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Full Length Research Paper

Degradation of organic wastes and recycling of nutrients enhanced by microbes in subterranean habitat

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Degradation of organic wastes and recycling of nutrients enhanced by various microbes of both bacterial and fungal species available at the entrance, twilight and dark zones of four different caves such as Pannian, Samanar, Ushman and KKB caves at Madurai was investigated. The roles of chemoheterotrophic and chemoautotrophic bacteria were found to be essential in cave ecosystem. The most important chemoheterotrophs were ammonifiers such as *Bacillus* sp., *Clostridium* sp., *Serratia* sp. and *Pseudomonas* sp. Whereas Nitrite bacteria like *Nitrosomonas* sp., *Nitrosococcus* sp. and *Nitrobacter winogradskyi*, *Nitrococcus mobilis* derived energy from the oxidation of ammonia and nitrite respectively. Many heterotrophs thrive on the organic releases from nitrifying bacteria. Such heterotrophs involved in the nitrogen cycle were nitrate-reducing bacteria while denitrifiers were sparse in comparison to nitrate reducers due to absence of an anaerobic atmosphere. A total of 15 mesophilous and four thermophilous fungi were isolated from the cave soil samples. Among 15 mesophilous fungi, 11 species belong to Deuteromycetes, 3 species are zygomycetes and 1 species belongs to Ascomycetes. The number of species and CFU of thermophilous fungi also decreased towards dark zone. *Serratia* sp. and *Bacillus* sp. were isolated from all the samples at the E, T and D zones of the studied caves. These two bacterial colonies were subjected to quantify enzymes such as cellulose and amylase respectively. The production of cellulose by *Serratia* sp. at 35°C, pH 6.5 and in substrate glucose is considerably more. The production of amylase by *Bacillus* sp. seem to be more at 35°C and the substrate ammonium nitrate is considerably ideal for the synthesis of higher quantity of amylase by *Bacillus* sp. In all the caves, D zone samples had more cellulase and amylase enzymes than the E and T zone samples. Apart from cellulase and amylase, the presence of lipase, catalase and RuBisCo also were identified from the study caves. The nutrient NO_3 was found to be more in all the samples. In the caves, nitrogen (N) compound was more than P and K. The FT-IR spectrum results showed that stretching of mineralized compounds were more at the D zone samples compared with E and T zone samples, due to microbial role at D zone with the aid of their enzymes which eventually degrade the complex organic matter into simpler forms.

Key words: Ammonifiers, RuBisCo, anaerobic, lipase, catalase.

INTRODUCTION

Cave represent a distinctive habitat with complete darkness, relatively constant air and water temperatures and humidity, and a poor supply of oxygen with heap of organic debris of cave dwelling animals, consequently, most cave ecosystems depend on allo-chtonous (energy derive from outside) organic material for energy (Poulson and Lavoie, 2000; Simon et al., 2003). Each cave

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arbitrarily divided into three zones the entrance zone at the cave mouth where the temperature and humidity fluctuates every day, the twilight zone, up to this level light can be traveled which perceive feasible and favourable environmental condition for most of the cave dwelling animals and the last zone is the everlasting dark region where typical cave adapted troglobitic animals with several peculiar features survive. Apart from these cave dwelling animals, cave accommodates a variety of microbes which play crucial role in decomposing organic debris of cave animals and recycle the food chain in the cave environment. The microbial population such as bacteria and actinomycetes fluctuates over the seasons that are reported to carry by seeping water during rainy months in Harlburg cave at Pennsylvania (Fawley and Long, 1997). The other reason of microbial population in cave was the trogloxene mammals which frequent the cave every day (Long and Fawley, 1981). The cave food recycling totally depended on the catabolic and anabolic microbial processes of bacteria and fungi. Microbial processes occurring in the absence of light have generally been considered insufficient to support ecosystem level processes; the dogma has been that life processes in the subsurface are dominated by heterotrophic consumption of surface derived carbon (Caumartin, 1963; Pedersen, 2001; Naeem, 2002; Alfreider et al., 2003). The paucity of information regarding degradation of organic wastes by microbes, recycling of nutrients with the aid of enzymes of microbial origin in subterranean environment is documented here.

MATERIALS AND METHODS

Sample collection

The soil samples were collected from dark zones of these two caves. The samples were brought to the laboratory and kept in the refrigerator then analyzed in order to determine the number of bacteria, Mesophilous fungi and thermophilous fungi belonging to different physiological and morphological groups. The soil samples were serially diluted and plated on nutrient agar and incubated at room temperature for 3 to 4 days. The biochemical tests for the identification of bacteria were carried out as described in Bergey's manual of systematic bacteriology (Garrity et al., 2001).

Study area

Cave 1 (Samanar cave)

The cave is perched on the northern slopes of the hill near a village Keelakuyilkudi. The cave is situated at an elevation of 108 m, from the ground level. It is typically having only one entrance, which faces northwest. A colony of about 500 individuals of the bat *Hipposideros speoris* and about 15 to 20 *Hipposideros fulvus* of both sexes inhabit this cave. Wild cats and different types of spiders, flies and lizards are abundant inside the cave. The cave has several labyrinthine ramifications 15 to 50 m from the cave mouth. Some nooks of the cave are almost inaccessible to humans. The bats and spiders use several of these pockets as their dwelling sites. Most of the areas, especially the deeper recesses are very damp and slippery with the layers of bat guano, and one has to crouch on one's heels since the roof of the cave is very low.

Cave 2 (Pannian cave)

The cave placed on the top of the Nagamalai ridge near Pannian village at an elevation of about 200 m from the ground level. The cave mouth faces the zenith. One should climb down using a rope to reach the floor of the cave. The passage inside the cave goes on both west and east directions from the entrance of the cave. The length of the cave extends about 69 m towards west and about 62 m towards east. The daylight can penetrate a maximum distance of about 10 m on either side of the cave entrance. After that total darkness persists forever. Some nooks were in the cave through which one must crawl to go beyond that level. A colony of 20 to 30 bats of *R. hardwickei* at the entrance near the cave mouth, 100 to 150 *Megaderma lyra* at the dark zone, 300-350 *H. speoris*, 350 to 400 *H. fulvus* inhabited invariably in all parts of the cave. Apart from bat colonies, frogs, geckos, spiders (*Artema* sp., *Philoponella* sp. and *Theridian* sp.), moths and flies are also inhabited various parts of the caves.

Caves 3 and 4 (Usman cave and KKB cave)

These two caves are located 10 km away from Madurai Kamaraj University campus. The study caves 3 and 4 are situated in Nagamalai hill at the elevation of 50 and 150 m respectively from the ground level. Both the caves are open towards west. The former has wider mouth and the latter has narrow entrance. About 150 and 400 individuals of bats *Rhinopoma hardwickei* and *H. speoris* occupy in cave 3 and cave 4 respectively. The spiders *Artema* sp., and *Theridian* sp. inhabit at the entrance of both the caves. The tineid moths and their maggots and cockroaches are found in all parts of the caves.

Collection of soil samples from study caves

In terms of the light, temperature and humidity each cave arbitrarily divided into three zones namely; (1) the entrance (E) zone near the cove mouth into which the environmental light penetrates with varying temperature and humidity, (2) the twilight zone (T), into which light is available with moderate conditions and (3) the dark zone, characterized by constant temperature and humidity with total darkness. The cave soil samples were collected in clean sterilized containers from entrance, twilight and dark zones of study caves.

Cultivation media

A pre-culture was prepared by inoculating a single colony in 10 ml of peptone medium consisting of (gl^{-1}) peptone 30, yeast extract 10 and NaCl 5 (pH 7.0) and inoculated on a rotary shaker (240 rpm) for 24 h. The numbers of aerobic, heterotrophic, chemo-lithoautotrophic bacteria were determined on nutrient agar media for the isolation, growth and purification of microorganisms (Malik, 1992). Selective media with $(NH_4)_2SO_4$ or Na NO_2 were used in order to determine the most probable number of nitrite and nitrate bacteria respectively and peptonated water was used for ammonifying bacteria (Pochon, 1954; Gerhardt et al., 1994).

Assay of amylase and cellulase activity was carried out using DNase method and lipase production was carried out by olive oil as a substrate using titrimetric method. Other enzymes that is, catalase and RuBisCo were identified. The soil nutrients were analysed by Agricultural Research Institute at Kovilpatti, Tamilnadu. FT-IR spectra of entrance, twilight and dark zone samples of all the

Table 1. Geo-physical characteristics of the dark zone of study caves.

Caves	Zone	Distance from entrance (m)	Temperature (°C)	Humidity (%)	Maximum light intensity (lux)	Major food source
Samanar	Dark	> 15	27	95	Constant darkness	Bat guano
Pannian	Dark	> 10	27	95	Constant	Bat guano
Usman	Dark	> 15	27	95	Constant	Bat guano
KKB	Dark	>20	27	95	Constant	Bat guano

four caves were taken using a Perkin Elmer. FT-IR spectrometer in the spectral range of 4000 to 400 cm^{-1}. Each sample mixed with spectroscopic grade KBr and made in the form of pellets at a pressure of about 13 mm in diameter and 1 mm in thickness.

RESULTS AND DISCUSSION

The result of the present study provide an information about degradation of organic wastes and recycling of nutritive elements especially nitrogen by the action of enzymes of microbial origin in cave ecosystem. The relationship of autotrophic productivity and heterotrophic cycling rates to bacterial species richness can significantly impact the diversity of higher tropic levels in chemolithoauto- trophically based cave ecosystems, with the systems possessing the highest productivity and supporting abundant and diverse macro invertebrate communities. Heterotrophic bacteria derive carbon from preformed organic compounds that are broken down enzymatically. For chemoheterotrophs, energy is derived through the oxidation of organic compounds via respiration. There are literally thousands of different types of chemoheterotrophs in the environment, and they are critical to many aspects of environmental microbiology including bio geo-chemical cycling, waste treatment and bioremediation. In the present study, the result depicts the fact that the ecosystem in study caves totally dependent on chemoautotrophic and chemoheterotrophic microbes (Table 1). It can be noted that high population of the bacteria found were the heterotrophs and they were more abundant in the dark zones of the caves since they do not fix carbon unlike autotrophs (Atlas, 1986). This is far less than that in epigean soil as reported earlier (Manolache et al., 1997). The most important and abundant group of heterotrophs were the *ammonifers* (2.62×10^5, 3.7×10^5, 2.36×10^5 and 2.72×10^5 CFU/g of dried matter). The sole carbon source for the ammonifiers were urea, uric acid, guanine, creatine and other organic nitrogenous excretory and digestive wastes of troglobites, troglophiles and trogloxenes. *Bacillus* sp., *Clostridium* sp., *Pseudomonas* sp. were prominent among the ammonifiers that produced and fixed ammonia as ammonium compounds. As observed in the Romanian caves, ammonifiers being heterotrophs dominated over other groups of bacteria involved in the nitrogen cycle of Chemoautotrophs are essentials for the functioning ecosystem and they are involved in the process of nitrification. *Nitrosomonas* sp., *Nitrosococcus* sp. (Nitrite bacteria 2.4×10^4, 1.64×10^4, 2.2×10^4, 1.49×10^4 CFU/g of dried matter) and *Nitrobacter winogradskyi*, *Nitrococcus mobiles* (Nitrate bacteria 1.53×10^4, 1.24×10^4, 1.49×10^4 and 1.33×10^4 CFU/g of dried matter) derive energy from the oxidation of ammonia and nitrite respectively. This energy released by oxidation of NH_4^+ and NO_2^- ions can be used to fix carbon dioxide and produce organic molecules. The acidic pH of the soil in this ecosystem proved the process of nitrification which has been defined as the transformation of ammonium to nitrite ions resulting in a change in charge from positive to negative (Watson et al., 1981). Many heterotrophs thrive on the organic releases from nitrifying bacteria. Such heterotrophs involved in the nitrogen cycle were the nitrate reducing bacteria (4.2×10^4, 3.46×10^4, 4.2×10^4 and 3.36×10^4 CFU/g of dried matter) the results were noted in Table 2. These organisms carry out assimilatory reduction of nitrate to ammonia. Owing to the long generation time of nitrifiers (8 to 24 h), the heterotrophic bacteria as usual outnumbered the nitrifying bacteria in an enriched nutritive environment of bat guano (Focht and Vestraete, 1977). This is true as heterotrophic nitrifiers produce very low levels of nitrite and nitrate and often use organic sources of N_2 rather than ammonia (Sarbu et al., 1994). Another group of nitrate dependant heterotroph was the denitrifiers that carry out dissimilatory reduction to produce nitrogen. Denitrifiers were sparse (1.4×10^1, 2.2×10^1, 1.4×10^1 and 2.1×10^1 CFU/g of dried matter) in comparison to nitrate reducers due to absence of an anaerobic atmosphere. In spite of this cave ecosystem being aerobic the presence of denitrifiers as supported by denitrification suggests the occurrence of anoxic microhabitats (Watson et al., 1981). Yet another group of heterotrophs pertaining to the nitrogen cycle are the nitrogen fixers. The absence of nitrogen fixing organisms indicates the limited role of atmospheric nitrogen in the ecosystem. No other kind of chemoautotrophs was observed. This was probably due to lack of appropriate conditions for their survival. About 15 mesophilous fungi and four thermophilous fungi were isolated from the study caves. The fungi *Aspergillus flavus* and *Aspergillus niger* were more predominant due to their cosmopolitan distribution in nature. The number this ecosystem (Manolache et al., 1997).

Table 2. Population of different physiological group bacteria in study caves.

Bacterial class	CFU*/g of dry matter in caves			
	Samanar	Pannian	Ushman	KKB
Chemoheterotrophs				
Ammonifiers	$2.62x10^5$	$3.7x10^5$	$2.36x10^5$	$2.72x10^5$
Nitrate reducers	$4.2x10^4$	$3.46x10^4$	$4.2x10^4$	$3.36x10^4$
Denitrifiers	1.4×10^1	$2.2x\ 10^1$	1.4×10^1	2.1×10^1
Chemoautotrophs				
Nitrite bacteria	2.4×10^4	1.64×10^4	2.2×10^4	1.49×10^4
Nitrate bacteria	1.53×10^4	1.24×10^4	1.49×10^4	1.33×10^4

Table 3. Occurrence of mesophilous fungi from the soil of entrance, twilight and dark zones at four caves. + indicates presence, - indicates absence of the species of fungi.

Fungi	Entrance	Twilight	Dark
	S P U E	S P U E	S P U E
	CFU x 10^4/g	CFU x 10^4/g	CFU x 10^4/g
Deuteromycetes			
Aspergillus flavus	14 7 5 12	10 7 6 7	6 4 3 5
A. versicolor	15 7 - 7	8 - 13 -	- - - -
A. tamari	12 13 5 4	14 9 - 8	- - - -
A. sydowii	7 - - -	- - - -	- - - -
A. chevalieri	9 - - -	- - - -	- - - -
A. ochraceous	8 - - 13	- - - -	- - - -
A. niger	19 12 16 8	12 5 12 8	10 11 13 7
A. parasiticus	4 - - -	- - - -	7 2 1 -
Curvularia brachyspora	4 6 1 8	5 4 - -	- - - -
Penicillium cyclopium	15 4 12 8	5 13 5 7	4 - - -
P. fellutanum	- 6 - -	- - - -	- - - -
Zygomycetes			
Absidia corymbifera	2 - 6 1	1 - 4 3	- - 4 -
Mucor sp.	- 3 - 4	- 2 1 3	- 1 - -
Rhizopus stolonifer	5 7 - -	7 1 - -	2 - - -
Ascomycetes			
Chaetomium sp.	5 14 13 12	5 13 13 10	7 13 10 8

S represents Samanar cave; P- Pannian cave; U- Usman cave and E- KKB cave.

of mesophilous and thermophilous fungal species as well as CFU in each species was more at E zone than T and D zones (Tables 3 and 4). This result in accordance with the finding of Koilraj et al. (1999a) which state that the CFU and fungal species higher at D zones compared to T and D zones in caves. The absence of thermophilous fungi in the dark zone of cave soil samples depict the essence of light and other favourable physical factors for sporulation (Koilraj et al., 1999b). The microscopic observation of mesophilous and thermophilous fungus morphological characterization showed in Plates 1, 2, 3 and 4. The production of cellulase and amylase by *Serratia* sp. and *Bacillus* sp. respectively were assayed in different dilutions (0.2 to 1.0 ml) of samples at pH 6.5 and 7.5; at temperatures 35 and 45°C and on the substrates glucose and lactose (for cellulase by Serratia sp.) ammonium nitrates and sodium chloride (for amylase by *Bacillus* sp). The pH of 6.5, 35°C temperature and the substrate glucose is considerably ideal for the production of cellulose by *Serratia* sp. Such a way, the temperature

Table 4. Occurrence of thermophilous fungi in study caves (E- entrance; T- twilight; D- dark zones).

Fungi	CFU x 10^4 / g dry sample											
Thermophilous fungi	Pannian			Samanar			Ushman			KKB		
	E	T	D	E	T	D	E	T	D	E	T	D
Thermoascus aurantiacus	7	5	-	4	2	-	2	-	-	6	3	-
Chaetomium thermophile var. dissitum	4	2	-	2	-	-	6	4	-	5	3	-
Humicola grisea var. thermoidea	5	4	-	5	4	-	7	4	-	5	2	-
Humicola stellata	3	3	-	2	-	-	4	2	-	5	-	-

Plate 1. Microphotograph of Mesophilous fungi. *Aspergillus terreus* (X 260).

35ºC and the substrate ammonium chloride are suitable for production of amylase by *Bacillus* sp. which was isolated from cave soil samples. These two enzymes cellulase and amylase were screened and quantified from dry soil samples of E, T and D zones of all four study caves. The dark zone samples incredibly had more enzymes than twilight and dark zone samples. Other enzymes like lipase, catalase and RuBisCo were identified from the cave soil samples. Amylase, cellulase and lipase activities in the cave samples indicated the presence of heterotrophs (Manolache et al., 1997). Presence of catalase and its absence in streptomycin treated samples establish its microbial origin (Gnittke et al., 1971). This result suggested that the dominating bacterial population is aerobic bacteria and hence an aerobic environment in this ecosystem. In spite of this, denitrifiers and anaerobes like *Clostridium* sp. and *Pseudomonas* sp. were identified. Denitrification has been considered as anaerobic respiration associated with otherwise strictly aerobic chemoheterotrophs (Randall and Ingraham, 1981) taking place in anoxic microhabitats (Watson et al., 1981) existing in soil sediments having an interface between aerobic and anaerobic layers favoured by reduced oxygen tension (Aragno and Hans Schlegel, 1981). RuBisCo (Ribulose–1,5–bis-carboxylase), an enzyme active in carbon dioxide fixation is an indicator of autotrophy, since heterotrophs lack this enzyme (Sarbu and Kane, 1995). The RuBisCo activity in the caveZ

Plate 2. Microphotograph of a Mesophilous fungus. *A. versicolar* (X 260).

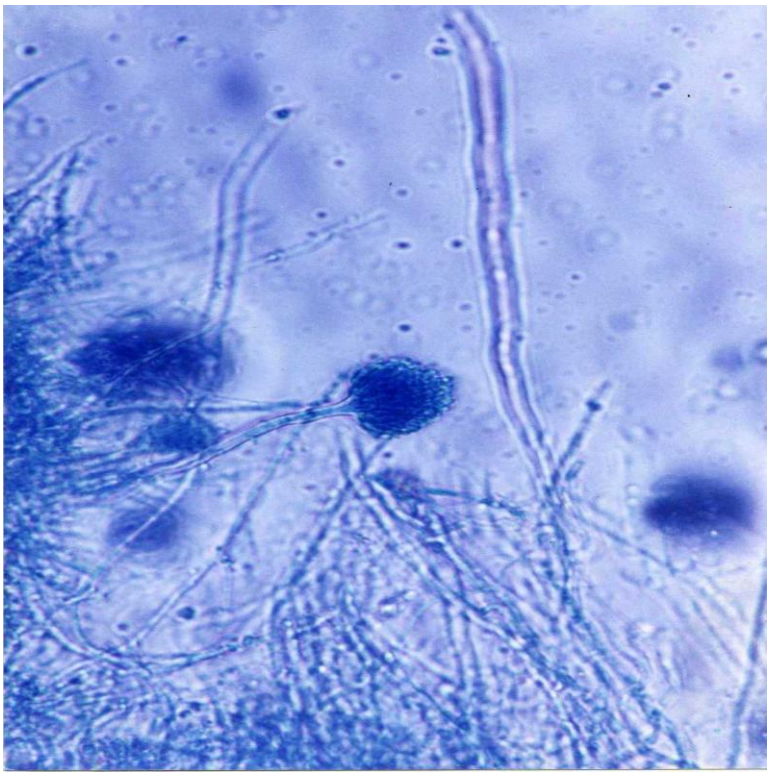

Plate 3. Microphotograph of a Mesophilous fungi. *A. sydowii* (X 260).

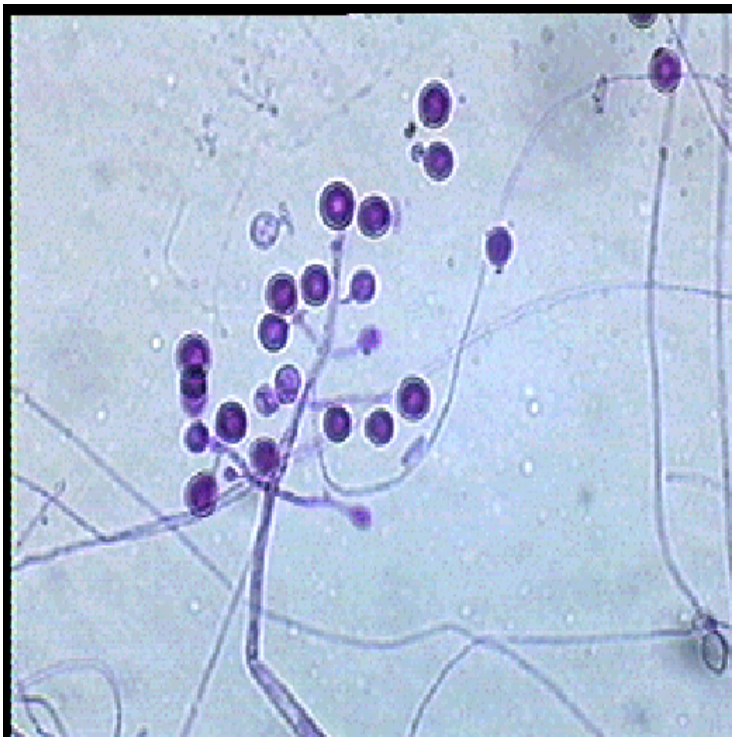

Plate 4. Microphotograph of a Thernwphilous fungi. *Humicola stellate* (x260).

sample suggested the presence of chemoautotrophic bacteria in the caves. The presence of NO_3 and high ratio of N (55.83, 48.73, 83.27, and 57.99%) in NPK further support the denitrification process and the subsequent nitrogen cycle by microbes in cave environment. The relationship of autotrophic productivity and heterotrophic cycling rates to bacterial species richness can significantly impact the diversity of higher trophic levels in chemolithoautotrophically based cave ecosystems, with the systems possessing the highest productivity supporting abundant and diverse macro invertebrate communities. Various active groups of biochemical compounds in organic wastes at E, T and D zone samples in four caves are analyzed with the help of FTIR. From the available information it is clear those nitrogenous compounds available in all the three zones in cave environment. Since, trogloxenes primarily occupy at the dark zone of caves huge organic debris accumulate at the D zone facilitate action of cave microbes. The microbial enzymes produced by microbes degrade the complex organic wastes and mineralize the compounds into simpler substances. Therefore, stretching of mineralized compounds like nitrate, nitrite, chloride, fluoride, iodide, disulfur, pyrol 2-acyl are more at the D zone samples support the chemoautotrophic and chemoheterotrophic mode of nutrient cycle in cave environment. The present study support the concept of mineralization process which is carried out by catabolic enzymes of microbial origin in vermicompost samples (Ravindran et al., 2008).

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