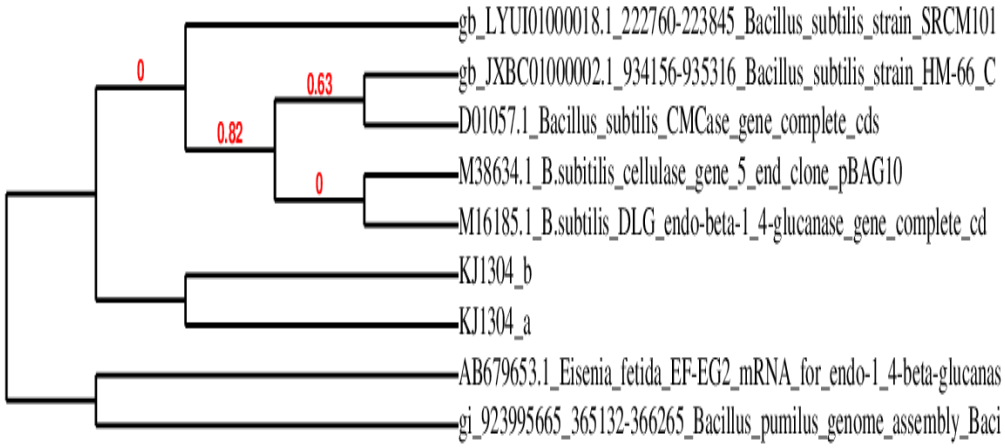

Figure 1: Cladogram representation of the phylogenetic analysis.
KJ130415.1_a– *Bacillus subtilis* strain N22_cellulase_gene; **KJ130415.1_b** - *Bacillus subtilis* strain N22_endo-beta-1,4-glucanase _gene

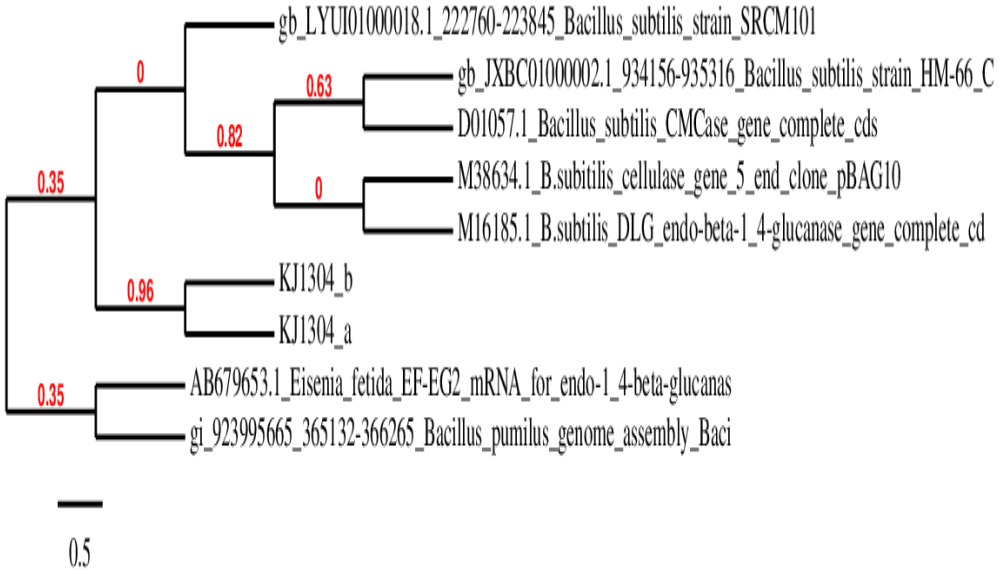

Figure 2: Phylogram exhibiting phylogenetic affiliation for cellulase gene in *Bacillus subtilis, Bacillus pumilus* and *Eisenia foetida*.
KJ130415.1_a– *Bacillus subtilis* strain N22_cellulase_gene; **KJ130415.1_b** - *Bacillus subtilis* strain N22_endo-beta-1,4-glucanase _gene

Jyotsana *et al*., (2010) isolated twenty species of bacteria from the gut of earthworm (*Eisenia foetida*) and all the isolates were found positive for cellulase production. One isolate showing maximum activity was identified as *Lysini bacillus sphaericus* (formerly *Bacillus sphaericus*). Mishra *et al*., (2011) isolated *Bacillus sp., Pseudomonas sp., Staphylococcus sp., Bacillus subtilis, Bacillus lentus, Azotobacter sp., Micrococcus sp., Tlavobacterium sp., Brevibacterium sp.* and *Thiobacillus sp.* from the tropical earthworm *Glyphodrillus tuberosus*, and recorded higher microbial load in gut section of the worm than undigested soil. Shankar *et al*., (2011) isolated cellulolytic bacteria from *Eudrilus eugeniae* and assessed the cellulolytic activity in the microbes, isolated from the gut.

A similar finding by Nozaki *et al*., (2009) detected the cellulosic activity of the extracts from the intestinal tissues and contents of *Pheretima (Metaphire) hilgendorfi*as a single band with molecular weight of 51k Da, which indicates that one major cellulase function is in degrading cellulose. The cellulase gene detected has a species specific cellulase produced by species specific symbiotic microorganism. A study similar to the present study by Nancy and Tyler, (2010) proved that the lateral transfer of genes from fungi underlies carotenoid production in aphids.

Conclusion

The study reveals an increased activity of cellulose on the 45th day of the experimental period in T1. On the 60th day, the treatments T1 and T2 exhibiting higher cellulase activity was degraded efficiently than their control C1 and C2 respectively. The *Eisenia foetida* gut isolate on the 60th day was found to be of the *Bacillus* genera in both the treatments. The phylogenetic analysis of the retrieved sequences for cellulase gene of *Eisenia foetida*, *Bacillus subtilis* and *Bacillus pumilus* unveiled a closer relatedness of *Bacillus pumilus* to *Eisenia foetida* for cellulase gene leading to an inference of the possibility of horizontal gene transfer (HGT) between the bacteria's and the earthworm's genome for cellulase gene in the course of evolution. Thus, vermicomposting using *Eisenia foetida* is an unconventional method of degrading the cellulosic paper waste for its management.

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