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Cyllamyces icaris sp. nov., a new anaerobic gut fungus with nodular sporangiophores isolated from Indian water buffalo (*Bubalus bubalis)*

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Rumen;
fungal taxonomy;
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Chytrid;
Zoospore.

A B S T R A C T

A new species of anaerobic rumen fungus (*Neocallimastigales*) *Cyllamyces icaris* was isolated from the dung sample of Indian water buffalo (*Bubalus bubalis)*. This fungus belongs to the genus *Cyllamyce* and can be easily distinguished from the genus *Caecomyces* by the presence of rhizoids as well as multiple intermittent bulbous rhizoids. *C.icaris* resembles the type species *Cyllamyces aberensis* in multiple oval sporangia, nodular branched short sporangiophores and uniflagellate zoospores but differs from it in a polycentric development, the presence of distinct rhizoids, multiple bulbous holdfast and fastidious growth pattern (complete life cycle within 14-16 hrs). The sporangium though fewer in number (1-3 on each sporangiophore) had a diameter of 19.33 ±6.78µm , much larger compared to that of *C. aberensis* (14.7 µm). Zoospores were observed to have a diameter range of 7.7 - 14.13 µm (11.13 ± 1.39 µm; $n_{= 480}$) while the average length of the flagella was 59.50 ± 10.33 µm ($n_{= 410}$). From an initial diameter of 14 µm (14.63 ± 0.26 µm [Mean ± SD]; $n_{=100}$), the bulbous holdfast grew up to 86 µm (56.64 ± 13.42; $n_{= 150}$) after 24 h. The Internal transcribed spacer 1 sequence analysis revealed the unique motif sequence in Variable region I and thus a ninth motif sequence in Variable region I for *Cyllamyces* icaris is included. The motif sequences for variable regions II, III and IV were obtained at 10, 13 and 8, respectively and were similar to those described earlier for *Cyllamyces aberensis*. As per morphology, *Cyllamyces icaris* could be considered as the interconnecting link between the rhizoidal and the non-rhizoidal fungus. The rhizoid penetrates while the intermittent bulbous rhizoid helps in the rupturing of fibrous feed particles. This fungus holds special significance in the degradation of poor quality feed in ruminants.

Introduction

Anaerobic gut fungi are normal inhabitants of the digestive tract of wild and domesticated ruminants and large monogastric herbivores. They are important as they produce highly active enzymes for degradation of lignocellulose (Gordon and

Phillip, 1998; Mountfort and Orpin, 1994) and have a unique ability to break and penetrate the fibrous feed particles through fungal mycelium, providing more surface area for the action of other microbes. All the anaerobic fungi studied so far are cellulolytic and are able to degrade structural carbohydrates of plant cell walls playing a vital role in the digestion of high fiber poor- quality forage. Thus in tropical regions where forages are generally fibrous and of poor quality, the development of methods to manipulate superior strains of fungi, naturally selected or genetically engineered, in the rumen would afford a means of improving the digestion of poor – quality fodder by large ruminants particular lactating cows and buffaloes.

Initially rumen fungi were classified as Chytridiomycetes, in the order, Spizellomycetales, by Heath et al., (1983) but later through sequence analysis of the 18S nuclear rRNA region and other numerical taxonomic methods they were assigned to the order Neocallimasticales of Chytridomycota (Li and Heath, 1992; Li et al., 1993). The family Neocallimasticacea comprises six genera which include *Neocallimastix, Piromyces, Caecomyces, Orpinomyces, Anaeromyces,* and *Cyllamyces* (Ho and Barr, 1995, Ozkose et al., 2001). The first three genera contain monocentric species while the latter three are polycentric in nature. *Cyllamyces* genus was recently included in the family Neocallimasticaceae with the characteristic features of the type species *Cyllamyces aberensis* being a multisporangiate thallus, single bulbous holdfast, multiple spherical sporangia, branched sporangiophores and uniflagellate zoospores (Ozkose *et al.,* 2001).

Earlier, we reported the occurrence of the *Cyllamyces* genus first time in India and Asia in buffalo (Sridhar *et al.,* 2007). The stable morphological features similar to the type species *Cyllamyces aberensis* are multiple spherical to oval sporangia, branched sporangiophores and uniflagellate zoospores. The dissimilar features include the presence of distinct elongated rhizoids and multiple bulbous holdfast. Here we report the life cycle ,morphology and molecular characterization of a new species of *Cyllamyces* obtained from the dung of the Indian water buffalo *Bubalus bubalis,* and the features which distinguish it from the hither to known species *Cyllamyces aberensis.*

Materials and Methods

Source of Samples

The Buffalo dung samples were obtained from Veterinary College Farm, Chennai. The sample was transported immediately to the laboratory and processed for isolation of anaerobic fungi (Sridhar et.al.,2010).

Isolation

The dung samples were 10 fold serial diluted with anaerobic diluting solution before inoculation into anaerobic culture medium based on the description of Phillips & Gordon (1988, 1989). Inoculated Hungate roll tubes (Belco Germany) were kept at 39^0 C for 72 hr. and individual developed fungal colonies were then transferred into broth tubes under a gentle steam of CO_2. The inoculated broth tubes were incubated at 39^0 C for 72 h and sub cultured weekly. Stock cultures of the isolates were maintained in 10% glycerol stored under liquid nitrogen. Aliquots taken with the help of sterile syringes from the broths were observed under a Nikon Eclipse 50i microscope to identify the isolated fungi. Further culture of

Cyllamyces was carried out inside on anaerobic chamber (Coy Laboratory, USA) having an atmosphere of 90-95% carbon-di-oxide and 5-10% hydrogen.

Morphological characterization and life cycle studies

For the examination of zoospores a small amount of culture medium was removed from the culture tube by the help of a syringe and placed on a clean microscope slide. For examining the vegetative growth fully grown biomass was removed and teased apart with a sterile loop before placing suitably small pieces on the slide for observation. Once the sample was placed on the slide it was overlaid with a cover slip before being observed by phase contrast microscopy. The overall appearance of the fungi was observed at 100-200X while details of structure, such as the numbers of flagella on zoospores, were observed at 400 X magnification. The method described by Ozkose et al. (2001) was adapted for the life cycle study of the isolate with suitable modifications. Representative photographs were taken every 1h up to 6 h and thereafter at every 2h interval for up to 24 h using a Nikon Coolpix 5400 digital camera fitted to a Nikon Eclipse 50i microscope (Nikon, Japan). The measurements were done after calibration with a stage micrometer.

Nuclear Staining

Nucleus of the zoospores were observed by ultraviolet fluorescence microscopy (Nikon Eclipse 50i microscope fitted with 575nm excitation and 590 nm barrier filters) after staining with fluorochrome, Propidium Iodide (Sigma, USA) 0.01% w/v in Phosphate buffered saline. One mL of 25% (v/v) glutaraldehyde solution was added to kill the zoospores before staining. The fluorochrome (10µl) was added to the samples and left for 10 min to stain.

Enzyme assays

Endoglucanase, xylanase and β-Glucosidase activities were estimated as described earlier (Sridhar et.al.,2010). The activities of Endoglucanase and xylanase were expressed as µmole of reducing sugar released per min under the assay conditions while the activity of β-glucosidase was expressed as µmole of p-nitrophenol released per min under the assay conditions. One international unit (IU) of the enzyme activity was defined as the amount of enzyme that released 1 µmol of reducing sugar (expressed as glucose or xylose) and 1 µmol of p-nitrophenol per min. Protein was estimated as per the method of Lowry et al., (1951). All enzyme assay were carried out in three replicates and the data were analyzed by one way ANOVA and the differences between the means were compared by the Duncan multiple range test (Steel and Torrie, 1980). The action of the fungi on paddy straw fermentation was also observed by inoculating fresh culture into media containing 1% straw and observing the changes in straw particles under a using a Nikon Coolpix 5400 digital camera fitted to a Nikon Eclipse 50i microscope (Nikon, Japan) .

Molecular characterization

The fungal biomass was initially obtained by growing it in 100ml anaerobic broth medium in thick-walled bottles (Scott-Duran, Germany) in anaerobic chamber (Coy Laboratory, USA). After 5 days of incubation at 39°C, the inner surfaces of the bottle were scrapped by the help of a sterile cell scrapper to remove the adhered fungal biomass and centrifuged at 5000 X g . The

supernatant was removed carefully and the pellet ground under aseptic conditions using liquid nitrogen. The genomic DNA of the isolate was extracted by CTAB method (Brownlee, 1994). The ribosomal Internal transcribed spacers 1 and 2 were amplified by PCR using primer set: 18S forward primer JB206 (GGA AGT AAA AGT CGT AAC AAG G) and the 28S reverse primer JB205 (TCC TCC GCT TAT TAA TAT GC). The ribosomal fragments were ligated with pGEMT-Easy vector® (Promega, USA) and *E.coli* (DH5α) competent cells were transformed by heat shock method (Sambrook and Russell, 2001).

Multiple transformed clones (white colonies) were selected by colony PCR with JB205 and JB206 primers. Plasmid extracted (Plasmid Miniprep®, Qiagen,) from the PCR positive colonies (Overnight cultures) were reconfirmed by restriction digest with *EcoR* I. (New England Biolabs, U.K.). The sequencing of the positive plasmids was carried out by MWG-Biotech (Bangalore, India) using M13 universal forward and reverse primers.

Sequence analysis

The sequence of *Cyllamyces icaris* (Genbank accession no: EU043229) was analyzed as per Tuckwell et al., (2005) and Brookman et al., (2000). Multiple sequence alignments with the existing sequences from Genbank (Table 1) were generated using CLUSTAL (Thompson et. al. 1997). The sequences were manually edited by MEGA software (Kumar et. al. 2004) and only the Internal transcribed spacer region 1 was considered for the Phylogenetic analysis. Maximum parsimony and minimum evolution analyses were performed by MEGA software. In maximum parsimony analysis, ‘‘close-neighbor interchange (CNI) with search level 1’’ was opted. The consensus tree was generated by 1000 bootstraps replicated with two random addition trees for each replicate. The minimum evolution result was obtained by the neighbor joining method with 1000 bootstrap replication (Brookman *et al.,* 2000). Evolutionary relationships of 30 ITS I sequences of anaerobic gut fungus were retrieved from Genbank using the Neighbor-Joining method and phylogenetic analyses were conducted in MEGA4 (Tamura *et. al.*2007).

Result and Discussion

Cyllamyces icaris

(Sridhar, Kumar et Anandan ,sp.nov.)

Strictly anaerobic fungus with polycentric multi sporangiate thallus, multiple bulbous holdfasts, indeterminate as well as intermittent bulbous rhizoids, terminal ovoid sporangia, nodular short branched sporangiophores, and zoospores uniflagellate rarely biflagellate.

Type species

The isolate (NIANP#CB3B1) is held both as fresh isolate as well as frozen in liquid nitrogen and at -80^{o}C in the microbial culture collection of the Bioenergetics and Environmental Science Division, National Institute of Animal Nutrition and Physiology, Bangalore, India.

Etymology

The word “icaris” is from the abbreviation, meaning “ Indian Council of Agricultural Research.”, New Delhi, the headquarters for agricultural research in India.

Habitat

The rumen of *Bubalus bubalis.*

Morphological characterization and life cycle studies

The zoospores of *Cyllamyces icaris* were usually spherical in shape and mono-flagellated (Fig1A) and propidium iodide staining revealed the zoospores to be uni-nucleated (Fig 1B). Occasionally, biflagellate zoospores were also obtained (Fig 1C) (2% n=200). Some other shapes of zoospores also obtained were oval (Fig1D), elliptical (Fig1E) or tripod shaped (Fig1F) but these were observed with a percentage of less than 1% each. Zoospores were observed to have a diameter range of 7.7 - 14.13 µm (11.13 ± 1.39 µm; *n*= 480) while the average length of the flagella was 59.50 ± 10.33 µm (*n*= 410) .The zoospores became inactive and shed the flagellum and encysted within 1 h of release from sporangia and developed into a holdfast (Fig.2A). The origin of the growth of rhizoid from the encysted zoospore was observed within 2 h and could be uni-polar or multi-polar (Fig.2B). At 3h the typical rhizoid with an apical bulge (dumbbell-shaped appearance) was observed (Fig.2C). The bulge could probably become a rhizoid/holdfast at its later developmental stages .The formation of the holdfast and emergence of rhizoid was seen at 4h.(Fig.2D). The rhizoid elongated to 40.04 ± 7.2 µm (*n* = 10) in length within 4h of the zoospore release. The rhizoid elongated further and more or less doubled in length at 5h (Fig.2E).Invariably, the encysted zoospore gave rise to a holdfast from which one or more rhizoids originated (Fig.2F). Formation of sporangiaphores from rhizoid was observed at 7h (Fig. 3.A). Formation of another holdfast and emergence of another indeterminate rhizoid was seen at 8h (Fig.3.B). Maturation of sporangia and release of zoospore at tip was obtained at 10h (Fig.3.C). Further multiplication was evident at 12h, development at 14h and complete maturation at 16 h (Figs.3.D). The rhizoid length increased further to 67.77 ± 13.6 (*n* = 5), 102.87 ± 14.37 (*n* = 22), 136.57 ± 15.5 (*n* = 29) and 206.32 ± 31.46 µm (*n* = 22) at 6, 8, 10 and 12 h of the zoospore release. The increase in rhizoid length was accompanied by the intermittent formation of holdfasts. The formation of sporangium (19.33 ± 6.78 µm; *n*= 45) and the maturation of zoospores occurred between 10 to 12 h of the parental zoospore release. The fully mature isolate with developed sporangia, hold-fast, nodular and bi-furcated sporangiophores, immature and mature sporangia is shown in Fig.4 . From an initial diameter of 14 µm (14.63 ± 0.26 µm [Mean ± SD]; *n*= 100), the bulbous holdfast grew up to 86 µm (56.64 ± 13.42; *n*= 150) after 24 h at full maturity.

The role of the anaerobic gut fungus in the rumen as the primary invader of the fibrous feed particles is well established (Orpin, 1975). The mode of degradation of the plant fiber differs between the rhizoidal (*Neocallimastix*, *Piromyces*, *Orpinomyces* and *Anaeromyces* sp.) and the non-rhizoidal (*Caecomyces*, *Cyllamyces aberensis*) fungi. The rhizoidal fungi are reported to be potent degraders, producing an extensive network of branched and tapering rhizoids to aid in substrate colonization. The non-rhizoidal fungi contribute in fiber degradation by penetrating and rupturing, respectively. *Caecomyces* spp. produce a more limited thallus, and it is proposed that they contribute to degradation by expanding from within and rupturing colonized tissues (Joblin, 1989). Morphologically, the missing link between rhizoidal fungi and *Caecomyces* spp. was fulfilled by *Cyllamyces aberensis*. Even though it needs further investigation, the new isolate *Cyllamyces icaris* certainly

Fig.1.AtoF Zoospores of *Cyllamyces icaris* A. Mono-flagellated Phase contrast image (⟶= flagellum), bar=10µm.B. Uni-nucleated (Fluorescent imaging) (⟶= nucleus) bar=10µm.C.Bi-flagellated zoospore(⟶= flagellum) bar=10µm. D. oval mono-flagellated zoospore (⟶= flagellum) bar=10µm .E. elliptical mono-flagellated zoospore (⟶= flagellum) bar=10µm and F. tripod shaped mono-flagellated zoospore(⟶= flagellum) bar=10µm.

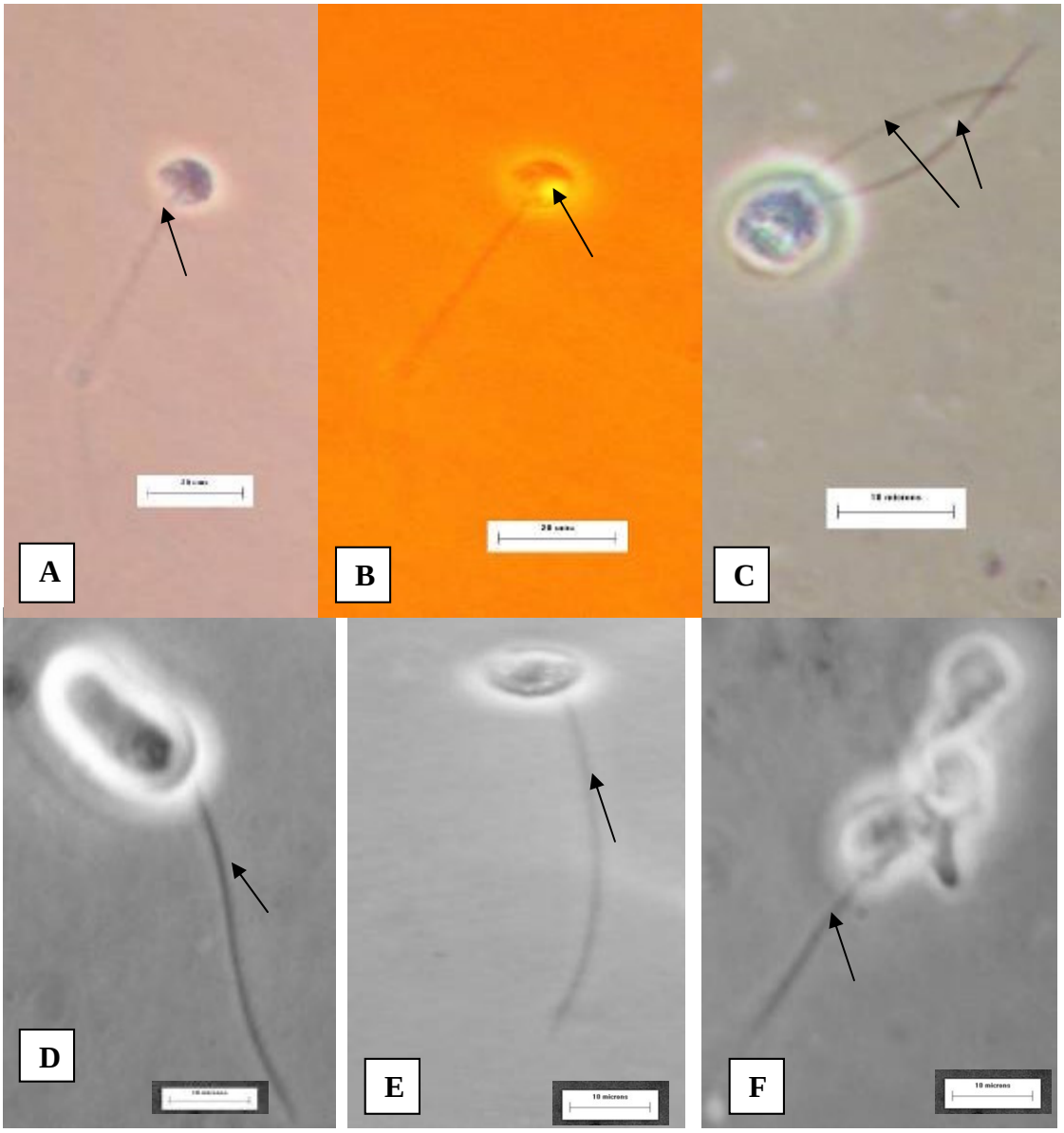


Fig.2 A to F Early life cycle of *Cyllamyces icaris* A. Encysted zoospore after release of flagella within 1h bar=10µm.B.Initiation of growth of the rhizoid/holdfast at 2h bar=10µm. C. Formation of dumbbell shaped sporangium from the holdfast at 3h bar=10µm. D. Formation of holdfast and emergence of rhizoid at 4h bar=10µm.E. Elongation of rhizoid 5h bar=10µm.F . Multiplication of rhizoid at 6h bar=10µm .

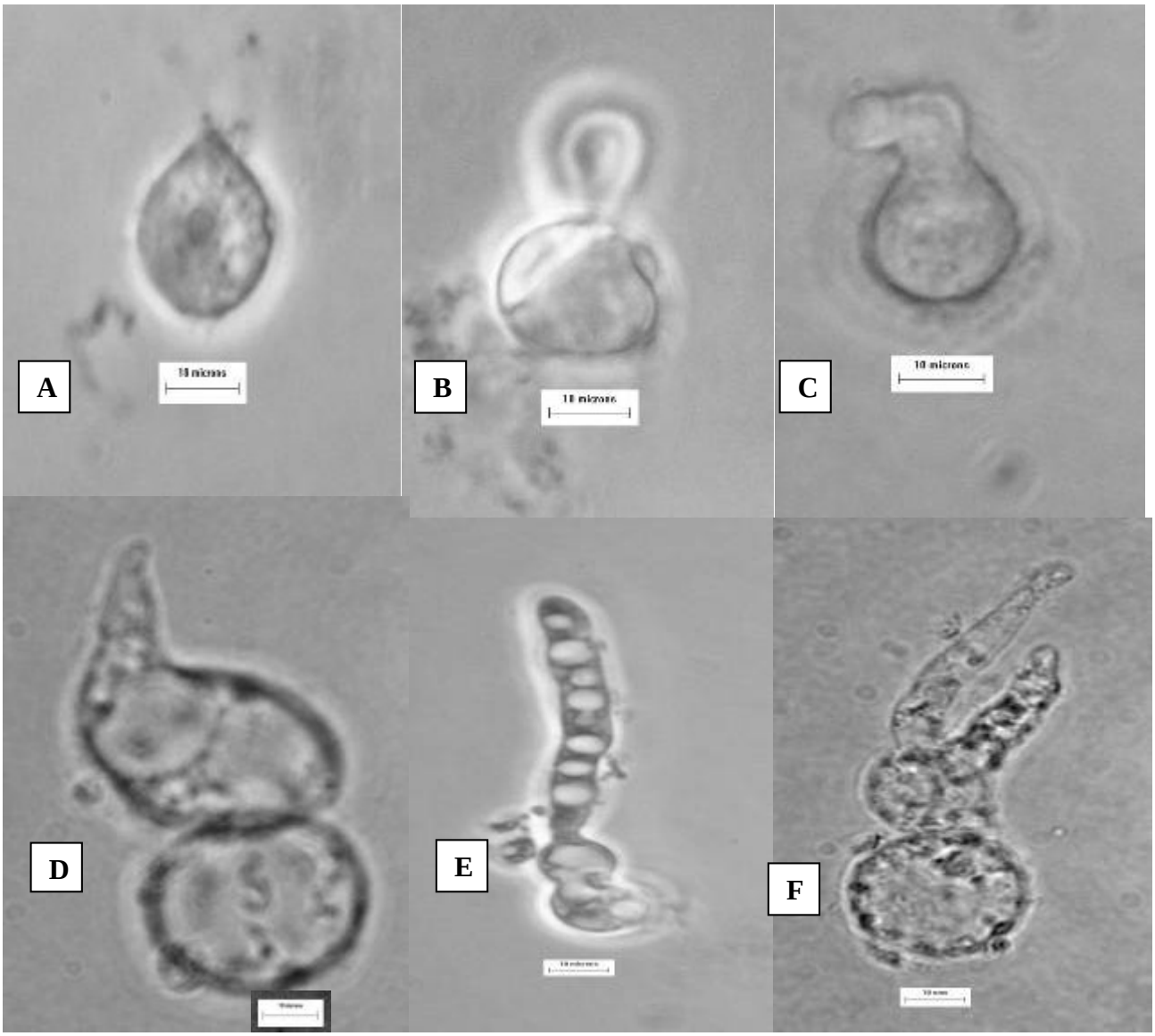


Fig.3 A to D Maturation of *Cyllamyces icaris* bar=10µm. A. Formation of sporangiaphores from rhizoid at 7h bar=10µm .B. Formation of another holdfast and emergence of rhizoid at 8h. bar=10µm C. Further multiplication and complete maturation at 16 h. bar=10µm.D. A. Fully matured *Cyllamyces icaris* at 24h, bar=10µm

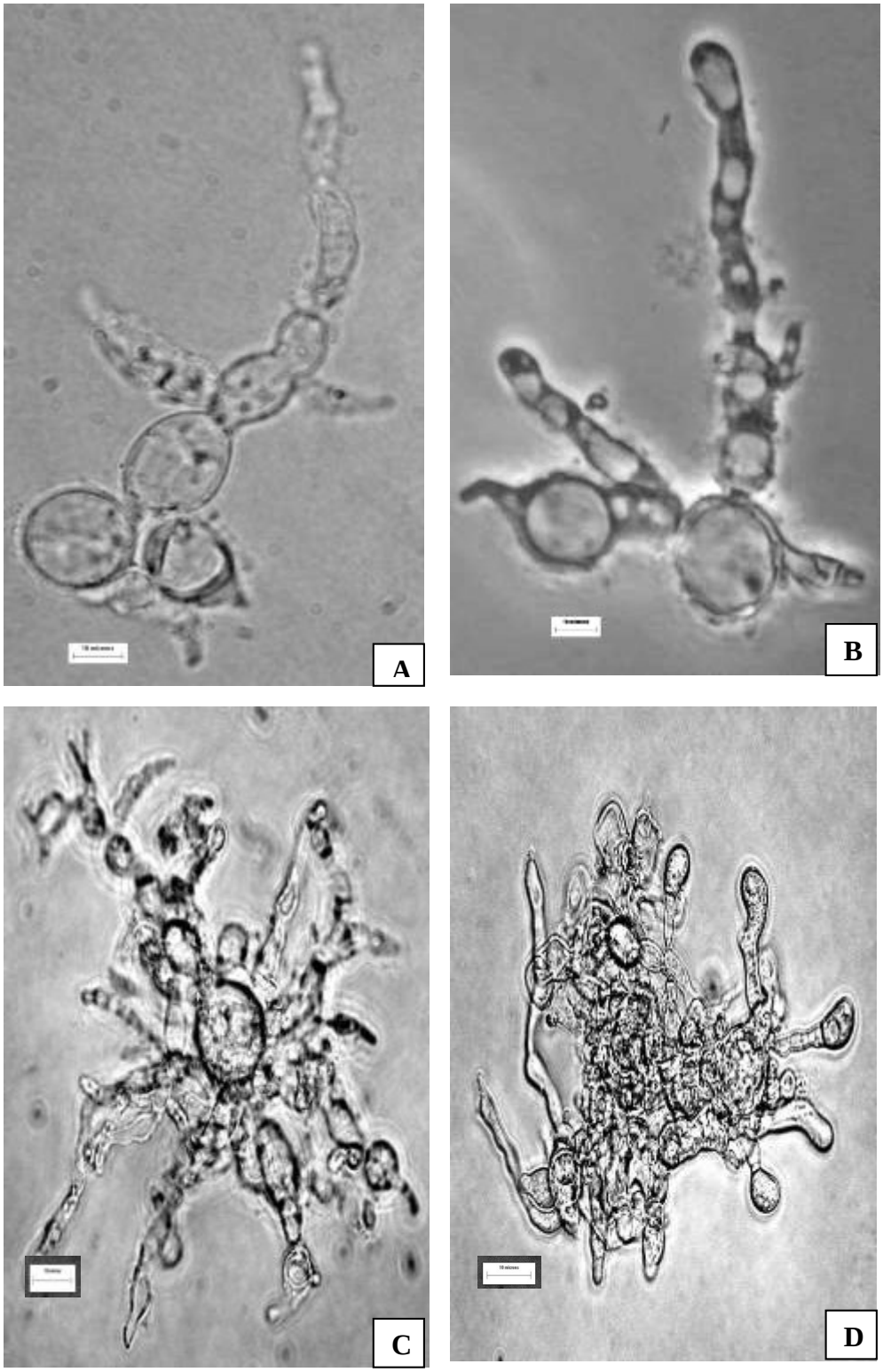
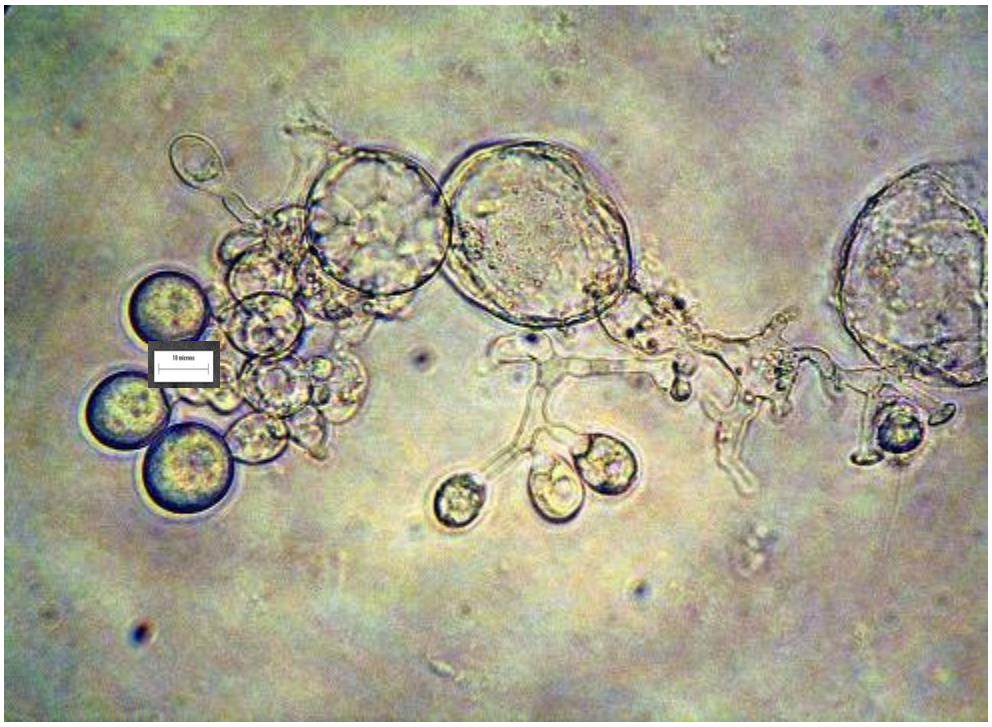


Fig.4 Fully mature isolate showing the development of sporangia 1= Hold-fast,2= Bi-furcated sporangiophore 3= Immature sporangia 4= mature sporangia 5= sporangial sac after zoospore release 6=released zoospore, bar=10µm



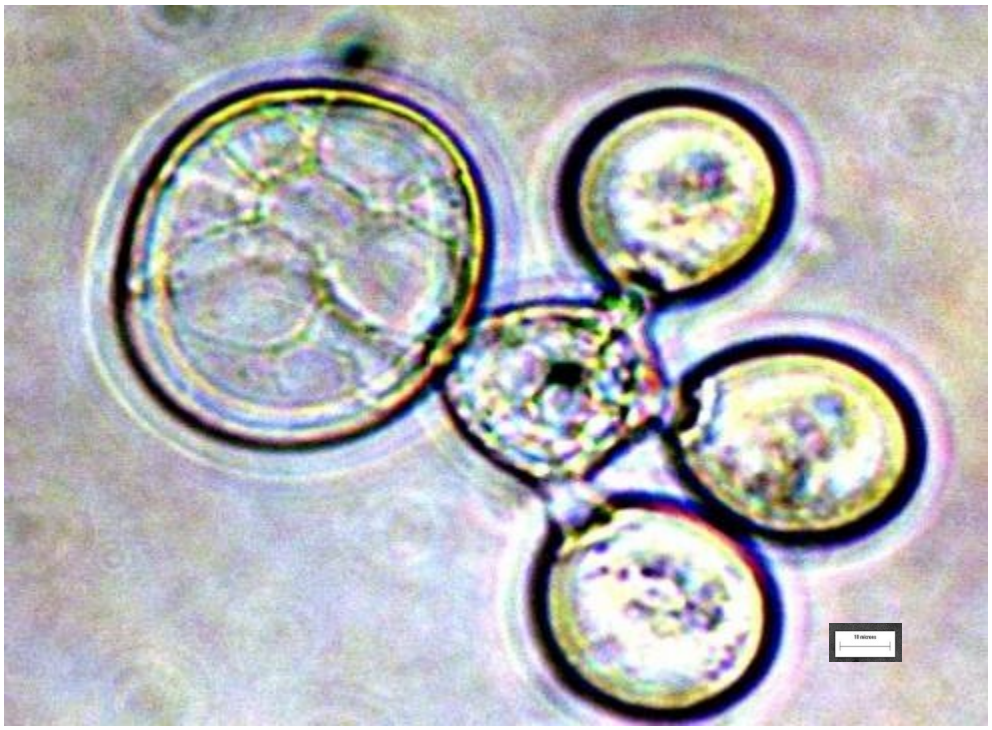


Fig.5 Degradation of paddy straw by *Cyllamyces icaris* A. at12 and B.at 24 after incubation, bar=10µm

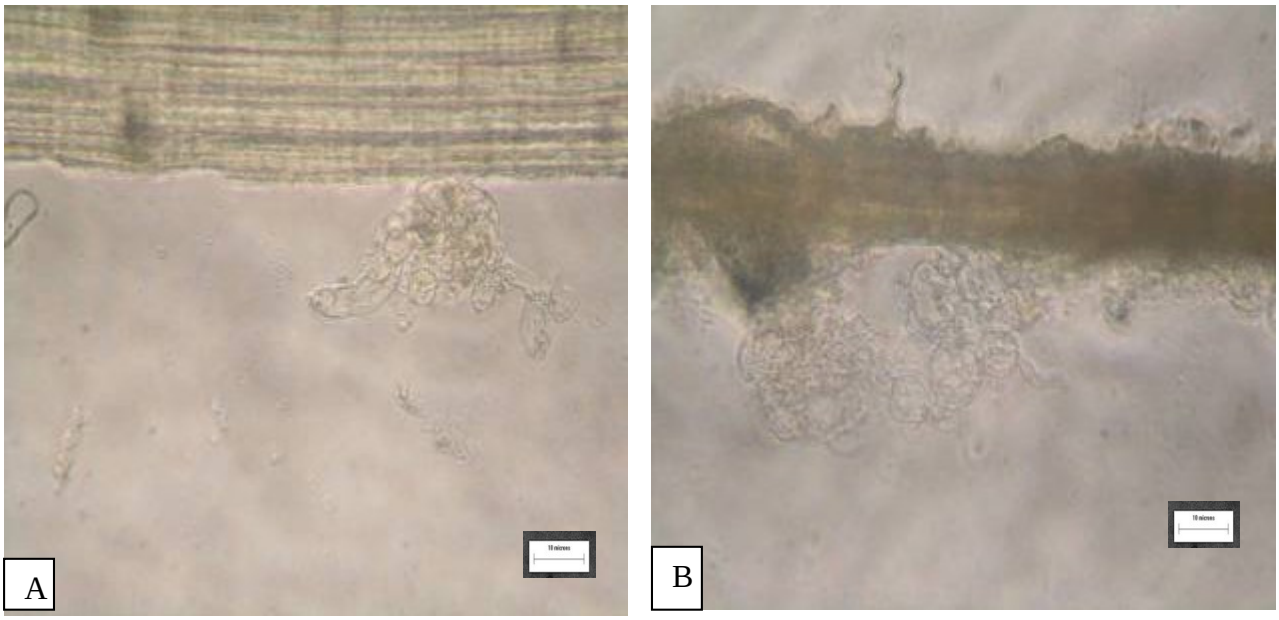
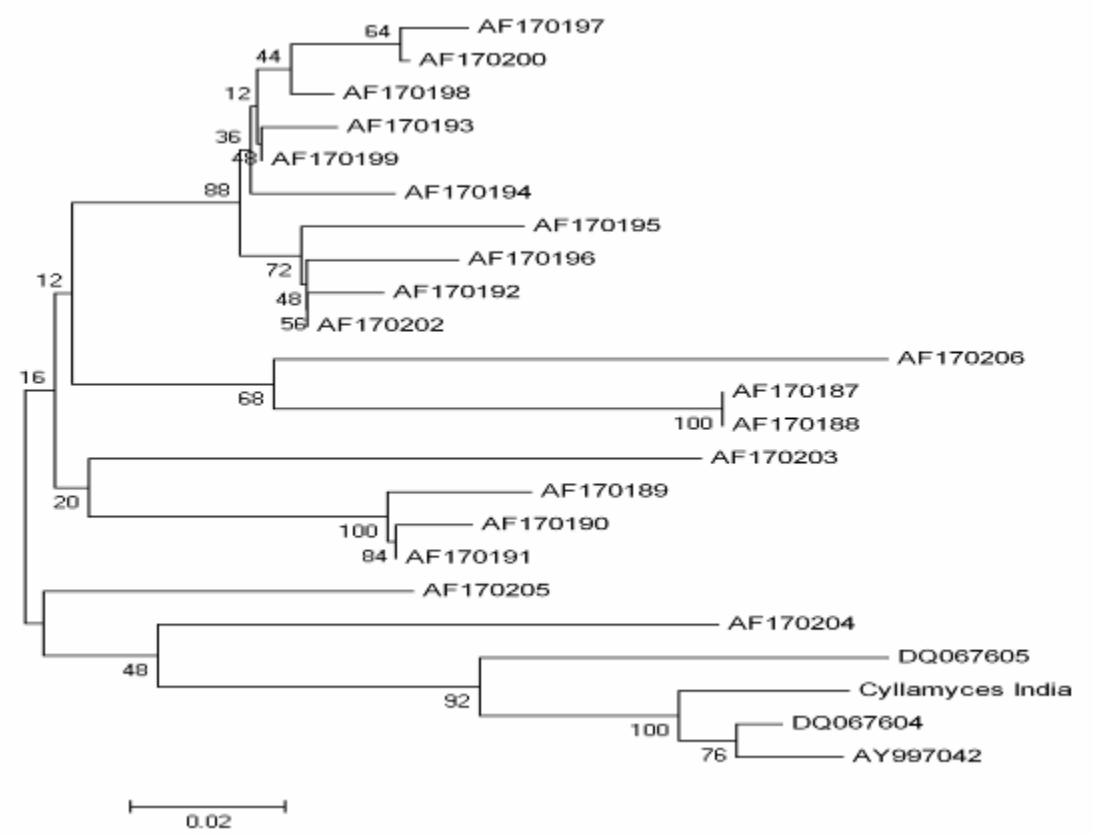


Fig.6 Evolutionary relationships of 30 ITS I sequences of anaerobic gut fungus retrieved from Genbank using the Neighbor-Joining method. Phylogenetic analyses were conducted in MEGA4 (Tamura *et. al.*, 2007).



has the cumulative effects of fiber penetration (as rhizoidal fungi), rupturing (like in *Caecomyces* spp.) and a fastidious growth pattern.

The genus *Cyllamyces* is characterized by multisporangiate thallus, single bulbous holdfast multiple spherical sporangia, branched sporangiophores, and uniflagellate zoospores (Ozkose et al 2001). Morphologically, *Cyllamyces icaris* resembled the type species *Cyllamyces aberensis* in multiple oval sporangia, branched sporangiophores and uniflagellate zoospores and differentiates in the presence of distinct rhizoid, shorter nodular sporangiophores, fewer sporangia (one to maximum three) multiple bulbous holdfast and fastidious growth pattern (complete life cycle within 14-16 hrs). The sporangium diameter of *C. icaris* (19.33 µm) was found to be larger than that of *C. aberensis* (14.7 µm) and also the bulbous holdfast which grew up to 86 µm (56.64 ± 13.42; *n*= 150) after 24 h at full maturity was much larger than the single bulbous holdfast reported in *C. aberensis* which grew to a diameter of up to 54 µm (33.9± 6.0 µm; *n* = 40) after 24 h (Ozkose et.al., 2001). Also our isolate produced fewer sporangia, as well as shorter branched sporangiophores as observed by Ozkose et al., 2001. Apart from the distinctive branched sporangiophores and polycentric development of *C. icaris*, a number of other features also set this organism apart from the wide range of morphological forms observed among *Caecomyces* spp. *Cyllamyces icaris* can be easily differentiated from *Caecomyces equi* (Gold *et al.*, 1988) and *Caecomyces communis* (Orpin , 1976) by the presence of rhizoid and multiple intermittent bulbous rhizoids. The sporangia of *C. icaris* are much smaller (19.33 µm as against 40 µm) than that of *Caecomyces* sp. (Wubah and Fuller, 1991). However, differentiating it from *Caecomyces sympodialis* requires further morphological characterization. In *C. sympodialis*, the germinating stalk from the bulbous holdfast is a sporangiophore which gives rise to sympodial sporangia; while, in *C. icaris* the stalk may be a rhizoid or sporangiophore which elongates further to produce bulbous rhizoid or terminal sporangium, respectively.

Enzyme assays

The degradation of a paddy straw particle by *C. icaris* at twelve and end of twenty four hr period is shown in Fig.5A&B.The extensive colonization is evident. This is another distinguishing fact between *Caecomyces sympodialis* and *Cyllamyces icaris* as the former cannot utilize xylan or clump paddy straw (Chen et.al.,2007) while in the case of the latter clumping of particles was evident and clumping of paddy straw was observed within 24 hrs of fungus inoculation (filtered through 11µm micro filter). Lack of rhizoid may be the reason behind the inability of *Caecomyces* to clump paddy straw. Most of the *in vitro* studies on the location of fiber-degrading enzymes produced by anaerobic fungi indicate that they are predominantly extracellular and free in the culture fluid (Mountfort and Asher, 1989; Pearce & Bauchop, 1985; Teunissen *et al.*, 1992).

The anaerobic fungi produce a wide range of polysaccharide degrading enzymes and are known to have high cellulolytic and xylanolytic activities which have been found associated with the rhizomycelium and are also secreted into the surrounding environment. The enzymes estimated in the present experiment are the major enzymes responsible for the degradation of lignocellulosic feed and the results are recorded in Table-2. The highest β-D-

glucosidase activity of 3459.00±104.39 units was obtained with starch followed by 593.00±49.66 units with cellobiose as substrate while lowest activity of 2.04 units was obtained in the case of filter paper.β-endoglucanase ranged from 9.00±06.21 with glucose to 01.60±0.09 units with filter paper as substrate. Cellobiose yielded the highest xylanase activity 66.70±11.54 units while the lowest activity of 00.82±0.04units was obtained with filter paper. *C.icaris* was thus characterized by high endoglucanase, xylanase and β - glucosidase activities which act on plant structural polysaccharides.

The role of the anaerobic gut fungus in the rumen as the primary invader of the fibrous feed particles is well established. The mode of degradation of the plant fiber differs between the rhizoidal (*Neocallimastix*, *Piromyces*, *Orpinomyces* and *Anaeromyces* sp.) and the non-rhizoidal (*Caecomyces*, *Cyllamyces aberensis*) fungi. The rhizoidal fungus and *Caecomyces* sp. (Joblin,1989) contribute in fiber degradation by penetrating and rupturing, respectively. As per morphology, *Cyllamyces icaris* could be considered as the interconnecting link between the rhizoidal and the non-rhizoidal fungus. The rhizoid penetrates while the intermittent bulbous rhizoid helps in the rupturing process of the fibrous feed particles.

Molecular characterization and Sequence analysis

DNA-based techniques have been adopted for understanding the phylogenetic relationships and diversity of micro-organisms in natural ecosystems as they introduce considerably fewer biases in sampling than culture-based methodologies. They can be generated directly from DNA and are considered more representative of the entire community than culture-derived data alone (Ward *et al.*, 1990).

Favored indicators of genetic diversity are the rRNA encoding gene sequences, particularly the internal transcribed spacers ITS1 and ITS2 and the intervening 5-8S rDNA; these can be used both to identify micro-organisms and to determine phylogenetic relationships within communities (Hausner *et al.*, 2000; Vainio and Hantula, 2000; Ranjard, *et al.*, 2001), including the gut fungi (Brookman ,*et al.*, 2000). There is a high degree of conservation in 18S rRNA gene sequences across the *Neocallimastigales*, for which morphological criteria have been used as the principal means of classifying the six genera and their species that constitute the order. There has, however been more recent progress on the use of internally transcribed spacer region sequences as a reliable means of identifying anaerobic fungi to the genus level (Brookman, et.al.,2000., Tuckwell et.al., 2005).Thus for phylogenetic analysis only the ITS I region was taken into consideration.

Variable Region sequence motifs for the Caecomyces and Cyllamyces ITS1 sequences as per Tuckwell *et al.* (2005) are enlisted in Table III. Motif sequences are given in the form for PERL regular expressions, such that the symbols or represent any character or space, and where variable numbers of residues are shown as e.g. T{2,4}, representing any number of T residues between 2 and 4. Motifs were drawn from sequences 62–96 for Variable Region I; 130–196 for Variable Region II; 198–266 for Variable Region III and 264–318 for Variable Region IV. Motif sequences for Variable Regions II–IV for the *Cyllamyces* genus fingerprint were modified to incorporate the additional sequence data (Table 3). As indicated, the

Table.1 Fungal isolates listed for ITS1 sequence analysis

Code	Accession No.	Fungus Isolate (*Neocallimasticaceae*)
NCS1	AF170194	*Neocallimastix frontalis* isolate NCS1
NCS2	AF170195	*Neocallimastix* sp. JB-1999 isolate NCS2
MCH3	AF170192	*Neocallimastix frontalis* isolate MCH3
NMG2	AF170196	*Neocallimastix frontalis* isolate NMG2
NMW2	AF170198	*Neocallimastix frontalis* isolate NMW2
NMW3	AF170199	*Neocallimastix* sp. JB-1999 isolate NMW3
NMW4	AF170200	*Neocallimastix* sp. JB-1999 isolate NMW4
NH	AF170193	*Neocallimastix hurleyensis*
NP	AF170197	*Neocallimastix patriciarum*
NUC1	AF170202	*Neocallimastix frontalis* isolate NUC1
PAC1	AF170203	*Piromyces* sp. JB-1999 isolate PAC1
PAK1	AF170204	*Piromyces* sp. JB-1999 isolate PAK1
PCG1	AF170205	*Piromyces* sp. JB-1999 isolate PCG1
PCS1	AF170206	*Piromyces* sp. JB-1999 isolate PCS1
AUC1	AF170187	*Anaeromyces* sp. JB-1999 isolate AUC1
AUC2	AF170188	*Anaeromyces* sp. JB-1999 isolate AUC2
OUC1	AF170190	*Orpinomyces* sp. JB-1999 isolate OUC1
OUC2	AF170189	*Orpinomyces* sp. JB-1999 isolate OUC2
OUS1	AF170191	*Orpinomyces* sp. JB-1999 isolate OUS1
Cyll	AY997042	*Cyllamyces aberensis* isolate AFTOL-846
W101	DQ067604	*Caecomyces sympodialis* isolate W101
CY50	DQ067605	*Caecomyces communis* isolate CY50
CB3B1	EU043229	*Cyllamyces icaris* Indian isolate CB3B1

Table.2 Activities of β-D-glucosidase, β-endoglucanase and Xylanase obtained upon growth of *Cyllamyces icaris* in the various media

Enzyme activity (Units/ ml)			
Media	**β-D-glucosidase***	**β-endoglucanase ****	**Xylanase *****
Glucose	13.50±2.62	9.00±06.21	55.00±3.91
Cellobiose	593.00±49.66	6.60±1.40	66.70±11.54
Xylan	157.00±19.73	4.70±0.92	28.90±1.04
Starch	3459.00±104.39	5.50±0.32	29.30±2.63
Filter paper	2.04±1.04	01.60±0.09	00.82±0.04

* Activity expressed as μ mole p-nitrophenol released min^{-1} ml^{-1} of culture filtrate

** Activity expressed as μ mole of glucose released min^{-1} ml^{-1} of culture filtrate

*** Activity expressed as μ mole of xylose released min^{-1} ml^{-1} of culture filtrate

Table.3 Variable Region sequence motifs for the Caecomyces and Cyllamyces ITS1 sequences. Motifs were drawn from sequences 62–96 for Variable Region I; 130–196 for Variable Region II; 198–266 for Variable Region III and 264–318 for Variable Region IV Tuckwell et al. (2005).

Variable Region	Motif	Motif sequence	Sequence group
I	7	GAGACCTC(T{2,4})GAAGGTC	*Caecomyces*
	8	GAGACCT(C|)GATT(T{1,3})GAAGGTC	*Cyllamyces*
II	9	TGTTTGAAATAATTTTTTT(T|)CCAAAAAAA(A|)TTATTGAA TGTCTTT	*Caecomyces*
	10	({T1,2})(G|)({T{2,5})(A{1,2})({T{1,3})(A{1,3})(C|)(T{1,2})(G|)(T|)GTC(T{2,3}	*Cyllamyces*
III	11	CCCTGTTTGGTGGAGCTTGTAAAAGTGAAACCATCAGGG)	*Caecomyces*
	12	CCCTGT(T|C)(T|)GGTTTGAAGCTTTGTTAAAAGTTTCG(A|G) ACCTTCAGGG	*Cyllamyces*
	13	CCCTG(G|T)TTTT(T|)GATGGAGCTTGT((T|C)|)(T|)(A{3,5})G(C |T)-	*Cyllamyces*
IV	7	(G|A)AAATC(A{2,3})(T{1,2})(C{1,2})A(G{2,3})	*Caecomyces*
	8	GGGGAATGGCAAACAATAA(T|)TTATTTTGAAAAA(A|)GAC CTT GGGGAATGGC(A|G)AAAC(T|C)T	*Cyllamyces*

Table.4 The ITS-I variable region motif sequences of *Cyllamyces icaris** as per Tuckwell et al., (2005).

Variable regions	Motif sequences	Position with respect to EU043229	Motif
I	GAAGGCCCGATGAGAGGGTC	61-80	New (assigned 9)
II	TTGTTTGTTTTTATAAACTGTGTCTTT	109-135	10
III	CCCTGCTTTTTGATGGAGCTTGTTAAAAAAGC AAAATCAAATTCAGGG	136-183	13
IV	GGGGAATGGCAAAACCT	181-197	8

***Genbank accession no: EU043229**

motif sequence for Variable region I of *Cyllamyces icaris* differs from the previous description proposed for *Cyllamyces* (Table 4) . Hence, we suggest here the inclusion of the 9th motif sequence of Variable region I for *Cyllamyces*. The motif sequences for variable regions II, III and IV were 10, 13 and 8, respectively.

As per the phylogenetic analysis, the published *Caecomyces sympodialis* isolate W101 (DQ0676040), *Caecomyces communis* CY50(DQ067605) and *Cyllamyces aberensis* isolate AFTOL-846(AY997042)as well as the Indian isolate CB_3B_1 (*Cyllamyces icaris*) are clearly grouped together (Fig. 6).

Lockhart *et al*., (2006) designed oligonucleotide primers for the 18S rRNA genes of members of the *Neocallimastigales* and used it in a nested PCR protocol to amplify 787-bp fragments of DNA from landfill site samples and demonstrated the occurrence of members of the *Neocallimastigales* outside the mammalian gut for the first time. Li and Heath (1992) used sequence data from a less well conserved ribosomal sequence, the internal transcribed spacer 1 region (ITS1) to compare and discriminate gut fungi. These authors were able to show that the genera *Orpinomyces*, *Neocallimastix* and *Piromyces* were closely related to each other and more distantly related to the genera *Anaeromyces* and *Caecomyces*. However, they failed to determine the relationships within the two cluster groups.

Li and Heath (1992) concluded that sequence data alone could not resolve the inter-relationships between these closely related genera. In a subsequent study, Li *et al*., (1993) used cladistic analysis of 42 morphological, ultrastructural and mitotic characters to attempt to determine the phylogenetic relationships of the anaerobic gut fungi. Dore and Stahl (1991) and Li and Heath (1992) both assessed the use of 18S rRNA sequences for the phylogenetic analysis of gut fungi. Dore and Stahl (1991) found very little difference between the 18S rRNA sequences of *Neocallimastix* and *Piromyces* ("97% identity) while a shorter 18S rRNA segment studied by Li and Heath (1992) could not distinguish between the genera *Orpinomyces*, *Neocallimastix* and *Piromyces*. Thus, while these studies were able to produce

trees showing the relationships between the gut fungi and other fungi} eukaryotes, the 18S rRNA sequence was clearly unsuitable for determining relationships within the group of gut fungi. The studies of Brookman, *et al.,* (2000) also showed, on average, pair wise identity of 97±4% for the 18S rRNA region which contrasts with the data from ITS1 sequences, which, as they show greater variation, are suitable for determining the relationships between gut fungi.

Conclusion

The role of the anaerobic gut fungus in the rumen as the primary invader of the fibrous feed particles is well established. The mode of degradation of the plant fiber differs between the rhizoidal (*Neocallimastix*, *Piromyces*, *Orpinomyces* and *Anaeromyces* spp.) and the non-rhizoidal (*Caecomyces*, *Cyllamyces aberensis*) fungi. The rhizoidal fungi are reported to be potent degraders, producing an extensive network of branched and tapering rhizoids to aid in substrate colonization. *Caecomyces* spp. produce a more limited thallus, and it is proposed that they contribute to degradation by expanding from within and rupturing colonized tissues (Joblin, 1989). Morphologically, the missing link between rhizoidal fungi and *Caecomyces* spp. was fulfilled by *Cyllamyces aberensis*. Based on both morphological and molecular analysis *Cyllamyces icaris* certainly has the cumulative effects of fiber penetration (as rhizoidal fungi), rupturing (as Caecomyces spp.) and fastidious growth pattern and is very different from *Cyllamyces aberensis* .

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