Journal of The Palaeontological Society of India
Vol. 49, 2004, pp. 151-157

ISSN 0522-9630

# BIODEGRADATION OF LEAF CUTICLES DURING FOSSILISATION: A PRELIMINARY STUDY

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## ABSTRACT

The role of micro-organisms (fungi and bacteria) in the degradation of dispersed and compressed leaf cuticles from clay, shale and carbonaceous shales have been studied under light scanning and transmission electron microscopes. To understand the mechanism of biodegradation through successive stages, a comparative study of surface morphological and histological features from least affected to severely altered cuticles was undertaken. Fungi and bacteria rapidly colonize the plant fragments including leaf litter when they are buried in soil or in sedimentary matrix. These colonizers enter the leaf tissues through stomata, pores and cracks. They first degrade non-lignified components then lignin-containing structures present between the cuticular layers and finally the cuticular membranes and its complex biopolymer contents. Observations under SEM and TEM show three characteristic stages in degradation of leaf cuticles affected by fungal attack, *viz.*, hyphal invasion, penetration and dissolution of tissues. In case of bacterial action, infestation, corrosion, cavity formation and tunneling represent main degradational phases. These processes ultimately contribute towards the formation of amorphous organic matter.

**Key words :** Biodegradation, leaf cuticles, fossils, SEM, TEM.

## INTRODUCTION

The study of biodegraded leaf cuticles from sedimentary deposits is an important source of information for evidences regarding activities of microbe over the thin extra cellular layer of leaves, which were buried in sediments and thus help to understand the phases of biodiagenesis. Microorganisms (fungi and bacteria) can tolerate a wide range of temperature, humidity, pH, eH and salinity variation in the depositional environment. They attack different kinds of plant fragments. Among fungi, particularly basidiomycetes and ascomycetes (microthyraceous members) are usually more aggressive than bacteria at the early phases of degradation (Bajpai and Maheshwari, 1988). Many bacteria viz., cocci, bacilli, thiobacilus etc. are more tolerant than fungi to anoxic condition and continue degradation even in conditions unfavourable to fungi. Kim and Singh (2000) have studied degradation of wood fragments by fungi and bacteria. They have identified several phases of degradation. Since foliage constitutes a major part of the organic matter that gets deposited in the basins along with the other clastic material; we have tried in the present work to understand the phases in degradation of fossil leaf cuticles.

## MATERIAL AND METHODS

Clay, shale and carbonaceous shale samples belonging to various geological horizons were selected from the collections available at BSIP, Lucknow. The selections were made on the basis of cuticle content in the sample macerates e.g. the cuticle of *Thinnfeldia* was selected for TEM studies as it showed good infestation of leaf cuticle by fungal hyphae and bacteria showing various stages of degradation. The study is based on the following samples: -

**Permian:** Lohapiti, Jharia Coalfields, Jharkhand. (Long. 86° 11' to 86° 13' E: Lat 23° 44'N.

**Late Triassic**: Grey argillaceous shale, Janar rivulet (23°40'53" N: 81°12' 40" E), and 1Km. South of Harai village, South Rewa Basin, Madhya Pradesh.

**Early Cretaceous:** 1) Carbonaceous shale and clay from Sehora, Jabalpur Formation, MP (22° 52'N:79° 29' E) ii) Grey shales from Naicolam, Sivaganga Formation (11°3' N:78° 49' E), Trichurapalli District, Tamilnadu.

**Miocene :** Lignitic clay from Amberiwadi (Lat 16° 30' 20" N: Long. 73° 23' 20" E) Sindhudurg District, Maharashtra.

**Pliocene:** Carbonaceous shale from Mahuadanr valley (23°23'5" N: 84°06'40" E), Jharkhand.

Well-preserved cuticles in both compressed and dispersed forms were isolated through standard palynological preparation techniques by using dilute HCl, HF and sieving through 500-mesh sieve.

These cuticles (structured/non-structured) were consequently treated with graded ethanol (25,50,80% and absolute). This dehydrated material is picked out with fine glass capillary and mounted on double scotch adhesive tape fixed on aluminium stub. Specimen is coated with 30-60 A° gold/palladium alloys and observed in Leo 430 SEM unit.

For TEM studies the structured and non-structured cuticle pieces were thoroughly washed with distilled water and dehydrated in graded ethanol for 4-5 minutes followed by the treatment with the acetone. The dehydrated specimens were fixed in gluteraldehyde (4%) prepared in 0.1 Mcacodylate buffer (pH 7.2). After fixation, cuticular materials were kept in the embedding medium in a mould at 60 °C for polymerization. After polymerization the molded blocks of each specimen were cut in to ultra thin sections at 600-700 °A and about hundreds of pieces were obtained. The cut pieces were placed in a grid for observation under TEM.

## BIODEGRADATION OF LEAF CUTICLES

Inspite of defensive features of cuticular layers (Kerp, 1990) several microbes (fungi, bacteria and viruses) attack leaf tissues and degrade them when they are buried in soil or in sedimentary matrix in aerobic or anaerobic conditions. The structured or weakly structured fossilized leaf cuticles preserve many features that show relationship with environment, while their degradation at the following categories enumerates anatomical changes during burial stage of cuticular pieces in sediments. These changes occur in several phases, which can be readily distinguished from parent morphological types. The terminology applied in present paper is based on Kim and Singh (2000). Here several sequential phases of degradation, e.g. invasion or infestation, penetration, corrosion, cavity formation, tunneling and formation of amorphous organic matter have been recognized in cuticle biodegradation. These categories follow two pathways a) decay by fungi and b) by bacteria. The bio-degradational activities over fossil cuticles were recognized with the comparison of original histological details and changes thereafter. During the process of degradation the microbial activities were controlled by i) fungi and bacteria, which are common inhabitants over the leaf fragments ii) their action mobilizes leaf tissues from structured to non structured amorphous types and iii) conditions involved during diagenesis, change the plant tissues into a humic mass.

It is difficult to imagine how the system actually operates to bring about this overall change. However, it is presumed that the combination of various processes and events bring this change in phases. Various phases of degradation are discussed below.

### Fungal action

The fungal degradational process affects slowly and is easily distinguishable due to the presence of fruiting bodies, hyphae and conidia etc., at the outer surface of the cuticular layer or inside the tissues. These Fungi perform their life cycles in saprophytic

## EXPLANATION OF PLATE I

SEM photographs of cuticle

A. fungal hyphae spreading over the cuticular layer (B-C) coccoid bacteria adhere on periclinal wall

B. showing binnary fission

C. magnified view of B

D-F LM photographs showing hyphae and fruiting bodies

D,F hyphal mat spread over cuticle layer

E. microthyraceous fruiting bodies and hyphae scattered over epidermal cells

G. biodegraded stomata of angiosperm cuticle showing corrosion of guard cells, subsidiary and accessory cells.

H – I TEM photographs showing corrosion and cavity formation

H. showing corrosion on periclinal wall

I. cavity formation on anticlinal wall

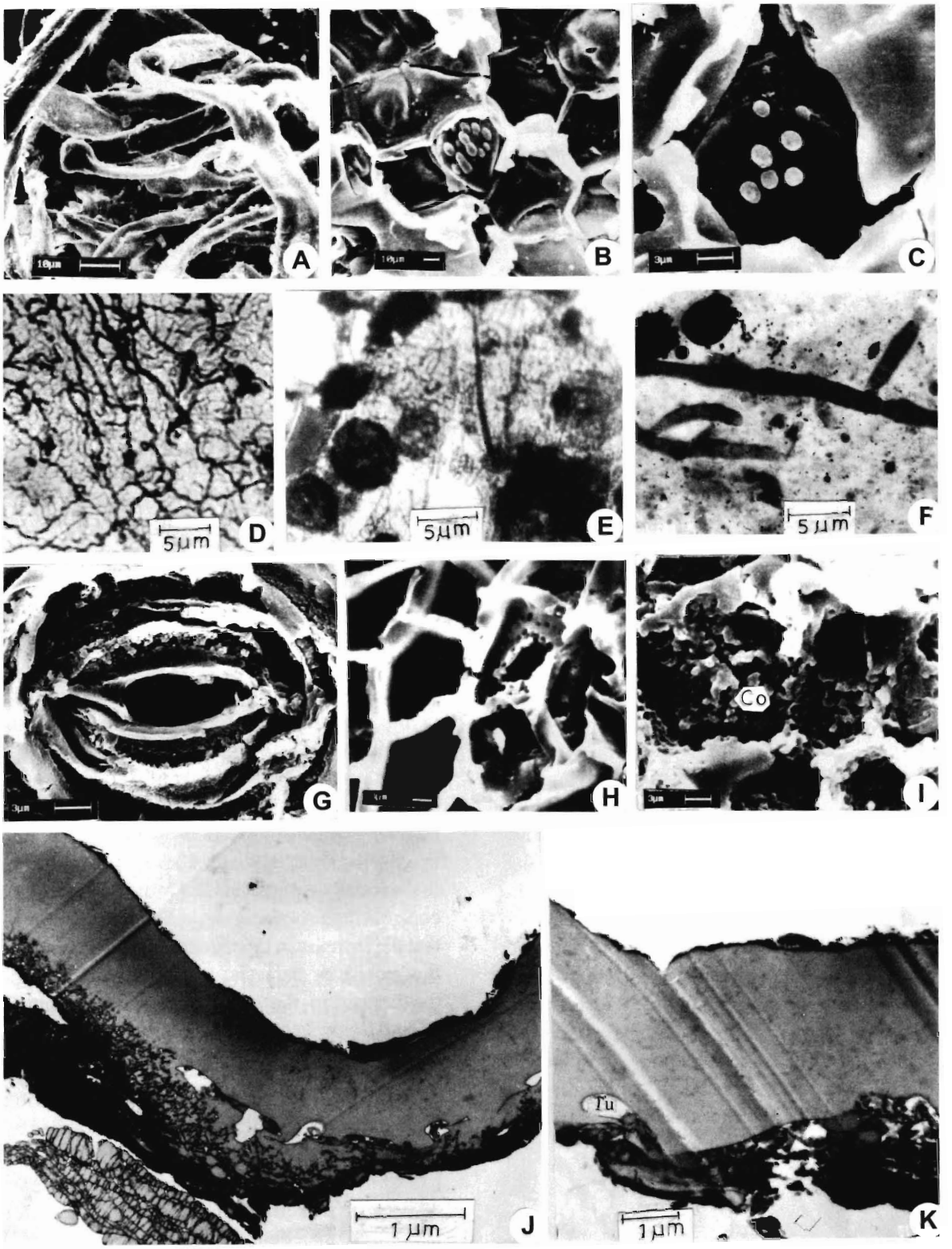
J-K TEM photographs of the genus *Thinfeldia indica*

J. showing osuter waxy layer, cuticular membrane and cavity formation

K. cavity formation and early stage of tunneling at lower cuticular layer.

Journal of The Palaeontological Society of India
Vol. 49, 2004

**Plate I**

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and parasitic phases. According to Schroth and Hildetbrand (1964) these fungi grew on plant walls and organic matters by allowing germination of chlamydospores (Pl. I, figs. E & F) and mycelial growth (Pl. I, figs. A & D) Earlier, the mode of penetration by fungi on cuticles had been a controversial phenomenon. It was believed that the mere physical force of the growth of infecting peg allows penetration of fungal bodies but contrary to this hypothesis, the involvement of hydrolyzing enzyme (cutinase) in this process has also been of prime significance (Webster, 1986). The following stages enumerate activity of fungi over the cuticular surface.

**Hyphal invasion**

Members of ascomycetes (Microthyraceae) and basidiomycetes have been observed to colonize the leaf epidermis during first attack and enter inside the upper and lower cuticular membranes through stomata, pore or cracks. The hyphae develop mesh like network (Pl. I, figs. A, D-E) over leaf surfaces and produce conidia, fruiting bodies, etc. The initial colonization of mycelia may also develop on cuticles through haustoria and/or zoosporangia, which enter through stomatal slit.

**Hyphal penetration**

A number of plant pathogen gain entry into the foliage entities slowly via stomata as an alternative to direct penetration of epidermal cells or tissues (Gupta *et al.*1999). Their SEM observation revealed that the mycoparasitism occurred when the antagonists and pathogen made a hyphal contact, entered inside the leaf epidermal layer through stomata or cracks and proliferated through coiling, grasping and penetration by producing hooks, haustoria and apresoria like structure. Sometimes they also grow outside the wall and dissolve outer non-waxy cuticular layer by releasing enzymes. Hyphae then dissolve epidermis and rupture the entire cuticular layer including the guard cells. Their longitudinal growth in palisade layer also causes degradation of inner tissues (Pl. I, figs. D-E).

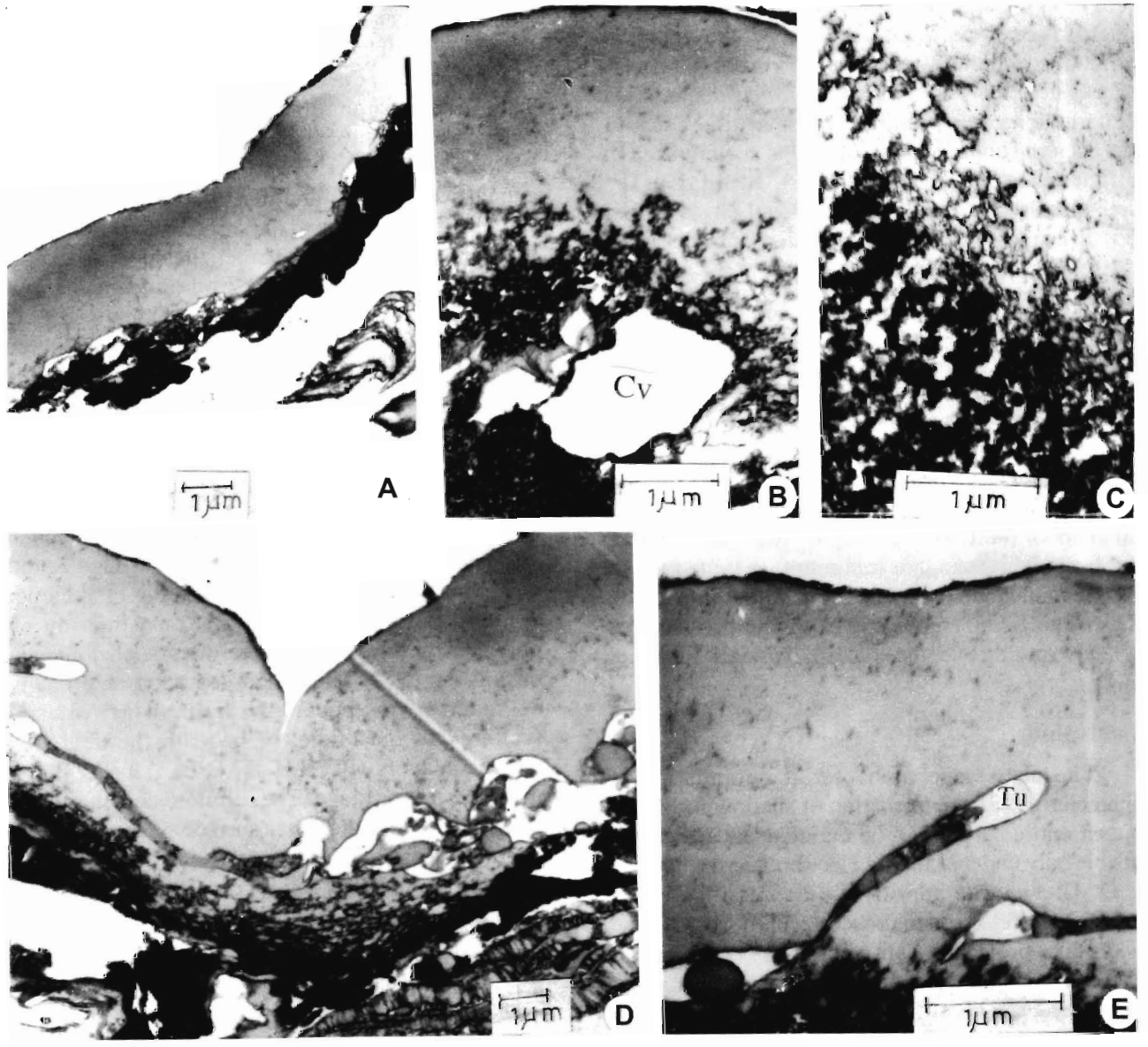
**Cavity formation**

The space between palisade and mesophyll layers develops cell lyses or cell separation in host tissues. During this stage the surrounding cellular parts are also dissolved by the continuously burrowing action of hyphae. These dissolved spaces appear like cavities on outer surface. In most of the cases hyphae develop in the cavities and produce branches and terminal fruiting bodies (Pl. I, fig. D, Pl. II, fig. B), which sometimes appear as scattered threads in or on the degraded tissues. During this degradational phase most of the tissues lose their cellular organization and appear like cavities from the outer surfaces.

**Bacterial action**

The identification of bacterial degradation is primarily based on SEM and TEM images of the cuticles. The degradation of leaf fragments causes direct breakdown of foliage and its constituents. This degradational phenomenon may also be termed as leaf litter decomposition, which is a part of the organic matter degradation. Bacteria may be highly selective with plant taxa, their biopolymer contents and histological features. In most of the cases bacterial degradation takes place after the partial decay by fungal hyphae. Sometimes both activities operate simultaneously, or fungi attack first and start degrading the organic matter by softening the tissues and make favourable conditions for bacterial attack (Demaison and Moore, 1980). The fungi and aerobic bacteria remain active till the availability of oxygen in the sediment. Anaerobic bacteria gradually replace this activity when the basin becomes deficient or depleted in oxygen. Thus, the oxidation- reduction potential in the sedimentary environment is the main factor, which controls the process of degradation. It is well known that the increase in the reducing conditions accelerates the growth of bacterial population. This is true for the deposition of organic matter under aquatic realm of both tropical and subtropical regions in wider sense.

The pattern of bacterial action is completely different from fungi. Fungi generally spread in many directions or along the cell walls by producing hyphae and its branches, while bacteria degrade at certain loci by increasing their population. The concentration of bacteria at certain places corrodes the cuticular layer and proceeds to form tubular structures. Bacterial attack on the anticlinal walls of weaker parts of parenchymatous tissues of the leaf is generally




## EXPLANATION OF PLATE II

(TEM photographs of the genus ***Thinfeldia indica*** from Late Triassic, South Rewa Basin)

A. waxy epicuticular layer and corroded lower layer.

B. cuticle showing cavities and degraded zone.

C. magnified view of the same.

D. highly degraded amorphous zone.

E. papillae showing inner degraded zone.

F. ramanents of waxy epicuticular layer and cavity at inner layer.

G. cuticular layer showing outer waxy layer, horizontal and vertical tunnels. Inner layer showing cavities and corrosions.

H. magnified view of tunneling phase in cuticle. Cavity showing degraded ball like biopolymer; (Cv = cavity, Co = corrosion, Tu = tunneling).

higher. Sometimes leaf fragments display a different style of degradation, almost without any evidence of fungal infection. These leaf fragments appear to begin degradation from areas adjacent to the venation without specificity to the level of vein architecture. (Gastaldo, 1994) also reported similar degradation pattern along veins. In due course of time most of the bacterial colonies were observed accumulated in pit chambers (Pl. I, figs. B-C, Pl. II, fig. E). The stepwise description of degradational phases is not possible in fossil forms. However, some characteristic phases showing bacterial degradation in fossils are described below:

**Infestation**

Before the onset of degradation of cuticle, bacteria first attack on outer membranous part (Pl. I, figs. B-C). During the colonization their population density varies with respect to the genetic and specific characters, cellular organization and biopolymer contents of the host. At hairy or papillate surfaces the bacterial attacks are minimum, as compared to the wet and smooth surfaces. The initial infestation may superficially appear due to the presence of selective pitting over the epidermal tissues (Pl. 1, fig. H-I)

**Corrosion**

The next stage of bacterial degradation is characterized by the formation of shallow forbs like pits at entire surface of the cuticular layer that are visible only under scanning electron microscope (Pl. I, G, I). The pits appear like depressions under transmission electron microscope (Pl. II, fig. D). The warts like projections are sometimes coalesced to each other. Thus, the degraded cuticle appears like granulated, weakly striated, conical or with v-shaped pits under scanning electron microscope and scabrate under light microscope. Under transmission electron microscope it appears like a small-branched capillary or shallow crescent shaped groove (Pl. II, fig. D).

**Cavity formation**

Bacterial attack at medium to advanced phases form cavities. Further, these cavities coalesce with each other and make outer cuticular surface non-structured. The decay at last phases form cavities of various diameters inside the cuticular membrane (Pl. I, figs. G-I, J, K; Pl. II, figs. B-C), which may be the effect of the consumption of biopolymer contents.

**Tunnelling**

Bacteria are selective during decay. They first attack on softer part of epidermis and enter to inner layer through burrowing and further form a tunnel like structure in the cuticle layer (Pl. II, figs. D-E). The bacterial tunneling is complex process in comparison to other stages of degradation. Here bacteria are more active in the middle part of the cuticular membrane and make tunnels that may be straight, crescent shaped or rarely branched. Bacteria when they are active in between the upper and lower layers (in compressed palisade and mesophyll tissues), they first corrode inner layer and penetrate ahead by making tunnels and leave residues in the shapes of microballs (Pl. II, fig.E). Under SEM it appear like holes from external view.

They are most active at the lignin concentrated parts of the leaf (mesophyll and palisade layers) beneath the cuticlar layer. They dissolve these layers by releasing the enzyme ligninases (Chui and Dolphin, 1989, Vidal *et al.*, 1989). After the initial degradation of lignin, bacteria may start degrading biopolymer contents up to some extent. Sometimes these polymers may not be completely degraded or remain unconsummated. These non-degraded or partially degraded parts appear like balls, which remain scattered in the tunnel or beneath the degraded cuticular layers (Pl. II, figs. C-E). This is called monomerization stage. The cellulose contents of the cell walls below the inner surface of the cuticle is degraded first by the bacterial enzyme *Cellulose* (Kilburn *et al.*, 1989) and is completely consumed.

Microbial activity on dispersed leaf fragments ultimately produces amorphous matter. This matter is characterized by the complete removal of morphological characters. The morphological changes in the cuticles from structured to nonstructured amorphous type lead to the formation of humic matter, which later influences the physical and chemical properties of the peat, shale or coal.

## DISCUSSION AND CONCLUSIONS

Microbial degradation of dispersed and compressed leaf cuticles represent various phases which are directly related with the ecological factors, defensive mechanism of parent leaf, grain size of sediment and redox potential at the burial stage. In the present communication it is not possible to provide

a substantial insight in to the entire mechanism through all the diagenetic stages of leaves in a sedimentary deposit. The interaction between leaves and other microorganism needs further detailed study in order to understand the role of various processes that operate at different levels of diagenesis. This knowledge can be utilized in understanding the genesis of fossil fuels.

The microbial activity and other diagenetic processes acting on the plant fragments play vital role in the preservation of particular part as some of which have transformed into finer particles and are mixed in humic detritus. Cuticles are more resistant than other plant parts mainly due to the biopolymer (Cutin, Cutan, Suberin) coating. Cutin in land plants is embedded in wax and in some instances the wax occurs in layers so that cuticle shows a lamellar structure. Martin and Juniper (1970) also reported similar lamellar layers. These features are clearly visible in the transverse section of chemically processed cuticular pieces.

Different types of bacteria occur over a mixture of leaves of various species and other plant derived organic matter in the soil. These plant cuticles have different kinds of defensive capabilities, but the bacteria with proper uptake of nutrients and minerals are able to degrade all of them at the favourable levels of pH of the sediments.

Generally, bacterial colonies are found in depressed areas on leaf surface or at any site (weaker membranous parts or lignin rich tissues) suitable for colonization. But fungi occur at the entire parts of epidermal layer and become active when their hyphae penetrate or enter through the cracks or stomata. Although bacteria, which are capable of degrading various polymeric organic compounds, are widespread in nature, the existing environmental conditions may limit their activity. TEM section of leaf cuticle shows that the extra cellular lignin is localized within the cell wall during bacterial decay. They generally enter to deeper layers of leaf dissolving the cuticular layer by tunneling and solublzing the polymeric compounds. Some non-degraded polymers, which were unaltered during diagenesis, lie near the tunnel and appear as ball like structures. Many environmental factors, e.g. temperature, relative humidity, pH value, salinity, etc., also affect degradational activity and the growth of microorganism over leaf cuticles.

## ACKNOWLEDGEMENTS

We are grateful to the authorities of Birbal Sahni Institute of Palaeobotany, Lucknow for providing infrastructural facilities. Thanks are also due to Director, B.S.I.P for encouragement during the course of the study and permission for the publication.

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Manuscript Accepted August 2004