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# A MORPHOLOGICAL AND PHYSIOLOGICAL STUDY OF THE GENUS *TERMITOMYCES* HEIM IN SOUTH AFRICA

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# A MORPHOLOGICAL AND PHYSIOLOGICAL STUDY OF THE GENUS TERMITOMYCES HEIM IN SOUTH AFRICA

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## I. Introduction, statement of problem and objectives of this investigation

An introductory review is presented which will provide information regarding the general biology of termites and the fungi they cultivate. This information was compiled from scientific publications by Batra & Batra (1979) and Bels & Pataragetvit (1982). Fungus-growing termites belong to the insect order Isoptera and more specifically, to the subfamily Macrotermitinae which comprises twelve genera. They have an imperfect metamorphosis and different castes constitute a typical colony for example : (i) soldiers (ii) workers (iii) larvae or nymphs which are immature forms and (iv) winged reproductive alates, a king and queen. The physogastric queen is an egg producing machine and is essential for the continued survival of the specific termite species. A colony is initiated by a winged couple which mate and spend the rest of their lives in a subterranean royal chamber. The soldiers are typical for each termite species and are used for identification purposes. Fungus-growing termite genera exhibit an obligate mutualistic relationship with the paleotropical mushroom genus *Termitomyces* Heim and have a wide distribution from African savanna to South Asian rain forest and desert. Apparently the symbiotic relationship originated in Africa from where speciation of fungi and termites took place. Termitaria may consist of subterranean nests with no distinguishable surface structures or, in addition to the underground nest, may possess a conspicuous above-ground part, the mound. Foraging workers collect living, dead or decomposed plant material which is used to build fungus combs. The combs are firm, moist, sponge, brain or coral-shaped faecal masses consisting of finely divided plant material. These are built in fungus chambers underground which have a flat base and a domed ceiling. A termite colony may construct one large central fungus comb or may add several adjacent accessory combs of smaller size. The mycelium of *Termitomyces* permeates the whole comb and produces small, white, round asexual fruiting structures called mycotêtes, spherules, synnemata, conidial spheres, sporodochia or bromatia. Comb sporodochia bear conidiophores and conidia and represent the imperfect phase of the genus *Termitomyces*

in the natural habitat. The highly nutritious and edible basidiocarps appear during the rainy season and perish within a short period of time because of beetle and fly larvae infestation. After decades of poor identifications and the creation of several synonyms by various authors, Heim (1942 a) settled the matter by creating the genus *Termitomyces* for all members of the Basidiomycotina with a true symbiotic relationship with the termite subfamily Macrotermitinae. The main characteristics of this genus comprise the following : (i) carpophores with a small to large pileus and prominent umbo (perforatorium) (ii) lamellae free to sub-adnate (iii) spores pink in mass, nonamyloid, hyaline, ellipsoid and smooth (iv) basidia cylindrical, four spored (v) stipe usually prolonged into a long pseudorhiza which rises from a subterranean fungus comb. Heim (1977) divided the genus into two subgenera, Praetermitomyces and Eutermitomyces.

A search of the literature revealed that only a few papers on the genus *Termitomyces* in South Africa have been published (Bottomley & Fuller, 1921; Bottomley & Talbot, 1954; Coaton, 1961; Doidge, 1950; Lastovica, 1974; Louwrens, 1964; Reid, 1975; Van der Bijl, 1920; Van der Westhuizen, 1983; Van der Westhuizen & Eicker in press). Consequently our knowledge of this genus in South Africa is limited. The papers on this subject with the exception of Bottomley & Talbot (1954), Doidge (1950), Reid (1975), Van der Westhuizen (1983) and Van der Westhuizen & Eicker (in press), have been written in a popular vein and contained only a limited amount of scientific data. The objectives of this study were therefore : (i) to determine which species of *Termitomyces* occur in South Africa and to provide detailed morphological descriptions of basidiocarps and pure cultures (ii) to identify the associated termite host where possible (iii) to ascertain the nutritional value of selected *Termitomyces* species in comparison with *Agaricus bisporus* (iv) to investigate the usefulness of biochemical methods in resolving species differences (v) to investigate the growth requirements of certain species (vi) and to conduct a preliminary investigation into the probable artificial cultivation of *Termitomyces umkowaani*.

# II. REVIEW OF LITERATURE

1. **Morphological studies**
2. **General biological and ecophysiological studies**
3. **Nutritive value of *Termitomyces* species**
4. **Growth requirements and cultivation studies**

## 1. Morphological studies

### 1.1 Descriptions of *Termitomyces* species from the Orient

Otani (1979) described a termite associated mushroom, *Termitomyces albuminosus* (Berk.) Heim growing in association with the termite *Odontotermes formosanus* Shiraki from Ishigaki Island, Japan. According to Otani this species also occurs in Ceylon, India, Singapore, Java, Borneo and Taiwan. Cheo (1942,1945,1948) studied this species in south-western China and made several observations of the termite-fungus relationship. Cheo (1942) concluded that the conidial phase growing on the fungus combs and described b Ciferri (1935) as *Aegerita duthei* Berk., was in reality the anamorph of *T. albuminosus*. Bels & Pataragetvit (1982) explored certain regions in Thailand and recorded the following species : *T. clypeatus* Heim, *T. fuliginosus* Heim, *T. globulus* Heim & Goossens and *T. mammiformis* Heim. Otani & Shimizu (1981) collected *T. clypeatus* growing in a subtropical rain forest on Iriomoto Island, Okinawa, Japan. Zang (1985) created a new genus, *Sinotermitomyces* Zang after examining undescribed termitophylous agarics in the province of Yunnan, China and he created two new species in this genus.

### 1.2 Records from India

Publications by Berkeley (1847,1882) and by Berkeley & Broome (1862, 1871) represented early attempts to describe fungi associated with termite nests. Several authors in succession described similar termite fungi and created in the process a series of synonyms. Petch (1906, 1913) placed thirteen of these synonyms under the name *Collybia albuminosa* (Berk.) Petch which grew in association with several *Odontotermes*

species in Ceylon. This mushroom together with *Entoloma microcarpum* (Berk. & Br.) Sacc. was collected by Bose (1923) on the island of Barkuda. The true symbiotic relationship between fungus and its termite host was not perceived until Bottomley & Fuller (1921) clarified the mystery. Aich, Roy & Samajpati (1977) collected *Termitomyces microcarpus* (Berk. & Br.) Heim, *T. eurhizus* (Berk.) Heim and *T. clypeatus* from West Bengal in India. Devi,Nair & Menon (1980) described *T. robustus* (Beeli) Heim from the Kerala state in India. Natarajan (1975, 1977a, 1979) in his series of four papers on South Indian Agaricales described the following species : *T. badius* Otieno, *T. clypeatus*, *T. microcarpus*, *T. indicus* Natarajan, *T. rabuorii* Otieno and *T. heimii* Natarajan. In another of his papers Natarajan (1977b) described another new species, *T. radicatus* Natarajan from India.

## 1.3 **Records from Africa and southern Africa**

The contribution of Heim to the genus *Termitomyces* is well known. In a series of papers stretching from 1940 to 1977, he produced an enormous amount of scientific data concerning all aspects of the termite-fungus relationship and taxonomy. Heim (1977) created two subgenera namely, Praetermitomyces and Eutermitomyces. He placed species lacking a subterranean pseudorhiza in the first subgenus and species with a true pseudorhiza in the second. Grassé & Heim (1950) described *T. medius* Heim & Grassé, a species which they considered should occupy an intermediate position between the two subgenera. Heim (1942a) used the following morphological characters to distinguish between different species : (i) basidiome size (ii) pileus colour and texture (iii) the shape of the central umbo or perforatorium (iv) and the presence or absence of velar remnants. According to Heim (1942b) these characters are subject to variation and are dependent on the structure, depth, size and nutritional value of the fungus combs as well as the resistance of the soil. Heim (1942b) considered the influence exerted by these sources of variation to be strong enough to create differences within and between species. Much of Heim's research conducted over many years has been condensed in his publication of 1977. Alasoadura (1966,1967) explored several vegetation zones in

Nigeria and he recorded six described *Termitomyces* species and four undescribed species. Alasoadura (1966) concluded that *Termitomyces* occurred in all the vegetation zones but seem to be more prevalent in the arid savanna regions than in the wet forest regions. Species recorded comprised the following : *T. clypeatus*, *T. globulus*,*T. mammiformis*, *T. microcarpus*, *T. robustus* (Beeli) Heim and *T. striatus* (Beeli) Heim. Zoberi (1973) provided a condensed description of these species and considered them highly palatable and an excellent source of protein. Pegler & Piearce (1980) and Piearce (1981) conducted a survey of the edible mushrooms of Zambia and provided detailed descriptions of seven *Termitomyces* species of which one was described by them as a new species, *T. titanicus* Pegler & Piearce. According to them this species produces the largest known basidiocarps, with pilei up to one meter in diameter and weighing approximately 3 kg. It is familiar to the local inhabitants who sell specimens at the roadsides. All collections were made in the copperbelt province of Zambia. Piearce (1987) provided a key to eleven species of *Termitomyces* for Zambia as well as an interesting discussion on the macromorphological variation which exists between the species of *Termitomyces*. He suggested that the macromorphology of *Termitomyces* basidiocarps depended on the identity and behaviour of the host termites as well as the peculiar nature of the symbiosis. Piearce (1987) stated that descriptions of *Termitomyces* species are only complete if such descriptions contain a reference to the termite host. Piearce (1987) observed that cultures of five different *Termitomyces* species prepared from the context of fruit bodies and comb sporodochia, were indistinguishable in the appearance of the colonies, the hyphal characters and the growth rate. He speculated that biochemical and genetical methods may assist in resolving species differences. Otieno (1964) reported the occurrence of several *Termitomyces* species in Kenya. He described five new species : (i) *T. narobiensis* Otieno (ii) *T. magoyensis* Otieno (iii) *T. biyii* Otieno (iv) *T. rabuorii* Otieno and (v) *T. tyleriana* Otieno. He placed *T. narobiensis* in the subgenus Praetermitomyces and the other species in the subgenus Eutermitomyces. Otieno (1964) provided condensed descriptions of a further nine species as well as an amended key for these species based on the key proposed by Heim (1958). In a later publication Otieno

(1968) described another new species *T. badius* from Kenya. *T. microcarpus*, *T. clypeatus* and *T. robustus* which also occur in the Congo (Heim, 1958) were collected by Otieno in Kenya. *T. magoyensis* can be found in Tanzania and *T. microcarpus* in Uganda and Tanzania. In all his descriptions of new *Termitomyces* species, Otieno omitted the identity of the host termite species which according to Piearce (1987), as already noted, is essential to include in any description of *Termitomyces* species. Pegler & Rayner (1969) provided taxonomic descriptions of *T.eurhizus*, *T. globulus* and *T. microcarpus* as their contribution to the agaric flora of Kenya. Morris (1986) and Williamson (1975) have recorded the *Termitomyces* species from Malawi.

Reid (1975) studied herbarium material of *T. umkowaani* (Cooke & Mass.) Reid comb. nov., collected in Natal near Durban in South Africa. Cooke and Massee described *T. umkowaani* in 1889 from specimens collected by Dr. Medley-Wood, as *Schulzeria umkowaani* (Cooke & Mass.) Sacc. To the indigenous population in Natal this mushroom is known as 'I' Kowe', while the names beefsteak or butter mushroom are more familiar to those of European extraction (Van der Bijl, 1920). Doidge (1950) mentioned the occurrence of this species at Grahamstown, Durban, Howick, Bloemfontein and Pretoria as well as the occurrence of *Entoloma microcarpum* on the soil of termitaria at Pretoria, Johannesburg, Klerksdorp and Pietermaritzburg in the Republic of South Africa. Reid (1975) provided a detailed description of *T. umkowaani* in his paper in which he mentioned the 'short inflated, thin-walled, barrel-shaped segments, up to 20,8 µm wide, lacking clamp connections' in the cuticular layer of the pileus. Van der Bijl (1920) observed the bell-shaped morphology of the pileus in immature specimens which tend to be club-shaped in the primordial phase. He stated that in mature specimens 'the cap frequently splits and the margin then turns upwards'. Bottomley & Talbot (1954) supplied a description of the I'Kowe, *Schulzeria umkowaani*. According to them this mushroom has a straw coloured umbonate cap with a long fusiform stipe and a blackish subterranean rooting base. Louwrens (1964) wrote a popular account on some of the mushrooms which she collected in the Transvaal. In this account she described and illustrated *Termitomyces cartilagineus*

(Transvaal varieties). Her description more or less resembles the description for *T.umkowaani* although she considered this species as a large variety of *T. cartilagineus.* Based on his own and Van der Bijl's descriptions, Reid (1975) decided that *Schulzeria umkowaani* is in reality a species of *Termitomyces* and therefore he created the new combination *Termitomyces umkowaani* (Cooke & Mass.) Reid comb. nov. Reid (1975) also studied herbarium material of *Entoloma sagittaeforme* (Kalchbr. & Cooke) Sacc. collected in Natal. He described the material in spite of the fact that the collection consisted only of immature fruit bodies with no basidiospores. The specific epithet, *sagittaeformis* refers to the arrow-shaped form of the fruit bodies as seen in section. This mushroom exhibited all the characteristics of the genus *Termitomyces*, therefore Reid created the new combination *T. sagittaeformis* (Kalchbr. & Cooke) Reid comb. nov. Heim (1977) placed *T. microcarpus* in the subgenus Praetermitomyces whereas Singer (1949) placed it in the genus *Podabrella* Sing. Singer (1962) recorded the following points in which *Podabrella* differs from *Termitomyces* : (i) carpophores lacked a pseudorhiza, veil and pigment (ii) primordia developed externally of termite nests (iii) epicutis of the pileus is a cutis and (iv) the carpophores are smaller in relation to species in the subgenus Eutermitomyces. Singer (1962) recommended that extensive cytological and electron microscopical research should be conducted on basidiospores to elucidate species differences. In an earlier publication Singer (1949) provided a key to the species based on Heim's (1958) key, with the addition of one species. The first report of the occurrence of *T. microcarpus* in South Africa was made by Bottomley and Fuller (1921), who observed the appearance of this small agaric in Pietermaritzburg, Natal and Pretoria and Klerksdorp in the Transvaal, where it grew on finely divided comb scattered over the soil surface. These authors noted that this mushroom was associated with *Odontotermes vulgaris* (Hav.), *O. transvaalensis* Sjöst. and *O.badius* (Hav.) at the three locations respectively. Coaton (1961) reported the presence of *T. microcarpus* and *T. albuminosus*(Berk.) Heim growing on the combs of active colonies of *O. badius* in Pretoria, Transvaal. Coaton (1961) probably incorrectly identified *T. albuminosus* since this species

only occur in Asia (Heim, 1977). Coaton (1961) mentioned that he observed the mass fructification of this species in a humid sub-floor area of a building in Pretoria where it grew from the combs of *O.badius*.

## 1.4 Cultural characters and development of *Termitomyces* mycelium

A search of the literature revealed that apart from Heim (1940, 1977) and Piearce (1987), only Batra & Batra (1979) conducted any cultural and developmental studies of *Termitomyces* mycelium. Batra & Batra (1979) described the appearance of the mycelium and the 'spherules' or sporodochia on the fungus combs as well as the ontogeny of a sporodochium. They described the conidia as 'narrowly ellipsoid, usually catenulate, thin-walled and borne in verticils on the distil swollen cells of the sperule'. They observed that the micromorphology and ontogeny of the sporodochia, conidiogenous cells and conidia seemed to be identical for all the species of *Termitomyces* in India, Pakistan and Thailand. Heim (1977) made similar observations for the species of Africa. Batra & Batra (1979) studied the micromorphology of the cultures and observed the 'bulbil-like' ambrosial masses which are characteristic of symbiotic fungi of insects (Batra & Batra, 1967) and consist of aggregated conidiophores and conidia. They described the growth characters of comb sporodochia cultures and reported the presence of yeast-like masses of sprout cells and conidia that coalesce to form farinose sporodochial masses. They stated that the conidia did not germinate on agar media but that other blastic cells did with one or more germ tubes. According to Heim (1942 b) conidia and blastospores did germinate on artificial media and produced identical cultures. Heim (1977) illustrated and described certain hyphal elements from the basidiome context cultures of *T. mammiformis* and recorded the presence of blastospores on various nutrient media.

## 1.5 Chemotaxonomic studies

Rawla, Prasher & Sarwal (1985) succeeded in differentiating between three species of *Termitomyces*, by utilizing qualitative analysis of

free and bound amino acids. On the principle of the presence or absence of these amino acids they found differences in their chromatograms between *T. microcarpus*, *T. striatus* and *T. microcarpus* forma *sentalensis* (Berk. & Br.) Heim.

## 2. General biological and ecophysiological studies

### 2.1 Studies on physical factors prevailing in termitaria

Geyer (1951) monitored temperature fluctuations in a fungus comb chamber of *Odontotermes* (Hav.) at a depth of 25 cm below ground level, and in adjacent soil at the same depth, over a period of twelve months in a termitarium located on the experimental farm of the University of Pretoria. He reported maximum and minimum temperatures in the fungus comb chambers of 24,3 $^{0}$C and 11,1 $^{0}$C and from his measurements arrived at the following conclusions : (i) the maximum temperature recorded in the soil never exceeded that of the comb chambers during the year (ii) the highest temperatures were measured in the termite nest during the four summer months and (iii) the temperature of the comb chambers did not remain the same during the year, but followed that of the soil. This investigation was followed by that of Lüscher (1961), who conducted similar research in the Ivory Coast of Africa. He took measurements of the temperature and the $CO_2$ content in the comb chambers of *Macrotermes natalensis* (Hav.) and recorded readings of 29,7 $^{0}$C and 2,6 % $CO_2$. Lüscher (1961) explained the mechanisms utilized by this termite to regulate the microclimate of the nest : the elaborate system of vents in the mound above ground level permits the replenishment of oxygen in the termitarium and the elimination of surplus $CO^2$, while the thin wall of the mound permits exchange of oxygen and $CO^2$ along a steep gradient. Matsumoto (1977) determined the $CO_2$ concentration and the respiration rate of fungus combs in termitaria of three species of *Macrotermes* and in the mound of a non-fungus growing termite which served as a control. These studies were conducted in southern and northern Thailand where Matsumoto reported temperatures between 24,1 $^{0}$C and 28 $^{0}$C and $CO_2$ concentrations between 1,2 % and 5,2 % for the fungus-growers, compared to 0,5 % to 1,6 % for the non-fungus grower. He concluded that the high respiration rate

of fungus combs may contribute to the equally high $CO_2$ concentration in the termitaria of fungus-growers in comparison with the much lower $CO_2$ concentration in the termitaria of the non-fungus grower. Lüscher (1951) came to a similar conclusion when he stated that the respiration of the termites and the fungus combs contribute to the high temperature and humidity in the termitarium.

## 2.2 **Distribution and composition of mycoflora in termitaria**

Thomas (1987 a,b) studied the distribution of *Termitomyces* and other fungi in the nests and in the gut of major workers of several Nigerian termite species representing the subfamily Macrotermitinae. She investigated termite manipulated soil, foodstores and fungus combs by direct isolation and dilution plate methods. From her results she made the following conclusions : (i) in the case of *Macrotermes bellicosus* (Smeathman) isolations of *Termitomyces* were restricted to the fungus comb and alimentary tract of the workers (ii) common soil fungi displayed a progressive reduction in species diversity from foodstore through the three zones of the aging comb (iii) the number of fungal colonies isolated decreased from foodstore to the comb (iv) in the case of the other termite species, *Termitomyces* was also isolated from forager faecal pellets and foraged food material in the gut, which proved that these termites incorporate *Termitomyces* into their food material and (v) the composition of the mycoflora isolated from the soil, termites and fungus combs was identical and this evidence proved that common soil fungi were being carried into the nest by the workers as contaminants. Thomas (1985) developed a selective medium for the isolation of *Termitomyces* from the termite habitat which enabled her to conduct her excellent research. Mohindra & Mukerji (1982) performed a physical-chemical analysis of termite manipulated soil from a mound of *Odontotermes obesus* (Rambur) in India and proved that termite soil has a higher clay, organic and inorganic content than the surrounding soil. They concluded that the high organic, carbon and nitrogen content of the termite manipulated soil was caused by the saliva and faecal material used by the termites to cement soil particles together. Because of the warm, humid conditions which prevail inside

the termitarium and the faecal material which provide an ideal substrate for the growth of microfungi, the composition of the mycoflora in termite manipulated and surrounding soil was determined. Mohindra & Mukerji (1982) produced evidence that the common soil fungi isolated were more prevalent in the nutrient enriched termite manipulated soil, than in the surrounding soil on both a quantitative and a qualitative basis. They also determined that seasonal fluctuations exerted a direct proportional influence on fungal populations.

## 2.3 **Factors inhibiting growth of alien fungi in termitaria**

Thomas (1987 c) observed that extracts of foodstore and whole termites in methanol and termite guts in benzene and methanol, significantly reduced the number of fungal colonies developing on her soil suspension plates. She suggested that termite saliva or other secretions might suppress the growth of undesirable fungi while permitting the unimpeded growth of *Termitomyces* on the combs. Sannasi & Sundara Rajulu (1967) reported the presence of an antimicrobial substance in the exudate of the physogastric queen termite which exerted an antimicrobial effect on both bacteria and fungi. They established that the active component of the antimicrobial substance consisted of fatty acids and rendered immunity to attack from *Aspergillus flavus* to the different castes of the termite colony. Batra & Batra (1966) observed that the defensive oral secretion of soldiers and worker manipulated soil exerted a fungistatic effect. They conducted experiments with conidial suspensions of *Trichoderma* and *Cunninghamella* on water agar on which they placed oral droplets of the soldiers. Spore germination was inhibited for sixteen to twenty-four hours but a clear zone remained around the oral droplets. They reported that similar results were obtained when conidia suspended in drops of water agar where exposed to the mucilaginous oral droplets containing volatile substances. Another factor inhibiting spore germination is $CO_2$ (Cochrane, 1958). Lüscher (1961) reported a $CO_2$ concentration of 2,7 % in termitaria while Matsumoto (1977) obtained readings up to 5,2 %. Batra & Batra (1966) have established that conidiospore germination of *Cunninghamella* and *Trichoderma* was reduced in

an atmosphere with 5 % $CO_2$ while at 10 % $CO_2$ spore germination was suppressed altogether. Petch (1906) considered the mechanical or weeding activity of termites as an important mechanism in controlling unwanted fungal growth while Grassé (1944) favoured the nest microclimate, production of antibiotics and the chemical composition of the comb as controlling factors. In spite of many different species of common soil fungi isolated by Batra & Batra (1966), Das, Maheshwari, Nigam, Shukla & Tandon (1962) and Thomas (1987 a,b) from soil adjacent to termitaria, the fungus combs support an almost pure culture of *Termitomyces* mycelium throughout the year. Neither the foodstore, nor the saliva moistened soil lining the fungus comb chambers, royal chamber and runways are capable of supporting any fungal growth as long as the soldiers and workers actively maintain their nests (Thomas, 1987 c). With the favourable microclimate and the excellent growth substratum in termitaria, the fungistatic effect exerted by termites must be considerable to prevent the termites and or their natural habitat being enveloped by foreign fungi.

## 2.4 **Fungus comb construction and nature of building material**

The german naturalist König (1779) was first to describe the sponge-like fungus gardens of the subfamily Macrotermitinae, supporting the white mycelium of conidia-bearing sporodochia. Smeathman (1791) made a similar report after he investigated termitaria in West Africa. Usually the fungus combs occur below the ground level but Annendale (1923) reported that the combs of *Odontotermes redemanni* (Wasmann) also occur above ground level in the elevated structure, the mound. Two schools of thought exist concerning the true nature of the comb material. Bathellier (1927) was first to suggest that the fungus combs consisted of undigested, masticated plant material. Rohrmann (1978) and Martin & Martin (1978) confirmed his observations. Supporters of the non-faecal origin lost ground to the scientists who advocated the faecal origin of the comb. Alibert (1964), Becker & Seifert (1962) and Josens (1971) produced evidence enhancing the faecal origin theory. This was achieved by staining leaf confetti with a fluorescent dye and observing the appearance of the dye on newly constructed combs. Sands (1960) made the only direct observation of comb construction and

concluded that the combs were constructed of spherical balls, similar in appearance to the faecal pellets in the hindgut of the workers. According to Sands (1960) and Grassé (1978), fungus-growing termites produce two kinds of faeces : (i) little or non-digested solid droppings and well-digested fluid excrements. Solid pellets consisted of plant material which was masticated by the mandibles and quickly passed through the alimentary tract of the termite where it was mixed with intestinal juice. Faeces were excreted as short, thick sausages and were transformed by termite mandibles into pellets or mylospheres. These pellets were used by the termites to construct their combs. Bels & Pataragetvit (1982) considered only the final fluid excrements as proper faeces whereas the mylospheres were not. Batra & Batra (1979) investigated the rectal contents of actively foraging workers of *Odontotermes obesus* and they established that the mean particle size of plant debris in the rectum and comb was the same. They also observed *Termitomyces* conidia in termite guts and in comb pellets. Undigested wood contains a high cellulose to lignin ratio (Sands, 1969) while Becker & Seifert (1962) determined that this ratio was reversed in the faeces of termites. Abo-Khatwa (1976) found a high lignin to cellulose ratio in the combs of *Macrotermes subhyalinus* suggesting that partially digested plant material had been incorporated into the fungus combs. High levels of uric acid, known to occur in termite faeces, were detected in combs by Abo-Khatwa (1976). Sufficient evidence therefore exists to reject the non-faecal and support the faecal origin of the comb.

## 2.5 **Chemical composition of the comb and *Termitomyces* sporodochia**

Batra & Batra (1979) determined the chemical composition of fungus combs constructed by *Odontotermes obesus* and obtained a mean nitrogen content of 2,01 % , a cellulose content of 9,68 % to 25,60 % , a lignin content of 9,97 % to 16,6 % and a high cellulose : lignin ratio of 1,50. Mishra & Sen-Sarma (1979) determined the chemical composition of faecal matter, nest material and fungus comb of thirteen termite species representing nine genera. They obtained a cellulose content

of 8,0 % to 20,0 %, hemicellulose 18,8 % to 32,0 %, lignin 20,2 % to 29,2 % and nitrogen 1,24 % to 2,13 %. Rohrmann & Rossman (1980) found the chitin content of old and new combs of *Macrotermes ukuzii* Fuller to be 0,8 % and 1,0 % respectively. Rohrmann (1978) determined the lignin and cellulose chitin content of old and new combs of this termite. The lignin content was found to be 14,30 % and 2,43 % respectively and the cellulose-chitin content 20,82 % and 27,18 %. Mishra & Sen-Sarma (1986) and Rohrmann & Rossman (1980) executed an amino acid analysis of the sporodochia of *T. albuminosus* and a *Termitomyces* sp. They obtained between fifteen and seventeen different unbound amino acids, for example amino-butyric acid and aspartic acid. According to Rohrmann & Rossman (1980) *Termitomyces* sporodochia contained a cellulose-chitin content of 6,5 % , a chitin content of 2,7 % , a nitrogen content of 7 % (Matsumoto, 1976; Rohrmann, 1978) and a protein content of 38 %. Rohrmann & Rossman (1980) estimated that chitin contributed approximately 3 % to the total nitrogen present in sporodochia with the remainder being made up by protein. They concluded that at least 86 % of nitrogen in sporodochia was present as protein. In his paper Rohrmann (1978) also recorded sodium, potassium, phosphate, calcium and magnesium contents of sporodochia and nitrogen contents of new and older combs (1,52 % and 1,34 % respectively). Mishra & Sen-Sarma (1979,1986) determined the free amino acid content of *Odontotermes obesus* fungus combs and obtained nine different amino acids. Mohindra & Mukerji (1982) analyzed the soil texture of a fungus comb and obtained a high clay content of 64,5 %. The fungus comb had a moisture content of 34,6 % and a pH of 6,5 with an electrical conductivity of 0,150 m ohm.cm$^{-1}$. Arshad & Schnitzer (1987) conducted a very detailed analysis of the nitrogen-containing compounds of a fungus comb. In contrast to previous publications on this subject they did not detect any unbound amino acids prior to acid hydrolysis. Glycine, aspartic acid, serine and alanine were the most abundant amino acids. Amino sugars consisted of glucosamine and galactosamine, while amino sugar nitrogen contributed 1,5 %, amino acids 68,3 % and ammonia 30,2 % to the total nitrogen content. From these results Arshad & Schnitzer (1987) concluded that proteinaceous materials made a major contribution to the total nitrogen content of the fungus comb which therefore had a protein content of 8,9 %.

Various authors have determined the moisture content of fungus combs of different termite species (Abo-Khatwa, 1976; Batra & Batra, 1979; Cmelik & Douglas, 1970; Mishra & Sen-Sarma, 1979). They obtained values ranging from 45 % to 56 %, which is favourable for the growth of wood-rotting fungi. These authors also determined pH values of fungus comb material which ranged from 2,2 to 6,2. Pathak & Lehri (1959) and Goodland (1965) reported a high calcium carbonate content in the soil of a termitarium which rendered the soil lining the fungus comb chambers alkaline but in spite of this the comb material remained acidic. König (1779) mentioned the bitter taste of pieces of fungus garden. Abo-Khatwa (1976) and Cmelik & Douglas (1970) extracted D-mannitol from the fungus combs of *Macrotermes subhyalinus* and *M. goliath* and in quantities of 5,5 % to 6,9 %, as based on the dry mass of the comb. Reducing sugar content was low, 1 % to 2,4 % in relation to D-mannitol. Abo-Khatwa (1976) also determined the amount of reducing sugars, lipids, nitrogen, uric acid, chlorophyll, vitamin C and A in four zones of the comb of *M. subhyalinus*. Abo-Khatwa (1976) proved that extracts of *Termitomyces* conidia could oxidize mannose, galactose, methanol, ethanol, propanol and D-mannitol though not sorbitol. Mishra & Sen-Sarma (1979) detected glucose, galactose, mannose, arabinose, xylose and a uronic acid in the fungus combs of four termite species. Arshad & Schnitzer (1987) detected the same sugars with the addition of rhamnose and fucose. They estimated that glucose was present in the highest concentration followed by xylose, arabinose, galactose, mannose, rhamnose and fucose. Arshad & Schnitzer (1987) stated that these sugars originated from different polysaccharides in the comb as their monose subunits and constituted 39,2 % of the mass of the fungus comb.

Abo-Khatwa (1976) characterized the lipid fraction in the fungus combs of *M. subhyalinus* and detected mono and triglycerides. He obtained high amounts of phospholipids and free fatty acids in conidia of *Termitomyces*. The fungus comb contained 1,8 % of the total lipids and conidia 2,1 %. Cmelik & Douglas (1970) investigated the lipid content in the fungus combs of *M. goliath* and detected free fatty acids with palmitic, oleic and linoleic acids as the main components. Some of the fatty acids were saturated or mono-saturated with an uneven number of

carbon atoms. Cmelik & Douglas (1970) also determined the sterol content of the combs which consisted of cholesterol, stimagsterol, ergesterol and ß-sitosterol. Phenolic compounds were detected by Cmelik & Douglas (1970) and consisted of aromatic oxo-compounds. The inorganic contents of fungus combs constructed by different termite species were determined by several scientists. Rohrmann (1978) determined the sodium, potassium, phosphorous, calcium and magnesium contents of newer and older combs constructed by two *Macrotermes* species. Abo-Khatwa (1976) detected the same elements in the comb of *Macrotermes subhyalinus* and obtained an ash content of 12,1 % to 12,5 %. Higher values were reported for the combs of *Odontotermes* and *Microtermes*, which varied from 12,5 % to 22,9 % as determined on a dry mass basis (Mishra & Sen-Sarma, 1979). Matsumoto (1976) measured the ash content of fungus combs and *Termitomyces* sporodochia of two *Macrotermes* species. He obtained values which ranged from 8,0 % to 9,3 % and 2,9 % to 5,0 % respectively. He proceeded to determine the carbon to nitrogen ratio of fungus combs and sporodochia. He observed a drastic reduction in the C : N ratio which occurred from fungus comb to sporodochia, caused by an increase in the nitrogen and a decrease in the carbon concentration. The increase in nitrogen content from 1,7 % to 7,3 % between comb and sporodochia, supported his observation. Arshad & Schnitzer (1987) executed a general analysis and determined the elemental composition of fungus combs. They obtained the following values : (i) carbon 41,1 % (ii) hydrogen 6,9 % (iii) nitrogen 1,9 % (iv) sulfur 0,1 % (v) oxygen 50,0 % and (v) ash 13,6 %. Arshad, Schnitzer & Preston (1987) determined the chemical composition of the fungus comb of *Macrotermes michaelseni* and they identified three organic fractions : (i) humic acid (ii) fulvic acid and (iii) a humin fraction which constituted 40, 20 and 40 $w.w^{-1}$ % of the fungus comb. By utilizing $^{13}C$ NMR and IR spectroscopy they established that each fraction contained paraffins, protein, carbohydrates, aromatics and carboxylic acid groups. According to Arshad *et al.*, (1987) the humin fraction was rich in carbohydrates, while the humic acid and fulvic acid fractions contained higher amounts of aromatic compounds.

## 2.6 Degradation and utilization of the comb and the nature of the termite-fungus symbiotic relationship

Kalshoven (1936) observed that the fungus combs of *Macrotermes*, *Odontotermes* and *Microtermes* were consumed from below at a certain age of maturity by worker termites followed by the addition of freshly prepared material on the upper surface of the comb. Grassé & Noirot (1957,1958) confirmed Kalshoven's observations. They used staining techniques to prove that the fungus combs degrades lignin, because after a period of degradation the dark colour of the comb changes to a lighter biscuit colour. Josens (1971) established that a period of between five to eight weeks elapsed before the degrading action of *Termitomyces* mycelium rendered the comb material suitable for termite consumption. According to Josens (1971) the sporodochia were restricted to the 'middle-aged' zone of the comb. It has been proved that *Termitomyces* mycelium has the ability to degrade cellulose and probably lignin as well (Mishra & Sen-Sarma, 1979,1986; Rohrmann 1978; Rohrmann & Rossman, 1980). These authors established that *Termitomyces* was responsible for most of the lignin and cellulose degradation in the comb material. Lignase enzymes were detected in newly deposited comb material that was being colonized by *Termitomyces* mycelium. Apart from comb material, sporodochia of *Termitomyces* were also consumed by nymphs and workers of fungus-growing termites (Batra, 1975). Although certain termites investigated by Mishra & Sen-Sarma (1980) were capable of digesting lignin with the aid of gut symbionts this mechanism has proved to be only partially effective. The degrading action of *Termitomyces* lignase liberated nitrogen-containing phenolic compounds as well as lignin bound proteins, which then became available for termite consumption. These nutrients were then incorporated into fungal tissue and increased the nutritive value and nitrogen content of the sporodochia (Rohrmann & Rossman, 1980). During the process of comb decomposition and fungal respiration carbon was removed from the comb material in the form of $CO_2$, therefore the nitrogen content of comb material and fungal tissue inevitably increased (Collins, 1982). Matsumoto (1976) confirmed these observations by proving that the carbon : nitrogen ratio progressively

decreased from leaf litter, to fungus comb, to the sporodochia. Abo-Khatwa (1978) and Martin & Martin (1978) investigated the origin and mechanism of cellulose digestion in *Macrotermes natalensis* and *M. subhyalinus*. They located the entire set of cellulolytic enzymes in the midgut where the $C_x$-cellulases and the ß-glucosidases were being produced by the midgut epithelium and the salivary glands. They concluded that the $C_1$-enzyme which is essential for the hydrolysis of crystalline cellulose was acquired by the workers when fed on *Termitomyces* sporodochia. The conidiophore-bearing sporodochia contained all three cellulolytic enzymes with high $C_1$, ß-glucosidase and $C_x$-activity. Osore & Okech (1983) extracted, purified and characterized two cellulolytic enzymes of a *Termitomyces* sp. They extracted these enzymes from lyophilized conidiophores and basidiocarps followed by partial purification and activity determination. Although they did not test for $C_1$-activity, high $C_x$ and ß-glucosidase activity was detected. Rouland, Civas, Renoux & Petek (1988 a,b) purified and characterized $C_1$ and $C_x$-enzymes from the digestive tract of *Macrotermes muelleri* and from sporodochia of the associated *Termitomyces* sp. They concluded that these enzymes from both termite and fungus, have identical physical-chemical characteristics. A hemicellulolytic enzyme (1,4-ß-D-xylan xylanohydrolase) was isolated, purified and characterized from the culture filtrate of *T. clypeatus* and was capable of hydrolyzing xylan, arabinoxylan and dextrin (Ghosh 1981; Ghosh, Banerjee & Sengupta, 1980; Khowala, Mukherjee & Sengupta 1988; Mukherjee & Sengupta, 1985). *T. microcarpus* has been thoroughly screened for the production of different enzymes (Parent & Skelton, 1977; Skelton & Matanganyidze, 1981; Skelton, 1986). Twenty-four different enzymes were detected which included the stable hydrolases, such as cellulase, esterase, proteinase and an oxidase, polyphenol oxidase. Other enzymes screened were intracellular respiratory enzymes. A multisubstrate specific enzyme amylase from the culture filtrate of *T. clypeatus* was purified and characterized by Ghosh & Sengupta (1987). They established that this enzyme was active against amylase, xylan, amylopectin, glycogen, arabinogalactan and arabinoxylan. Mishra & Sen-Sarma (1986) measured the activity of various carbohydrases, chitinase, protease, esterase and lignase in the sporodochia of *T. albuminosus*. Only chitinase and

lignase activity could be detected in the sporodochia and the authors stated that the presence of an enzyme was also an indication of the corresponding substrate in the fungus comb. Mishra & Sen-Sarma (1986) elaborated on the advantage of *Termitomyces* produced enzymes for the nutritional and digestive physiology of the termites. They stated that the presence of various enzymes in the fungus sporodochia reduced the metabolic energy expenditure of the termites by eliminating the need to produce and secrete large quantities of enzymes. Martin & Martin (1978) and Abo-Khatwa (1978) emphasized the role of acquired digestive enzymes which enabled the termite worker to degrade crystalline cellulose as a source of energy. The importance of the comb for the wide distribution and survival of fungus-growing termites is stressed by many authors (Batra & Batra, 1966,1979; Collins, 1982; Rohrmann, 1978; Rohrmann & Rossman, 1980). These authors explained that fungus-growing termites were dependent on the degradation of plant material in the comb by *Termitomyces* mycelium enriching it nutritionally and concentrating nitrogen in the sporodochia. The construction of combs in warm, humid fungus chambers underground created a favourable atmosphere for plant litter decomposition by *Termitomyces* mycelium which, in turn, rendered this valuable food source palatable to the termites. The fungus-comb combination accelerated the decomposition process which permitted the digestion of a larger amount of organic material, increased the nutritive value of the leaf litter and supported a bigger termite colony than could be accomplished by the termites alone in a semi-arid, adverse environment. Additional functions of the comb were temperature and humidity control in the mound (Lüsher, 1951; Rohrmann, 1978). Heat and water were generated through the cell respiration of fungus mycelium while the high mannitol content in the comb assisted in regulating temperature in the fungus chambers (Abo-Khatwa, 1976). The combs provided energy and nutrients for the production of basidiocarps during the rainy season. Bathellier (1927) and Heim (1977) observed that the sporodochia were also the primordia from which the pseudorhizae of basidiocarps developed. Batra & Batra (1979) established that combs which had borne a flush of *Termitomyces albuminosus* contained less cellulose and more lignin than combs which had not borne basidiocarps. Fungus comb

material was very thoroughly degraded by the mycelium of *Termitomyces*, consequently almost all of the mycelium was eventually converted into termite tissue with only a small amount of faecal matter returned to the soil (Collins, 1982).

## 2.7 **Inoculation of the comb**

Because of the intimate relationship between termite and fungus the symbiotic partners are unable to exist independently. Consequently the symbiotic relationship has become obligatory. It has thus became imperative to perpetuate this relationship. Two possible methods exist whereby the inoculation of the primordial comb in a newly found colony can occur. Inoculation may be effected by transportation of *Termitomyces* conidia by the reproductive castes or the collection of the basidiospores by foraging workers. Johnson, Thomas, Wood & Swift (1981) and Johnson (1981) demonstrated that in laboratory reared colonies the primordial fungus comb was inoculated with *Termitomyces* conidia by the female alate which ingested conidia from the comb before she commenced on the mating flight. The female alate carried the conidia in the form of a bolus in the foregut where the spores survived for ten to eleven weeks after colony foundation had occur. The comb was then inoculated by the workers. Johnson *et al.*(1981) proved that in the genus *Macrotermes* the male reproductives carried the conidia while in the case of *Microtermes* the female alates performed this function. Akhtar (1978) and Grassé & Noirot (1955) conducted similar research on species of the genera *Macrotermes* and *Microtermes* which inoculated their combs spontaneously. According to Lüscher (1951) and Sands (1960) this behaviour was probable if the alates carried conidiophores from the parental nest. Batra & Batra (1966) located conidia in the guts and integument of swarming alates. Sands (1969) rejected the theory of Johnson (1981) and Johnson *et al.* (1981) that fragile, thin-walled conidia could remain viable for two to three months between the swarming of the alates and the inoculation of the 'sterile' comb. It was suggested by Sands (1960) that for those species where the alates did not carry conidiophores, basidiospores along with plant litter might be collected by foraging workers and

brought into the nest. Sieber (1983) proved this hypothesis of Sands. He offered filter paper and soil with basidiospores to young workers of two termite species of fungus-growing termites, after which the workers constructed and inoculated a fertile fungus comb. Sieber (1983) assumed that the basidiospores passed through the termite gut before germination occurred. Johnson *et al.* (1981) failed to germinate basidiospores of *Termitomyces* on a selective agar medium. According to them basidiospores of *Termitomyces* did not germinate readily due to inadequate nutrient conditions. They speculated that passage through the termite gut was essential for germination. Although Sieber (1983) advocated this method of comb inoculation the question of whether the foraging workers purposely seeked out the basidiospores or collected the spores incidentally remain unanswered. According to Johnson *et al.* (1981) this method of comb inoculation seemed highly improbable because of the scarcity of basidiocarps in unfavourable and variable climatic conditions as well as the perishableness of the basidiocarps. The emergence of *Termitomyces* basidiocarps above the parent nests rarely synchronizes with the construction of fungus combs in incipient colonies.

## 3. Nutritive value of *Termitomyces* species

### 3.1 Nutritive value of African species

There seems to be general agreement among scientists and consumers alike as to the superior nutritive value of this mushroom. Mukiibi (1973) investigated the nutritional value of *T. microcarpus* and four *Termitomyces* spp. from Uganda. He determined the crude protein, carbohydrate, fat, crude fiber, total ash and moisture content as well as the essential amino acid and biological values of the mushrooms. Ogundana & Fagade (1981) in Nigeria compared the nutritive value of *T. robustus* and *T. clypeatus* with that of *Pleurotus tuber-regium*. They established that the *Termitomyces* species had a protein content of over 31 % and a carbohydrate content of 32 % of which 26 % were reducing sugars. *Pleurotus* contained 14,6 % protein and 18,6 % carbohydrates of which 2,9 % were reducing sugars. Both *Termitomyces* species contained a higher ascorbic acid content (10 mg % and 14,3 mg %) than *Pleurotus*

(3,3 mg %), while the hydrocyanic and oxalate contents of all the mushrooms were low. Zoberi (1973) mentioned in his paper that all the *Termitomyces* species in Nigeria were eagerly sought and consumed by the local population. Sharp (1983) reported the occurrence of *Termitomyces* in Zimbabwe while Skelton (1984) issued a warning regarding the raw consumption of *T. microcarpus*. This little mushroom according to Robertson (1983) contains a group III toxin which is a gastrointestinal irritant. Skelton (1984) advised that it should be boiled in water, drained and then cooked in vegetable oil. Parent & Thoen (1977,1978) investigated the nutritive value of several *Termitomyces* species from the Upper-Shaba region in Zaire. They determined the protein, lipid, ash, crude fiber, carbohydrate, calorific value as well as the calcium, phosphate and iron contents of the mushrooms. Lastovica (1974) reported the appearance of *T. umkowaani* and *T. microcarpus* in a garden at Winterskloof near Pietermaritzburg in Natal. They described their method for cooking these mushrooms with onions, butter, salt and milk. They concluded that the texture of *T. microcarpus* was too fibrillous and not worth the effort of collecting and cooking. According to them *T. umkowaani* was superior in both texture and flavour while the dimensions and mass of a single mature specimen warranted the effort of preparing a meal from it.

### 3.2 **Comparable nutritional studies on Indian species**

The protein content and amino acid composition of five edible mushrooms including *T. eurhizus*, were determined by Purkayastha & Chandra (1975,1976) and a similar analysis was executed on a *Lepiota* sp. and *Termitomyces* sp. by Bano, Ahmed & Shrivastava (1964). Chandra & Purkayastha (1976) conducted feeding experiments with albino mice by feeding them dry mycelium powder of five different edible mushrooms. They established that the gain in body mass of mice fed on mycelial powder of *Calocybe indica* or *T. eurhizus* was significantly higher than that of the others. Samajpati (1981) conducted a survey of the annual consumption of *T. eurhizus*, *T. microcarpus* and *T. clypeatus* in local markets of West Bengal, India. Samajpati (1981) established that all

three species were extremely popular among the local population and were consumed in substantial quantities. Crisan & Sands (1978) determined the nutritional value of six *Termitomyces* species as well as the nutritive value of several edible mushrooms. Although only one *Termitomyces* species was properly identified, the nutritional data in this study remain important. Crisan & Sands (1978) determined crude protein content, fat, carbohydrate, fibre and ash contents as well as the energy value of several edible mushrooms including some African and Indian *Termitomyces* species. This was followed by an analysis of the amino acid composition from which an Essential Amino Acid Index, the Biological Value, Amino Acid Score and the Nutritional Index were calculated. The limiting amino acids were also determined from data.

## 4. Growth requirements and cultivation studies

### 4.1 Growth requirements of some *Termitomyces* species

Samajpati (1981) investigated the effect of several complex and defined media on the growth of *T. eurhizus*, *T. microcarpus* and *T. clypeatus*. Batra & Batra (1979) in their monumental study of the termite-fungus relationship, tested the effect of several media on the growth of *T. albuminosus*. These media included oat meal, Sabouraud glucose, potato-dextrose, yeast and malt extract, fungus comb filtrate and comb residue suspensions. The growth response on a basal medium with various vitamins included or excluded was also determined. They concluded from their experiments that *Termitomyces* mycelium grew slowly on the basal medium, was heterotrophic for thiamine and utilized carboxymethyl cellulose and Walseth cellulose, though not cotton fiber as carbon sources. Mishra & Sen-Sarma (1986) established that *T. albuminosus* could utilize glucose, fructose, xylose, arabinose, mannose and galactose as well as proteins, lipids and the degradation products of lignin. Abo-Khatwa (1976) proved that extracts of *Termitomyces* conidia could oxidize D-mannitol, D-mannose, D-galactose, low molecular weight alcohols and several organic acids. Chandra & Purkayastha (1977) investigated the growth requirements of five species of edible mushrooms which included *T. eurhizus*. They determined the effect of the following factors on the growth of mycelium : (i)

pH and temperature (ii) carbon and nitrogen sources (iii) different C : N ratios (iv) macro and micro elements and (v) vitamins. Ghosh & Sengupta (1978) conducted experiments with *T. clypeatus* along with two other wild mushrooms collected in India. In addition to the physiological experiments of Chandra & Purkayastha (1977) Ghosh & Sengupta (1978) also determined the pH variation of culture filtrates caused by fermentation of *T. clypeatus* mycelium.

## 4.2 **Cultivation studies**

Sengupta, Naskar & Jana (1984) investigated the growth of *T. clypeatus* mycelium on various natural substrates such as sawdust, wheat bran, green coconut coir and bagasse. They detected several saccharifying enzymes in their cultures responsible for the degradation of the agrowastes as well as the end products of enzyme hydrolysis. They observed that all the agrowastes except sawdust supported good mycelium growth. No attempt was made to induce basidiocarp formation. Quimio (1977) determined the effect of several complex media on the growth of *T. cartilagineus* mycelium as well as spawn formation on twelve different natural substrates. Quimio (1977) observed that rice bran, corn meal, mud press and grated coconut meat pulp supported the best mycelium growth. To induce basidiocarp formation Quimio (1977) experimented with ordinary soil, termite house soil and mud press as casing materials which were watered with soil extract, termite extract, termite house extract and plain water. The cased materials were incubated at different temperatures and under alternating light and dark conditions for three months. Neither Quimio (1977) nor Heim (1942 b) who conducted similar experiments succeeded in their attempts to induce basidiocarp formation. In contrast with these attempts De (1983) succeeded in inducing basidiocarp formation by *T. microcarpus* in culture. He prepared cultures on malt agar slants which were exposed to different periods of light and darkness. Basidiocarps were produced only when a period of six hours darkness was preceded by eighteen hours light. A search of the literature revealed that the physical and chemical conditions which are necessary to induce basidiocarp formation in nature are only partially understood and very

superficially researched. What is known at this stage is that the comb material is impregnated with termite intestinal juices which might stimulate growth of *Termitomyces* mycelium (Batra & Batra, 1979; Grassé, 1978; Sands, 1960). According to Bels & Pataragetvit (1982) the termites abandon or partly abandon their combs at a certain developmental stage of the termite colony which is followed by a decrease in temperature and carbon dioxide concentration in the fungus comb chambers (Lüscher, 1961; Noirot, 1970). Bels & Pataragetvit (1982) speculated that the drop in temperature and carbon dioxide concentration in the comb chambers as well as the depletion of termite intestinal juice in the comb material might stimulate the formation of pseudorhizae and fruit bodies. They advised that much more research ought to be done on this subject before the situation can be clarified.

## III. MATERIAL AND METHODS

### A. NUTRIENT PHYSIOLOGY

#### (i) Acquisition of fresh material and preparation of cultures

Cultures were prepared from basidiocarps collected at several localities in the vicinity of Pretoria, Hoedspruit, Rustenburg, Piet Retief and Derdepoort in the Transvaal, Stanger in northern Natal and from the Kroonstad district in the Orange Free State in the Republic of South Africa. Basidiocarps appeared during the rainy season which stretched from the end of October to approximately the middle of April. Fruit bodies appeared above termite nests in connection with the fungus combs. Where possible, soldiers of the associated termite host were collected for identification purposes. Pure cultures were aseptically prepared by placing pieces of context tissue from the stipe or from the pileus on Hagem Modess medium supplemented with anantibiotic (see appendix 1). Cultures were made on the same medium as soon as sufficient mycelium developed from the primary inoculum. Subsequent isolates were made on yeast extract, soytone, soluble starch medium (YESS medium, see appendix 1) on which the mushroom mycelium was maintained for the duration of the study period. Cultures

were maintained on 5 cm agar plates, in a broth (300 $cm^3$ conical flasks containing 50 $cm^3$ medium per flask) and in a semi-solid medium (10 $cm^3$ in 20 $cm^3$ culture bottles). Termitaria of *Macrotermes natalensis* (Haviland) and *Odontotermes badius* (Hav.) were excavated to obtain fungus comb material and mycelium of comb sporodochia. No fruit bodies were encountered on the termitaria of *M. natalensis* during the period of investigation while fruit bodies of *T. reticulatus* were found growing on the termitaria of *O. badius* in direct contact with the combs at the time when sporodochia were removed from this termite's fungus comb. Some of the sporodochia found growing on the combs were removed aseptically and placed on Hagem Modess and cellulose nutrient media with an antibiotic (see appendix 1). Subculturing, the maintenance of cultures and the nutrient medium utilized were identical to those utilized for the context tissue cultures. Yeast extract, soytone, soluble starch medium served as inoculum medium for all subsequent experiments. Six species were isolated into pure culture : (a) *T. umkowaani* (b) *T. microcarpus* (c) *T. sagittaeformis* (d) *T. clypeatus* (e) *T. reticulatus* and (f) a *Termitomyces* sp. (sporodochia isolated from the combs of *M. natalensis* and referred to as the anamorph in Results and Discussion when reporting results from physiological experiments). Specimens of *T. schimperi* and *T. striatus* were investigated as dry herbarium material and subsequently no living cultures were prepared or physiological experiments conducted with these species. Six isolates of *T. umkowaani* were made in the Transvaal. Two were obtained from the University of Pretoria campus grounds, PRUM nos. 2254 and 2705 and one each from Hoedspruit, PRUM 2704 , Rustenburg, PRUM 2685, Derdepoort PRUM 2643 and Stanger. Two isolates were made of *T. microcarpus* in the Pretoria district collected on the University of Pretoria campus grounds and from Silverton. Two isolates were made of *T. sagittaeformis* both from the National Botanical Gardens in Pretoria, PRUM 2235 and PREM 49058 and only one isolate of *T. clypeatus* in the vicinity of Johannesburg, PRUM 2402. Three isolates were made of *T. reticulatus*, two isolates were collected on the University of Pretoria Experimental Farm grounds and one isolate from the Orange Free State, Kroonstad District PRUM 2693. Cultures were also prepared from natural

sporodochia isolated from the combs of *M. natalensis* situated in the grounds of the Agricultural Experimental Farm at Rietondale, Pretoria and from the combs of *O. badius* situated in the University Experimental Farm grounds. Fruit bodies were collected over three successive summer rainy seasons which stretched from 1986 to 1989. The following experiments were conducted with the species in culture : (i) the effect of three environmental factors on mycelium growth was determined for species a - f (ii) the effect of different carbon and nitrogen sources on mycelium growth was determined for species a - d (iii) the hydrolytic capabilities of the different mushrooms to degrade selected carbon and nitrogen sources were determined for species a - f and (iv) an extensive morphological and taxonomical investigation were executed on all the species.

(ii) **Preparation, inoculation of nutrient media and incubation of cultures**

The following procedures were used to prepare and inoculate nutrient media. Ingredients of the various media were measured on a balance and placed in suitable autoclavable containers. After hydration with double-distilled water the media were autoclaved at a pressure of 150 kPa and a temperature of 120 $^{0}$C for 15 to 20 minutes. The methods described in par. 7 were applied when cold sterilization procedures were necessary. Nutrient media were poured aseptically into sterile Petri dishes in a laminar flow cabinet. Liquid media were prepared in a similar manner and dispensed into 300 $cm^3$ conical flasks with 50 $cm^3$ per flask. Two procedures were followed for the inoculation of media. Liquid media were inoculated with ± 5 $mm^2$ mycelium pieces which were cut from mycelium growing on agar plates with a sterile dissecting-knife. These were attached on the inside of sterile conical flasks against the glass surface just above the level of the broth and cut into numerous pieces. Inoculation was accomplished by swirling, during which numerous inoculation centers were formed. Solid media were inoculated with mycelium from liquid cultures with the aid of a sterile inoculating loop. Stationary cultures were incubated at 26 $^{0}$C in a dark incubator and shake cultures were incubated under identical

in a dark incubator and shake cultures were incubated under identical conditions on a reciprocating table revolving at a speed of 100 rpm.

(iii) **Determination of growth**

The dry mass of the mycelium mat was used as a measure of growth. This was determined by filtering the liquid cultures through a 7 cm Whatman no. 1 filter paper, placed in a Büchner funnel on a suction flask. The filter papers were dried in an oven at 60 $^{0}$C for ten to twelve hours and the dry mass ascertained.

(iv) **Statistical methods**

Results were statistically processed to test for significant differences between treatment means. This was accomplished by constructing an Analysis of Variance table from which the value of $\sigma^2$ and the least significant difference were calculated. The multiple pairing procedure of Tuckey was applied to compare the different treatment means with the least significant difference (Clarke, 1980).

## 1. Environmental factors

### 1.1 Effect of pH

To ascertain the effect of pH on the mycelium growth of *T. microcarpus*, the following medium was prepared : $MgSO_4.7H_2O$ 0,75 g; $NH_4NO_3$ 2,0 g; KCI 0,3 g; D-glucose 10 g; 1 $dm^3$ double-distilled water. The utilization of a synthetic defined medium was essential to prevent excessive growth of the mycelium. The remaining species were cultivated on a semi-defined medium which consisted of : $MgSO_4.7H_2O$ 0,75 g; soytone 2,0 g; KCI 0,3 g; soluble starch 10,0 g; yeast extract 1,0 g; 1 $dm^3$ distilled water. A phosphate-citrate buffer of 0,5 $mol.dm^{-3}$ strength was employed to prepare the following pH series : 2,0 ; 3,0; 4,0; 4,5; 5,0; 6,0; 7,0; 8,0. Due to a slight retarding effect on mycelium growth of *T. sagittaeformis* and *T. reticulatus* caused by the 0,5 $mol.dm^{-3}$ buffer, the ionic strength was reduced to 0,25 $mol.dm^{-3}$ for these species. The buffer consisted of phosphate ($KH_2PO_4$;

$HPO_4$) and citrate stock solutions which were combined in a certain tio until the desired pH value was reached. The pH determinations re performed with a pH-meter and a glass electrode. The nutrient th was placed in 300 $cm^3$ conical flasks and the buffer solutions 100 $cm^3$ screw-cap laboratory bottles which were autoclaved rately. The two were combined to give a total volume of 200 $cm^3$. ... buffered medium was thoroughly agitated and then poured into 100 $cm^3$ conical flasks with 50 $cm^3$ medium per flask. The broths were inoculated according to the method described in par. A (ii) and where then incubated for 35 days at 26 $^0$C in complete darkness. At the end of the incubation period the mycelium dry mass was determined according to the methods already described.

### 1.2 **Effect of temperature**

Mushroom mycelium was cultivated on the nutrient media described in par. 1.1 with the addition of $K_2HPO_4$ 0,9 g and $KH_2PO_4$ 0,7 g per 1 $dm^3$ distilled water. The nutrient media were prepared and inoculated according to the methods described in par. (ii) after which the cultures (100 $cm^3$ conical flasks with 50 $cm^3$ medium each) were incubated at the following temperatures in complete darkness : 5 $^0$; 10 $^0$; 15 $^0$; 20 $^0$; 25 $^0$; 30 $^0$; 35 $^0$; 40 $^0$C. After an incubation period of 35 days the dry mass of the mycelium was determined.

### 1.3 **Effect of light**

Mushroom mycelium was grown in the YESS medium (see appendix 1) at 26 $^0$C under the following light conditions : (i) continuous light (ii) continuous darkness and (iii) alternating light and dark periods of twelve hours each. Light intensity was 1140 lux with an energy emission of 12,6 $\mu$E $m^{-2}.s^{-1}$ under a 30 watt fluorescent lamp . Dark conditions were created by enveloping the cultures in a black cloth inside a dark incubator. After 35 days incubation the mycelium dry mass was determined in the usual way.

## 2. **Effect of cellulose on the growth of mycelium**

*Termitomyces* mycelium was grown in a broth which consisted of : (i) the basal medium (appendix 1) (ii) carboxymethyl cellulose (CMC) or powdered cellulose 10 g and (iii) 1 $dm^3$ distilled water. The medium was prepared and inoculated in the usual way and incubated for 35 days at 26 $^0C$ in complete darkness. Cultures with crystalline cellulose were shaken at 100 rpm for 24 hours followed by a stationary period of 24 hours. This schedule was adhered to throughout the 35 days incubation period. The experiment was terminated at the end of this period and the mycelium dry mass was ascertained.

## 2.1 Determination of 1,4-ß-D-glucan cellobiohydrolase activity ($C_1$)

The method of Fernley (1963) was employed to assay $C_1$-activity (EC 3.2.1.91). Mycelium was grown on the nutrient medium described in par. 2.1 with powdered cellulose instead of CMC as the sole carbon source. Cultures were incubated for three weeks at 26 $^0C$ on a reciprocating table revolving at 100 rpm. Culture filtrates were prepared by filtering the cultures through filter paper (Whatman no. 1 of 7 cm) in a suction flask. All glassware was meticulously cleaned and rinsed with double-distilled water and enzyme solutions were kept at 4 $^0C$ on ice at all times. These precautionary measures were adhered to for all subsequent enzyme activity determinations. Culture filtrates were dialyzed against distilled water at 4 $^0C$ in 2 $dm^3$ wide mouth conical flasks for 18 to 24 hours. Reaction mixtures consisted of 1 $cm^3$ buffered substrate and 0,5 $cm^3$ enzyme solution. Cellulose-azure powder type II (cellulose covalently bonded with a water soluble reactive dyestuff) was suspended in 0,02 $mol.dm^{-3}$ citric acid, 0,04 $Na_2HPO_4$ buffer of pH 6,1 at a concentration of 4 $mg.cm^{-3}$. Reaction mixtures were incubated at 38 $^0C$ and measurements were taken after three, six, nine, twenty four and forty eight hours in the following way. The enzyme reaction was quenched by adding 5 $cm^3$ of a 2,4 $mol.dm^{-3}$ $K_2HPO_4$ solution of pH 9,0 to each tube and centrifuging at 300 rpm for 5 minutes. The reference cuvette contained 1 $cm^3$ buffered substrate, 0,5 $cm^3$ enzyme solution and 5 $cm^3$ $K_2HPO_4$ solution at zero time. Optical densities were measured spectrophotometrically at 595 nm. The optical

densities of standard solutions of original dyestuff (Remazol Brilliant Violet 5 R) in the phosphate-citrate buffer (pH 6,1) at various concentrations (50 to 500 $\mu g.cm^{-3}$) were determined and a standard graph constructed from these readings. The amount of $K_2HPO_4$ soluble cellodextrins produced in 1 $\mu g.cm^{-3}$ of the reaction mixture was calculated from the standard graph.

## 2.2 Determination of 1,4-ß-D-glucan 4-glucanohydrolase activity ($C_X$)

The method of Reese & Mandels (1963) with certain modifications was employed to determine $C_X$-activity (EC 3.2.1.4). Mushroom mycelium was grown on the basal medium with 10,0 g CMC.$dm^{-3}$ distilled water. The CMC utilized had a degree of substitution of 52 % and the degree of polymerization ranged from 100 to 200 glucose molecules. The basal medium was heated to boiling point before the CMC was added. The medium was dispensed into 300 $cm^3$ conical flasks in 50 $cm^3$ quantities followed by sterilization and inoculation according to the standard methods. The cultures were incubated at 26 $^0$C in complete darkness for two weeks. CMC was employed as substrate for the production of $C_X$-enzymes and was dissolved in a 0,1 mol.$dm^{-3}$ phosphate-citrate buffer of pH 5,0. Enzyme activity was assayed in 15 mm by 100 mm test tubes with a volume of 12 $cm^3$. The reaction mixture consisted of 9 $cm^3$ buffered substrate and 1 $cm^3$ culture filtrate. Test tubes were filled with substrate and placed in a water bath at 37 $^0$C. Each test tube received 1 $cm^3$ culture filtrate after which each tube was inverted to ensure proper mixing of enzyme and substrate. The reaction period was six hours and the amount of reducing sugars liberated was determined at one hour intervals. Three replicates were used for each measurement. Spectrophotometric methods and Miller's (1959) recipe for the dinitrosalicylic acid reagent (see appendix 1) were employed to measure reducing compounds in the reaction mixture. This was performed by pipetting 1 $cm^3$ of the reaction mixture and 2 $cm^3$ of the dinitrosalicylic acid reagent (DNS) into clean dry test tubes which were then boiled for 15 minutes in a water bath. The amount of nitro-aminosalicylic acid formed was directly proportional to the amount of

reducing compounds in each sample. The optical densities of samples was read in a single beam spectrophotometer at a wavelength of 540 nm. The samples were read in disposable polystyrene cuvettes of 1 cm light path. All subsequent spectrophotometric determinations were executed under these conditions. After the optical densities of the samples were obtained, the amount of reducing compounds produced in the reaction mixtures was read from a standard graph. This graph was drafted by preparing a serial dilution of cellobiose in 0,1 mol.dm$^{-3}$ phosphate-citrate buffer of pH 5,0 and ranged from 0,1 mg.cm$^{-3}$ to 1,0 mg.cm$^{-3}$. The reducing power and optical densities of the serial dilutions were determined according to the methods described above. The reference cuvette contained 1 cm$^3$ of buffered substrate and 2 cm$^3$ DNS reagent. Sample solutions with an optical density higher than 0,6 were diluted with the buffer and the values read from the standard graph were multiplied with the dilution factor. The reference solution was diluted in the same ratio as the samples. A portion of the culture filtrate was boiled for 5 minutes and incubated with the substrate to serve as a control. The presence or absence of reducing compounds in the control reaction mixture was determined in the usual way.

## 3. **Effect of xylan on the growth of mycelium**

Mushroom mycelium was cultivated in a liquid medium which consisted of the basal medium and a 1 % w.v$^{-1}$ suspension of xylan from oat spelts. The medium was prepared and inoculated according to standard methods using 100 cm$^3$ conical flasks with 50 cm$^3$ medium per flask. The cultures were incubated in complete darkness at 26 $^0$C for 35 days. At the end of this period the mycelium dry mass was determined.

### 3.1 **Determination of xylanase activity**

The xylan medium described in the previous paragraph was employed to cultivate mycelium and to stimulate the production of the endoxylanase enzyme (1,4-ß-D-xylan xylanohydrolase, EC 3.2.1.32). The liquid medium was prepared in 300 cm$^3$ conical flasks with 50 cm$^3$ medium per flask. Cultures were incubated for 18 to 21 days at 26 $^0$C in darkness under

static conditions. Cultures were then chilled to ± 4 $^{0}$C, filtered and centrifuged at 8000 rpm to remove unhydrolyzed xylan in suspension. The clear filtrates were dialyzed against 2 $dm^3$ distilled water at 4 $^{0}$C for 16 hours in boiled dialysis bags. Enzyme activity was assayed according to the methods described in par. 2.2 with the following modifications. Xylan from oat spelts served as substrate and was suspended in 0,1 $mol.dm^{-3}$ phosphate-citrate buffer of pH 5,0 which was autoclaved. The reaction mixtures were incubated in a water bath at 30 $^{0}$C and the amount of reducing compounds liberated during hydrolysis was read from a standard graph. This graph was drafted according to the method described in par. 2.2, except that a serial dilution of D-xylose was prepared in a concentration range of 0,1 $mg.cm^{-3}$ to 1,0 $mg.cm^{-3}$.

### 3.2 **Determination of xylobiase activity**

Mushroom mycelium was cultivated in the xylan medium described in par. 3.1. Cultures were incubated at 26 $^{0}$C in a dark incubator for 10 days and were then chilled, filtered and dialyzed. Xylobiase or 1,4-ß-D-xylan xylohydrolase activity (EC 3.2.1.37) was assayed according to the method of par. 3.1 with the following modifications. As substrate a 5 $mmol.dm^{-3}$ solution of o- nitrophenol-ß-D-xylopyranoside, dissolved in 0,1 $mol.dm^{-3}$ phosphate-citrate buffer (pH 5,0) was used. The reaction mixture consisted of 3 $cm^3$ buffered substrate and 1 $cm^3$ culture filtrate which were incubated in a water bath at 37 $^{0}$C. The reaction was visualized by pipetting 1 $cm^3$ of the reaction mixture and 2 $cm^2$ of a 2,5 % $w.v^{-1}$ solution of $K_2HPO_4$ into clean test tubes. The amount of o-nitrophenol liberated during the reaction was read from a standard graph. This graph was drafted by measuring the optical densities of ten standard solutions of o-nitrophenol, dissolved in buffer at various concentrations (10 $\mu g.cm^{-3}$ to 100 $\mu g.cm^{-3}$). Samples were read at a wavelength of 445 nm and the reference cuvette contained 1 $cm^3$ buffered substrate and 2 $cm^3$ $K_2HPO_4$ solution.

## 4. Effect of starch on the growth of mycelium

Mushroom mycelium was cultivated in a liquid medium which consisted of basal medium and a 1 % $w.v^{-1}$ starch solution. The medium was prepared and inoculated according to the standard methods and the cultures were incubated in complete darkness at 26 $^{0}C$ for 35 days. At the end of this period the dry mass of the mycelium was determined.

### 4.1 Determination of α-amylase activity

The YESS nutrient medium was utilized to stimulate the production of α-amylase (1,4-α-D-glucan-glucanohydrolase, EC 3.2.1.1). The medium was distributed into 300 $cm^3$ conical flasks with 50 $cm^3$ medium per flask. The cultures were incubated at 26 $^{0}C$ for 12 days under static conditions. Culture filtrates were processed and enzyme activity assayed according to the methods described in par. 2.2 with the following modifications. Soluble starch, 10 $g.dm^{-3}$ dissolved in phosphate buffer ($K_2HPO_4$ and $NaH_2PO_4.2H_2O$ 20 $mmol.dm^{-3}$; NaCI 10 $mmol.dm^{-3}$; pH 6,9) was utilized as substrate and was autoclaved before utilization. After the completion of dialysis, the culture filtrates were concentrated by replacing the distilled water with a nearly saturated solution of polyethylene glycol (mol.wt = 5000-7000). Two $cm^3$ phosphate buffer was added to the dialysis bags and 300 $\mu$l of this solution was pipetted into each tube containing 9 $cm^3$ substrate. Reaction mixtures were incubated in a water bath at 25 $^{0}C$ for six hours. The amount of reducing compounds liberated into the reaction mixture was read from a standard graph which was constructed from the optical densities of standard solutions of D-maltose in 20 $mmol.dm^{-3}$ phosphate buffer (pH 6,9) at various concentrations (0,1 $mg.cm^{-3}$ to 0,55 $mg.cm^{-3}$).

## 5. Effect of pectic acid substrates on the growth of mycelium

Mushroom mycelium was cultivated on a basal medium with a 1 % $w.v^{-1}$ solution of pectin (polygalacturonic acid methyl ester) or Na-polypectate (polygalacturonic acid, sodium salt, water soluble). The

pectin was added gradually and the medium was then agitated on a magnetic stirrer for one hour until the pectin was properly hydrated and the broth became viscous. The medium was autoclaved and after inoculation, the cultures were incubated at 26 $^{0}$C for 35 days. The dry mass of the mycelium was determined at the end of this period.

## 5.1 **Determination of activity of pectinolytic enzymes**

Mushroom mycelium was grown in the pectin (carboxylic groups of the galacturonate residues are methylated) medium described in the previous paragraph to stimulate the production of pectinolytic enzymes : (i) poly (1,4-α-D-galacturonide glycanohydrolase, EC 3.1.1.15) with substrate pectin and (ii) polygalacturonase EC 3.2.1.15 with substrate Na-polypectate (carboxylic groups are uncombined). The method of Bateman (1966) with certain modifications was applied to determine pectinolytic enzyme activity. After an incubation period of nine days at 26 $^{0}$C the cultures were filtered, the filtrates processed and the enzyme activity assayed according to the methods described in par. 3.1. A 0,3 % w.v$^{-1}$ solution of pectin was prepared in 0,3 mol.dm$^{-3}$ phosphate-citrate buffer of pH 5,0. Reaction mixtures contained 8 cm$^{3}$ buffered substrate, 1 cm$^{3}$ culture filtrate and 1 cm$^{3}$ of a 0,05 mol.dm$^{-3}$ $CaCI_2$ solution to stimulate enzyme activity. Enzyme hydrolysis proceeded at 30 $^{0}$C for a period of six hours during which the formation of reducing groups in the reaction mixtures was determined at one hour intervals. A 0,3 % w.v$^{-1}$ solution of sodium-polypectate was prepared in 0,3 mol.dm$^{-3}$ phosphate-citrate buffer of pH 6,0. Reaction mixtures contained 5 cm$^{3}$ buffered substrate and 1 cm$^{3}$ culture filtrate. Pectinesterase activity (i) was assayed under the same conditions as pectinase activity (ii). Samples with DNS reagent were centrifuged at 300 rpm for 5 minutes to remove unhydrolyzed substrate before the optical densities of the clear supernatants were determined. The amount of reducing compounds liberated during the reaction was read from a standard graph which was constructed from the optical densities of standard solutions of D-galacturonic acid in 0,3 mol.dm$^{-3}$ phosphate-citrate buffer (pH 5,0) at various concentrations (0,025 mg.cm$^{-3}$ to 0,25 mg.cm$^{-3}$).

## 6. Effect of lipids on the growth of mycelium

Mushroom mycelium was grown in the basal medium with a fatty substrate. Two nonionic detergents (synthetic lipids) namely polyoxyethylenesorbitan monooleate (Tween 80) and polyoxyethylenesorbitan trioleate (Tween 85) were included in the medium at a concentration of 1 % $v.v^{-1}$. The Tweens were autoclaved separately from the basal medium and were added aseptically to the basal medium with the aid of a sterile pipette. The medium was then thoroughly swirled to ensure proper solution. Each conical flask (250 $cm^3$ capacity) received 50 $cm^3$ medium and after inoculation the stationary cultures were incubated at 26 $^0C$ for 35 days. Olive oil represented a natural lipid and was added to the basal medium at a concentration of 1 % $v.v^{-1}$. Olive oil was emulsified beforehand according to the method of Bier (1955) with the aid of polyvinyl alcohol (PVA). Ten grams of PVA was added to 1 $dm^3$ of distilled water followed by the addition of 5 $cm^3$, 0,1 $mol.dm^{-3}$ HCI solution. This solution was stirred and heated to ± 85 $^0C$ until solution was completed after which the solution was cooled, filtered and the pH adjusted to 7,0 with 0,1 $mol.dm^{-3}$ NaOH. Olive oil was added to the PVA solution to achieve a final concentration of 1 % $v.v^{-1}$ and was then emulsified in a Waring blender for 5 minutes. The olive oil emulsion and basal medium were autoclaved separately after which the emulsion was added aseptically with the aid of a sterile pipette to the basal medium at a concentration of 1 % $v.v^{-1}$. Each 250 $cm^3$ conical flask received 50 $cm^3$ of medium and was then inoculated. Cultures were incubated at 26 $^0C$ on a shaker revolving at 100 rpm for 35 days. Mycelium dry mass was determined in the usual way.

### 6.1 Determination of lipase activity

Mushroom mycelium was grown on the olive oil emulsion described in par. 6.0 to stimulate the production of lipase (triacylglycerol acylhydrolase, EC 3.1.1.3). At the end of the incubation period the cultures were filtered and dialyzed against distilled water at 4 $^0C$ for 48 hours. Lipase activity with olive oil as substrate was

determined according to the method of Bergmeyer (1974). An olive oil emulsion in 1 mol.dm$^{-3}$ triethanolamine buffer (pH 5,6) with 10 mmol.dm$^{-3}$ $CaCl_2$ was employed as substrate. Reaction mixtures consisted of 1 cm$^3$ of substrate and 200 $\mu$l of culture filtrate which were contained in stoppered centrifuge tubes. The tubes were shaken for twenty-four hours at 25 $^0$C after which the reaction was stopped with the addition of 5 cm$^3$ chloroform. Tubes were shaken for a further 5 minutes to dissolve free fatty acids followed by the addition of 2,5 cm$^3$ copper reagent (see appendix 1). The tubes were shaken for 20 minutes and then centrifuged at 3000 rpm for 5 minutes to separate the phases after which 2 cm$^3$ of the chloroform layer was pipetted into clean tubes. To each tube 250 $\mu$l of a diethyldithiocarbamate reagent was added and mixed. The extinction of the samples were measured at 440 nm and was read against a reagent blank which consisted of 5 cm$^3$ chloroform and 2,5 cm$^3$ copper reagent prepared in the same way as the samples. A sample blank which consisted of 200 $\mu$l inactivated culture filtrate and 1 cm$^3$ substrate served as control. The amount of fatty acids liberated into the assay mixture during hydrolysis was determined with the aid of a standard graph. This graph was drafted by preparing a serial dilution of oleic acid in chloroform in the concentration range 10 to 80 $\mu$mol.cm$^{-3}$. The optical densities of the serial dilution samples were determined spectrophotometrically with the aid of the copper reagent according to the procedure described above.

## 6.2 **Determination of esterase activity**

Mushroom mycelium was grown on the Tween 80 medium described in par. 6 and served as substrate for the production of esterase (carboxylic-ester hydrolase EC 3.1.1.1). Culture filtrates were prepared and treated according to the procedure described in par. 6.1. The assay mixture consisted of 4 cm$^3$ substrate (0,5 % v.v$^{-1}$ solution of Tween 80 in 0,1 mol.dm$^{-3}$ sodium phosphate buffer, pH 6,0) and 1 cm$^3$ culture filtrate. These were incubated at 25 $^0$C for twenty-four hours after which 1 cm$^3$ was withdrawn from each tube and the amount of fatty acids

liberated during hydrolysis was determined with the copper reagent according to the procedures described in par. 6.1.

## 7. Effect of other carbon sources and vitamins on the growth of mycelium

Several carbon sources which might be utilized by *Termitomyces* mycelium were investigated and included the following: monosaccharides, disaccharides, sugar alcohols, amino sugars, deoxy sugars, sugar phosphates, sugar acids, organic acids, lipids and polysaccharides. The different carbon sources were added to the basal medium and the carbon content of each source was equivalent to 100 mmoles of D-glucose and in the case of polysaccharides the amount added was 10 $g.dm^{-3}$. The liquid media were dispensed into 100 $cm^3$ conical flasks with 50 $cm^3$ medium per flask. All the carbon sources with the exception of the polysaccharides, lipids and Tweens were cold sterilized by means of microfiltration to avoid heat damage during the autoclaving process. This was performed by dissolving the carbon source in 20 $cm^3$ distilled water which was then added to the basal medium under aseptic conditions with the aid of a sterile 20 $cm^3$ syringe and a 0,22 $\mu$m disposable filter unit. The same procedure was followed for the sterilization of the multi-vitamins solutions. This medium consisted of the basal medium, D-fructose 10,0 g ; thiamine hydrochloride 2,0 mg ; pyridoxine hydrochloride 4,0 mg ; riboflavin 2,0 mg ; nicotinamide 2,0 mg ; p-aminobenzoic acid 0,2 mg ; biotin 0,8 mg ; D-panthothenic acid 0,4 mg ; folic acid 20,0 $\mu$g ; ascorbic acid 4,0 mg and 1 $dm^3$ distilled water. The cultures were incubated at 26 $^0C$ in a dark incubator for 35 days under static conditions. The mycelium dry mass was determined at the end of this period. The basal medium without a carbon source served as growth control. The lipids, olive oil and Tweens were autoclaved separately form the basal medium and were added aseptically to the cooled basal medium before inoculation.

## 8. Effect of nitrogen sources on the growth of mycelium

The utilization of several nitrogen sources was investigated and included eight inorganic, four complex organic sources and five amino acids. The basal medium with D-fructose was supplemented with the different nitrogen sources and was cold sterilized according to the methods described in par. 7. except that both D-fructose and the nitrogen sources were microfiltered and added to the rest of the basal medium. Ammonium nitrate was replaced with the different nitrogen sources under investigation except when the effect of $NH_4NO_3$ on growth was investigated. The amount of nitrogen in each compound was equivalent to 50 mmoles of sodium nitrate and in the case of the complex organic sources, 2 $g.dm^{-3}$. The media were prepared, autoclaved, inoculated and the cultures incubated according to the methods already described. Mycelium dry mass was determined at the end of the incubation period.

## 8.1 **Determination of trypsin activity**

To establish the ability of *Termitomyces* to degrade proteins in general the hydrolysis of casein was measured according to the method of Kunitz (1947). Mushroom mycelium was grown in a casein medium (described in the previous paragraph) to stimulate the production of trypsin (EC 3.4.21.4). The medium was poured into 300 $cm^3$ conical flasks in 50 $cm^3$ quantities and incubated for twelve days at 26 $^0$C in a dark incubator. The culture filtrates were dialyzed at 4 $^0$C against distilled water for 16 hours and trypsin activity was measured according to the method of Kunitz (1947) which was followed precisely.

## 8.2 **Amino acid and crude protein determination**

The amino acid composition of three *Termitomyces* species was determined to evaluate the quality and quantity of amino acids and therefore the protein quality of these mushrooms. This data was compared with the protein quality of *Agaricus bisporus* ($C_4$ variety). The analysis was performed on mycelium grown on the basal medium with D-fructose as carbon source and the cultures were incubated at 26 $^0$C for three weeks on a reciprocating table at 100 rpm. The cultures were filtered and

the mycelium mats were thoroughly rinsed with distilled water to remove all traces of medium. The mycelium mats were lyophilized and ground to a fine powder with a mortar and pestle. The powder was hydrolyzed for 22 hours at 110 $^{0}$C with the vapor of 6 N HCI. The mycelium powder was dissolved in 5 mmol.dm$^{-3}$ $Na_2HPO_4$ solution of pH 7,4. Solvent A consisted of a solution of sodium acetate trihydrate (0,14 mol.dm$^{-3}$) and 500 $\mu$l triethylamine. The pH was adjusted with acetic acid to 6,4 and 940 cm$^3$ of this solution was combined with 60 cm$^3$ acetonitrile. Solvent B consisted of 600 cm$^3$ acetonitrile and 400 cm$^3$ distilled water. High performance liquid chromatography was utilized to determine the amino acid composition of the mycelium and the separation of amino acids was performed with a Pico.Tag amino acid separating column of Waters Associates. Separation was performed at a flowspeed of 1 ml.min$^{-1}$ and a pressure of 14,479 x 10$^3$ kPa. The nitrogen content of the mycelium powder was determined by micro-Kjeldahl analysis and the crude protein content was calculated with the aid of the conversion factor (N x 4,38). The Essential Amino Acid Index and the Biological Value of the mushroom protein were calculated with the aid of the formulas supplied by Oser (1951, 1959) and the Nutritional Index and Amino Acid Score according to the formulas of Crisan & Sands (1978).

## 9. **Preparation of natural substrates**

The growth of *T. umkowaani* on the following substrates was determined : (i) sorghum, wheat bran, sawdust (ii) wheat bran, sawdust (iii) rye, wheat bran, sawdust (iv) tobacco leaves (v) wheat straw (vi) sawdust and (vii) wheat bran. Substrates i to vi with the exception of wheat bran were pulverized in a Wiley mill and filtered through no. 20 mesh wire. The sieved particles were hydrated with different liquid media. Substrates i to iii were hydrated with the following liquid media : (i) YESS medium (ii) basal medium (iii) basal medium supplemented with several vitamins (see par. 6) and (iv) distilled water. Substrates iv to vii were hydrated with basal medium. The grounded substrates were placed in 300 cm$^3$ conical flasks with 167 g per flask. Substrates i to iii were combined in a ratio of : 7,5 : 1,5 ; 1,0 (sorghum, wheat

or rye : wheat bran : sawdust). Moisture content was 1,3 $cm^3.g^{-1}$ unhydrated substrate. The hydrated solid media were inoculated in the following way. Mycelium of *T. umkowaani* was grown in: (i) YESS medium (ii) basal medium with fructose and (iii) the basal medium with multi-vitamins. The broths were prepared and inoculated according to the methods already described and incubated on a reciprocating table at 100 rpm at 26 $^0$C for three weeks. The cultures were poured onto the natural substrates and excess medium was decanted. The spawned substrates were incubated at 26 $^0$C under dark conditions for four to twelve weeks. The amount of growth was assessed in a purely qualitative manner at the end of the incubation period. No attempt was made to induce basidiocarp production.

## B. MORPHOLOGY

### 1. Light microscopy and preparation of microscope slides of:

### 1.1 fresh and dried mushroom tissue

Intact fruit bodies were collected in the vicinity of termitaria where the fruit bodies grew in connection with the subterranean fungus combs. Spore prints were prepared by placing pieces of intact pilei on white paper. Spores were mounted in lactophenol blue. Fruit bodies were dried in a warm air oven at 80 $^0$C for 16 hours and were then deposited in the herbarium of the National Collection of Fungi (PREM) Plant Protection Research Institute Pretoria, or in the herbarium of the H.G.W.J.Schweikerdt-Herbarium (PRUM), University of Pretoria, Botany Department. Existing specimens deposited in the collections of the above-mentioned herbaria were also studied. Different parts of the fruit bodies were investigated by mounting free hand sections in a mixture of 1 % $w.v^{-1}$ aqueous phloxine and 10 % $w.v^{-1}$ KOH. These were examined microscopically at different magnifications and photographed with a Ilford FP 4 35 mm film.

### 1.2 plate cultures

Mycelium of *Termitomyces* was grown on YESS medium. Pieces of mycelium were removed from the advancing zone, aerial and submerged mycelium of cultures and mounted in lactophenol blue. These were macerated with an inoculating loop and covered with a coverslip. The coverslip plate culture technique of Ansell (1982) was utilized to study the development of conidiophores. Molten agar (YESS medium) was poured aseptically into a Petri dish to a depth of 5 mm. A channel of approximately 2,5 x 1 cm was cut in the agar whereafter the walls of the channel were inoculated and covered with a flame sterilized 22 $mm^2$ square coverslip, leaving about 2 mm uncovered for aeration. Cultures were incubated for 7 days at 26 $^0$C whereafter the coverslips were removed from the plate cultures and mounted in lactophenol blue. The microscopic characters were examined at different magnifications and photographed with an Ilford FP 4 35 mm film.

Macroscopic growth characters of the cultures were examined at low magnification with the aid of a dissection microscope equipped with a Tessovar photomacrographic zoom system. The cultures were illuminated with cold optic fibre light and photographs were taken at different magnifications with a Ilford 35 mm film. The macroscopic growth characters of the cultures were described with the aid of the terms utilized by Nobles (1948), the guaiacol reagent was prepared according to Davidson, Campbell & Blaisdell (1938) and the colours were described according to the terminology of Ridgway (1912).

## 2. **Scanning electron microscopy**

Cultures prepared from comb sporodochia and basidiome context were investigated three dimensionally with the aid of a freeze etching technique fully integrated with a scanning electron microscope. Material was prepared for scanning electron microscopy (SEM) according to the method of Hamilton-Attwell & Jooste (1988). Specially modified SEM buttons (bored out to form a hollow cup) were autoclaved and placed aseptically on water agar plates from which a block (± 15 $mm^2$) was excised to expose the Petri dish bottom. Molten nutrient agar (YESS medium) was poured aseptically with a sterile Pasteur pipette into the bottom of the hollow SEM button to form a 1-2 mm thick agar layer. A small amount of fungus material was inoculated onto this

layer and incubated for 7 days after which the SEM button was removed and immediately frozen in liquid nitrogen at -254 $^{0}$C from which the material was transferred to a precooled vacuum chamber. The frozen material was slowly heated to approximately -55 $^{0}$C to sublimate ice and then coated with a thin gold layer before the material was examined at -190 $^{0}$C at 10 KV, at a working distance of 20 to 27 mm. Hyphal structures were photographed with Ilford FP 4 120 mm film at different magnifications.

### 3. **Transmission electron microscopy**

Material was fixed in 2,5 % w.v$^{-1}$ glutaraldehyde in 0,07 Mol.dm$^{-3}$ phosphate buffer pH (7,4-7,6) for 2 hours under vacuum. The specimens were washed three times in the same buffer. Post fixation was in 0,25 % w.v$^{-1}$ osmium tetroxide for 2 hours. The material was then washed in distilled water and dehydration was in a graded acetone series. The specimens were embedded in 30 %, 60 % and 100 % Quetol succesively and polymerized at 60 $^{0}$C for 48 hours. Ultrathin sections were cut with a microtome diamond knife, mounted on copper grids and stained for 10 minutes in 40 % w.$^{v-1}$ uranyl acetate and 2 minutes in lead citrate (Reynolds, 1963). Sections were examined with a Phillips EM 300 transmission electron microcsope at different magnifications.

### 4. **Germination studies**

Germination studies were executed in a semi-solid minimal medium with the following composition : $(NH_4)_2SO_4$ 5,0 g; $KH_2PO_4$ 0,31 g; $K_2HPO_4$ 0,45 g ; $Na_2HPO_4$ 0,92 g; NaCI 0,1 g; $CaCI_2$ 0,05 g; Mg $SO_4.7H_2O$ 0,2 g; histidine 5mg; tryptophane 200 mg; methionine 20 mg; chloramphenicol 250 mg; 1 dm$^3$ distilled water. The medium was pipetted into culture bottles in 5 cm$^3$ quantities and autoclaved. The medium was inoculated with a suspension of conidia or basidiospores in a sterile 0,15 % NaCI solution with a sterile inoculating loop and incubated at 26 $^{0}$C for 72 hours. The cultures were investigated microscopically by placing a small amount of medium on a microscope slide with a sterilized inoculating loop at twenty-four hour intervals.

## 5. Collection and identification of host termite species

Termites associated with the specific *Termitomyces* species were collected simultaneously with fruit bodies and placed in 70 % ethanol. Soldiers were used for identification purposes and the distribution pattern of the specific termite species was supplied by the National Collection of Insects, Plant Protection Research Institute, Pretoria.

# IV. RESULTS AND DISCUSSION

## A. NUTRIENT PHYSIOLOGY

### 1. Environmental factors

### 1.1 Effect of pH on the growth of mycelium

Growth of *Termitomyces* at different pH values is graphically illustrated in Fig.1 and growth data is presented in Table 1. It is apparent that *T. microcarpus* displays the highest tolerance toward pH fluctuations, growing from 3,0 to 10,0 with optimal growth at or near 7,0 and no growth at 2,0 and 11,0. *T. sagittaeformis* has the lowest tolerance toward pH fluctuations, growing from 4,0 to 6,0 with optimal growth at or near 6,0 and no growth at 3,0 and 7,0. The pH range of *T. reticulatus* stretches from 3,0 to 7,0 with optimal growth at or near 6,0 and no growth at 2,0 and 8,0. *T. clypeatus* grows in the range 3,0 to 6,0 with optimal growth at or near 4,0 and no growth at 2,0 and 7,0. *T. umkowaani* grows in the range 3,0 to 7,0 with optimal growth at or near 4,5 and no growth at 2,0 and 8,0. The data in Table 1 indicate whether the different treatment means differ significantly. *T. clypeatus* and *T. umkowaani* seem to be more acidophilous with pH optima 4,0 and 4,5 respectively while the other three species display growth at or near neutral pH values.

These results are in accordance with those obtained by Batra & Batra (1979) and Mishra & Sen-Sarma (1979) who obtained pH values of 4,5 to 6,8 and 4,2 to 5,3 respectively for fungus combs of different termite species. The combs tend to be acidic to slightly neutral and

subsequently would not restrict growth of *Termitomyces* mycelium. Chandra & Purkayastha (1977) established that the optimum pH for growth of *T. eurhizus* was 4,0 while Ghosh & Sengupta (1978) obtained an optimal pH value of 2,5 for *T. clypeatus* which differs from the data in Table 1 which indicate an optimum pH value at or near 4,0.

### 1.2 **The effect of temperature on the growth of mycelium**

Results of the growth of *Termitomyces* mycelium at different temperatures are listed in Table 2 and illustrated in Fig.2. *T. microcarpus* grows over a broad temperature range with growth commencing at 5 $^0$C and terminating at 30 $^0$C. The optimal temperature is at or near 25 $^0$C with no growth at 35 $^0$C. *T. sagittaeformis* exhibits a lower tolerance toward temperature fluctuations growing from 15 $^0$C to 30 $^0$C with optimal growth at or near 30 $^0$C and no growth at 5 $^0$C to 10 $^0$C and 35 $^0$C. *T. reticulatus* shows a high tolerance toward temperature fluctuations and grows from 10 $^0$C to 35 $^0$C with optimal growth at or near 25 $^0$C and no growth at 5 $^0$C and 40 $^0$C. *T. clypeatus* has a much narrower temperature range and grows from 15 $^0$C to 30 $^0$C with optimal growth at or near 20 $^0$C and no growth at 5 $^0$C to 10 $^0$C and 35 $^0$C. *T. umkowaani* exhibits an equally limited temperature tolerance growing from 15 $^0$C to 30 $^0$C with optimal growth at or near 25 $^0$C and no growth at 5 $^0$C to 10 $^0$C and 35 $^0$C. *T. sagittaeformis* displays the highest temperature for optimal growth, ± 30 $^0$C, while *T. clypeatus* has the lowest temperature for optimal growth, ± 20 $^0$C. The remaining three species grow at or near 25 $^0$C.

Thomas (1985;1987 a,b) incubated isolates of *Termitomyces* spp. made from termite manipulated organic material at 28 $^0$C to 30 $^0$C which, according to her is the optimal growth temperature. Batra & Batra (1979) conducted preliminary growth experiments in the temperature range 5 $^0$C to 35 $^0$C with *T. albuminosus* and obtained an optimal growth temperature of ± 25 $^0$C. De (1982) incubated his cultures of *T. microcarpus* at ± 28 $^0$C during his efforts to induce fruit body formation. Quimio (1977) obtained an optimum growth

temperature of 30 $^{0}$C for *T. cartilagineus* with no growth at 10 $^{0}$C and 40 $^{0}$C. Samajpathi (1981) utilized an incubation temperature of ± 30 $^{0}$C for cultures of *T. clypeatus*, *T. microcarpus* and *T. eurhizus*. Purkayastha & Chandra (1975,1976) incubated their cultures of *T. eurhizus* at 26 $^{0}$C and 28 $^{0}$C to 30 $^{0}$C, while Ghosh & Sengupta (1978, 1987) preferred to incubate cultures of *T. clypeatus* at ± 30 $^{0}$C. Geyer (1951) recorded temperatures of 11,1 $^{0}$C to 24,2 $^{0}$C in fungus comb chambers of termitaria located in South Africa and in Thailand Matsumoto (1977) recorded temperatures of 25,8 $^{0}$C to 29,7 $^{0}$C, while Lüscher (1961) obtained a temperature of 29,7 $^{0}$C in the fungus comb chamber of a termitarium in the Ivory Coast. The data in Table 2 indicates that most of the *Termitomyces* species investigated with the exception of *T. clypeatus* prefer temperatures in the region of 25 $^{0}$C. The temperature measurements for optimal mycelium growth in Table 2 are in the temperature range recorded for fungus comb chambers of termitaria located in an African subtropical climate (Geyer, 1951), where a monoculture of *Termitomyces* mycelium is maintained by the worker termites throughout the year. The water saturated atmosphere of the fungus comb chambers are nearly always at a temperature higher than the environment. These conditions prevail throughout most of the year and are maintained by the high rate of respiration of the worker termite population and *Termitomyces* mycelium (Matsumoto, 1977). These favourable conditions explain the luxuriant growth of *Termitomyces* mycelium on the comb.

### 1.3 **Effect of light on growth of mycelium**

Results listed in Table 3 and graphically depicted in Fig. 3 indicate that mycelium growth of the species investigated are light insensitive. The mycelium yield attained does not differ significantly even at the 5 % level. However mycelium yield attained under dark incubation conditions is slightly higher for each species under investigation and may reflect the subterranean dark environment where optimal mycelium growth of *Termitomyces* occurs in the natural habitat. This at least represents the growth response of pure vegetative mycelium under artificial light conditions.

**TABLE 1.** GROWTH OF *TERMITOMYCES* AT DIFFERENT pH VALUES.

| | Mycelium dry mass (mg) pH values | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Species tested | 3.0 | 4.0 | 4.5 | 5.0 | 6.0 | 7.0 | 8.0 | 9.0 | 10.0 |
| 1.*T. microcarpus* | 53 | 167 | - | 278 | 401 | 402 | 278 | 156 | 133 |
| 2.*T.sagittaeformis* | - | 23 | - | 38 | 46 | - | - | - | - |
| 3.*T. reticulatus* | 14 | 32 | - | 51 | 92 | 21 | - | - | - |
| 4.*T. clypeatus* | 30 | 100 | - | 50 | 25 | - | - | - | - |
| 5.*T. umkowaani* | 21 | 34 | 40 | 23 | 23 | 17 | - | - | - |

| F-ratio | *P*≤0.05 | 0.01 | 0.001 |
|---|---|---|---|
| 1. F(7,24)=24,00*** | LSD 75,93 | 102,89 | 137,77 |
| 2. F(2,9) =3,29 *** | 4,22 | 6,06 | 8,92 |
| 3. F(4,15)=38,13*** | 15,13 | 20,93 | 28,92 |
| 4. F(3,12)=12,11*** | 30,42 | 42,65 | 60,29 |
| 5. F(5,18)=5,69** | 10,63 | 14,11 | - |

* significance at *P* ≤0.05
** significance at *P* ≤0.05; 0.01
*** significance at *P* ≤0.05; 0.01; 0.001.
n.s. denotes not significant.

**TABLE 2.** GROWTH OF *TERMITOMYCES* AT DIFFERENT TEMPERATURES.

| | Mycelium dry mass (mg) Temperature °C | | | | | | |
|---|---|---|---|---|---|---|---|
| Species tested | 5 | 10 | 15 | 20 | 25 | 30 | 35 |
| 1.*T. microcarpus* | 39 | 17 | 18 | 48 | 37 | - | - |
| 2.*T. sagittaeformis* | - | 16 | 27 | 41 | 51 | - | - |
| 3.*T. reticulatus* | - | 9 | 41 | 83 | 200 | 72 | 12 |
| 4.*T. clypeatus* | - | - | 12 | 69 | 55 | 45 | - |
| 5.*T. umkowaani* | - | - | 21 | 29 | 47 | 42 | - |

| F-ratio | *P*≤0.05 | *P*≤0.01 | *P*≤0.001 |
|---|---|---|---|
| 1. F(5,18)=18,48*** | LSD 11,80 | 16,16 | 22,03 |
| 2. F(3,12)=2,86* | 27,95 | - | - |
| 3. F(5,18)=57,63*** | 27,86 | 38,17 | 52,01 |
| 4. F(3,12)=3,32* | 40,62 | - | - |
| 5. F(3,12)=2,17 n.s. | - | - | - |

FIGURE 1(a). GROWTH OF *T. MICROCARPUS* AT DIFFERENT pH VALUES.

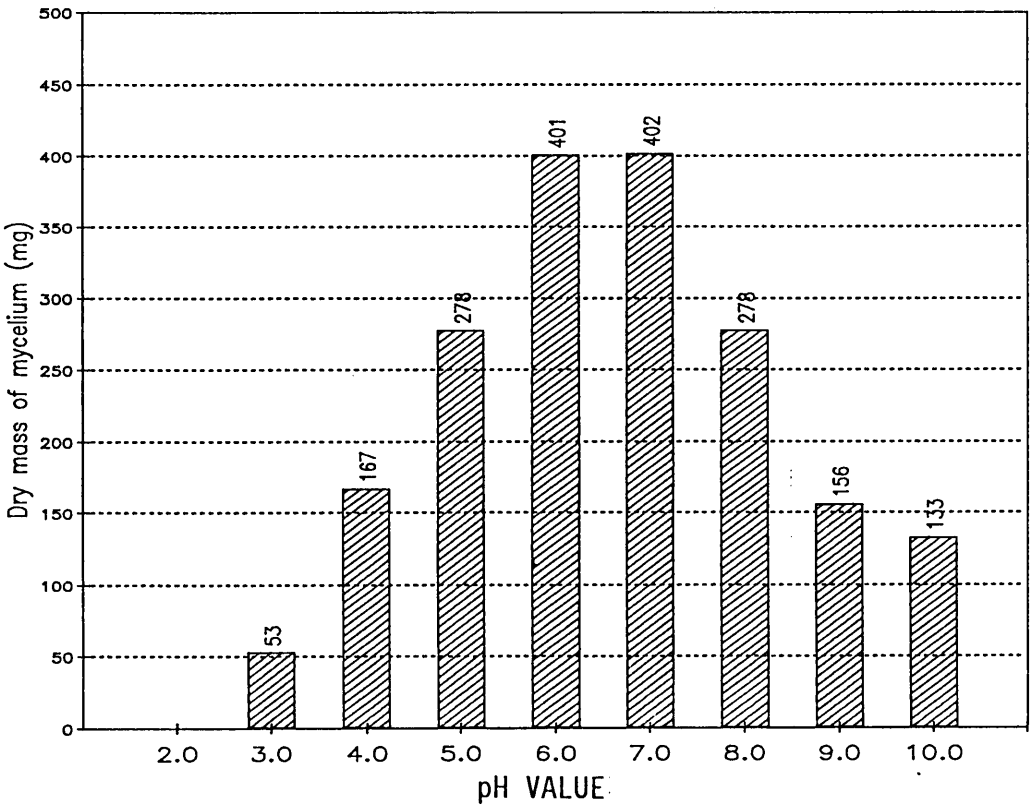



FIGURE 1(b). GROWTH OF *T. SAGITTAEFORMIS* AT DIFFERENT pH VALUES.

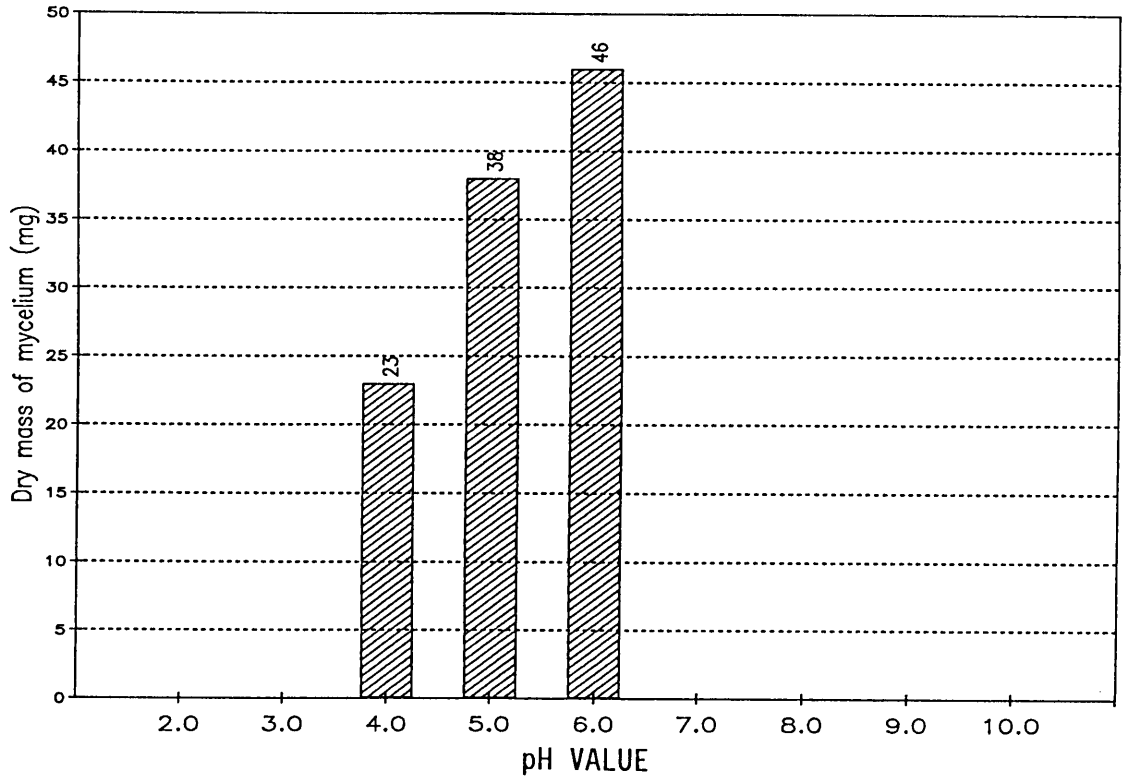


FIGURE 1(c). GROWTH OF *T. RETICULATUS* AT DIFFERENT pH VALUES.

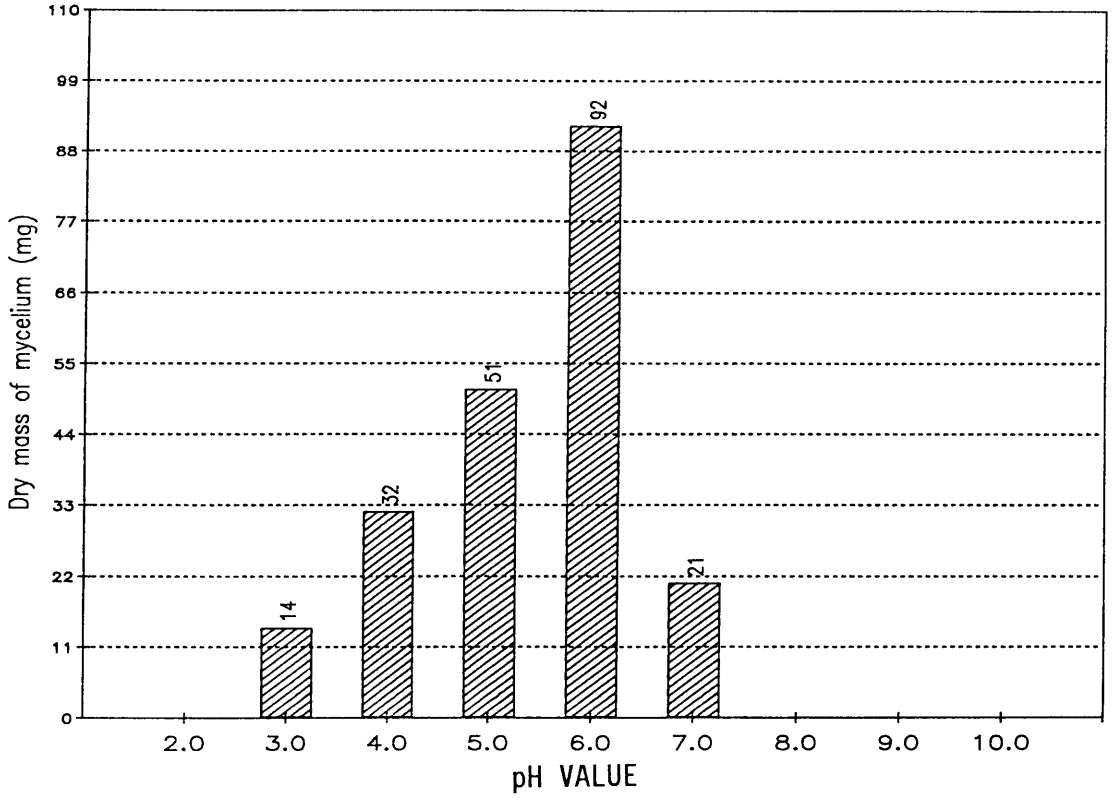



FIGURE 1(d). GROWTH OF *T. CLYPEATUS* AT DIFFERENT pH VALUES.

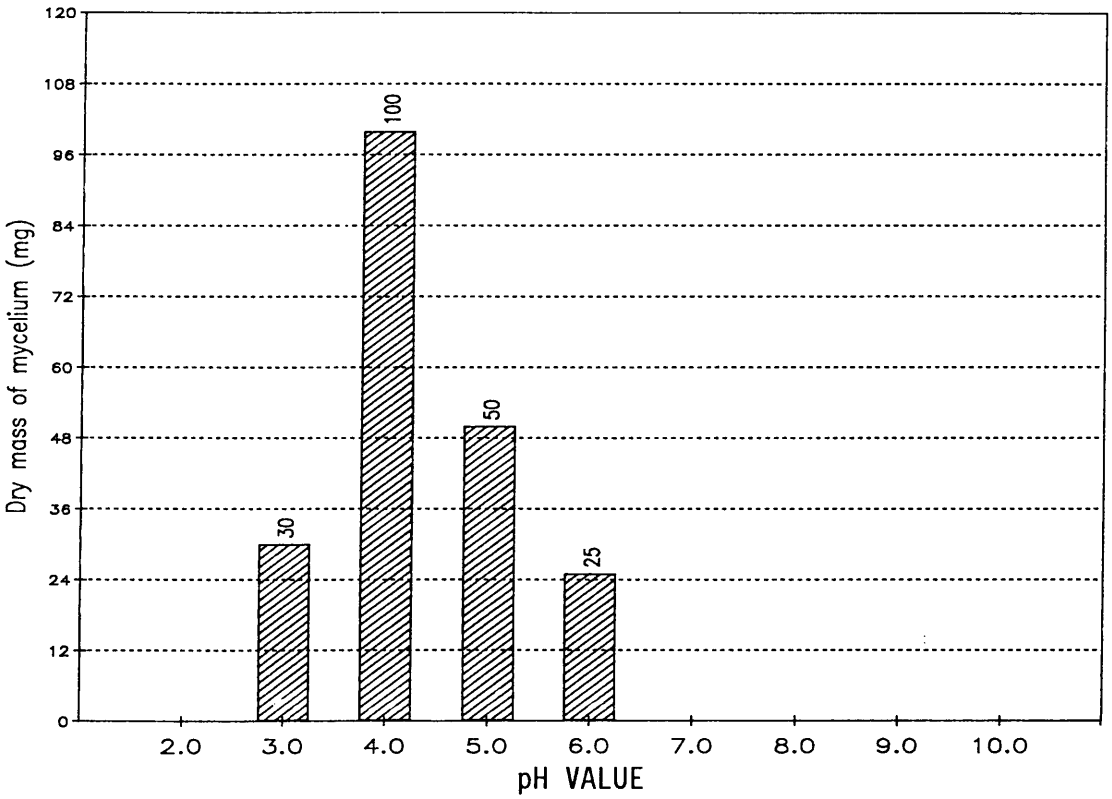


FIGURE 1(e). GROWTH OF *T. UMKOWAANI* AT DIFFERENT pH VALUES.

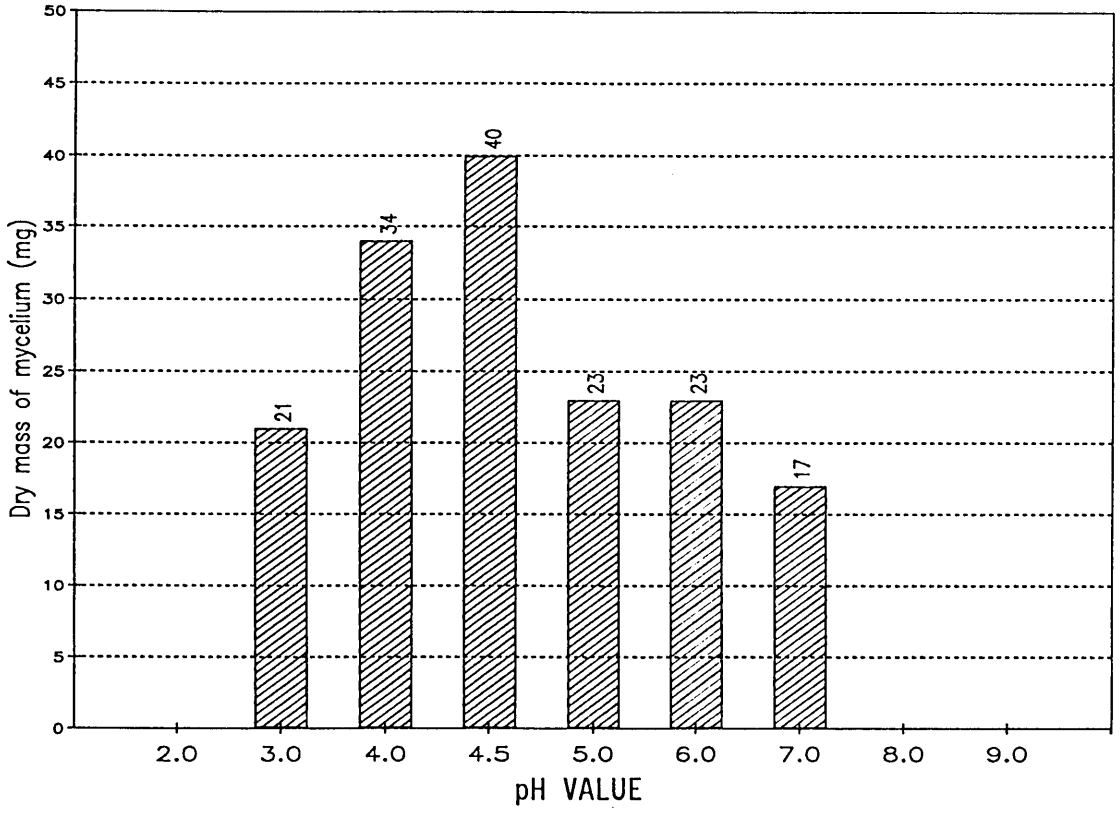



FIGURE 2(a). GROWTH OF *T. MICROCARPUS* AT DIFFERENT TEMPERATURES.

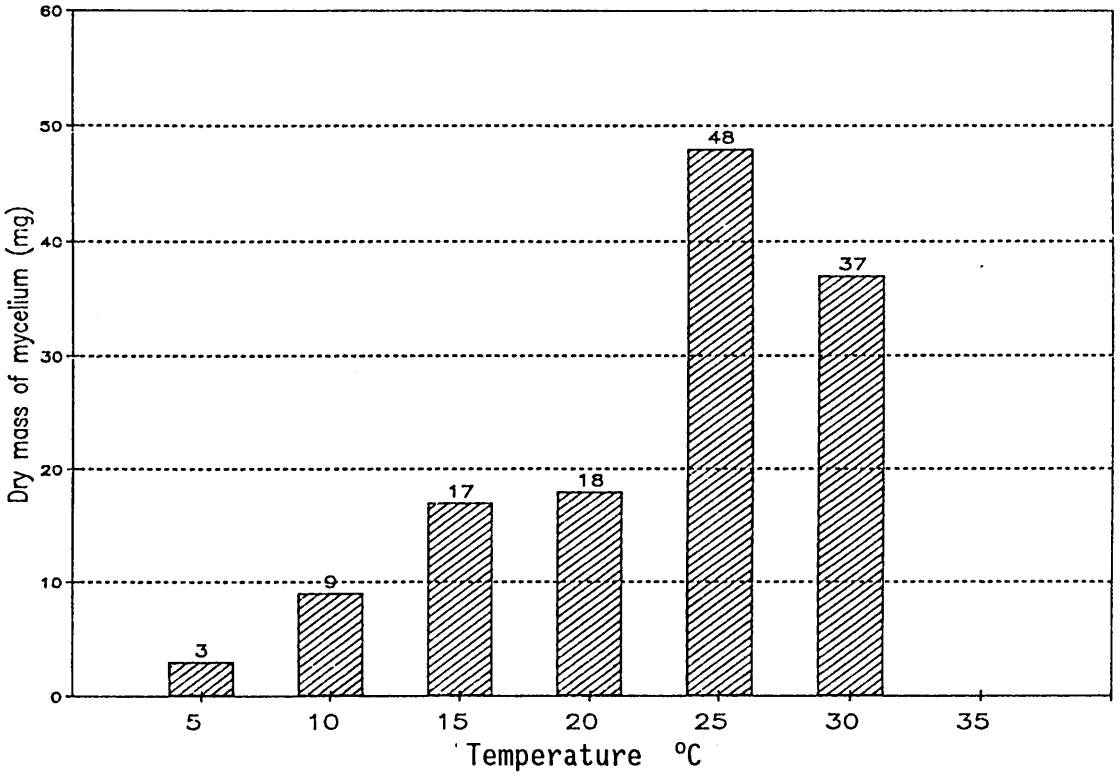


FIGURE 2(b). GROWTH OF *T. SAGITTAEFORMIS* AT DIFFERENT TEMPERATURES.

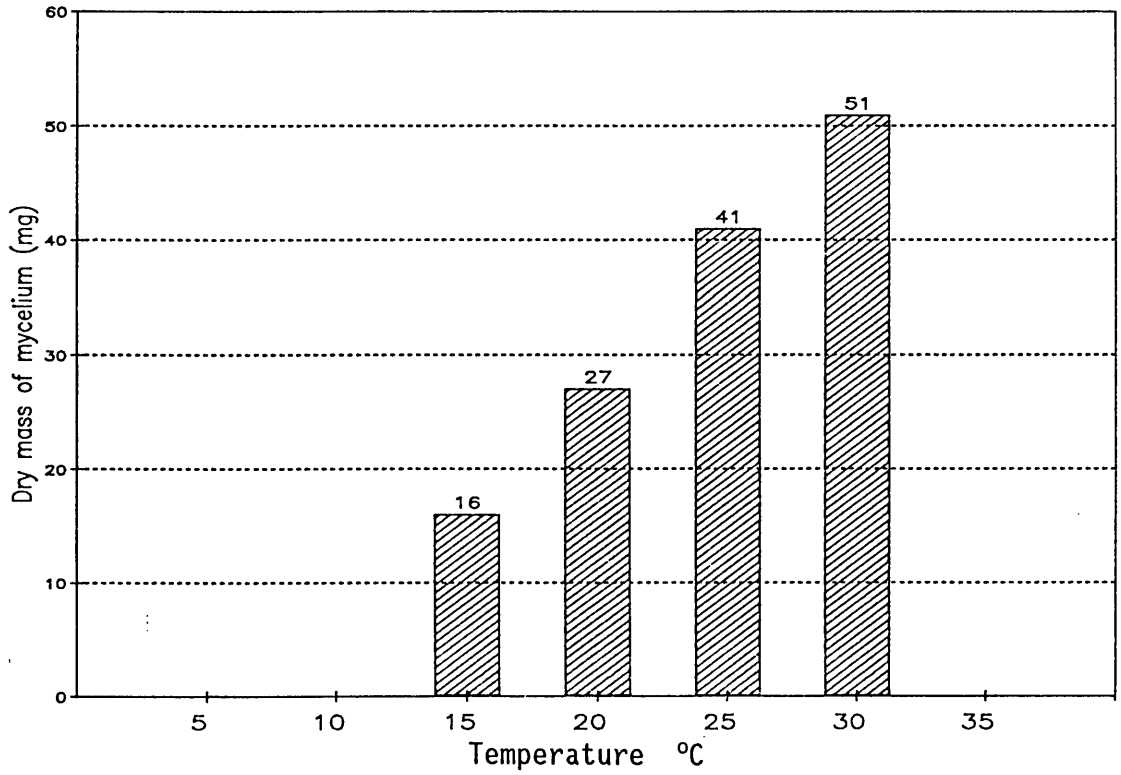



FIGURE 2(c). GROWTH OF *T. RETICULATUS* AT DIFFERENT TEMPERATURES.

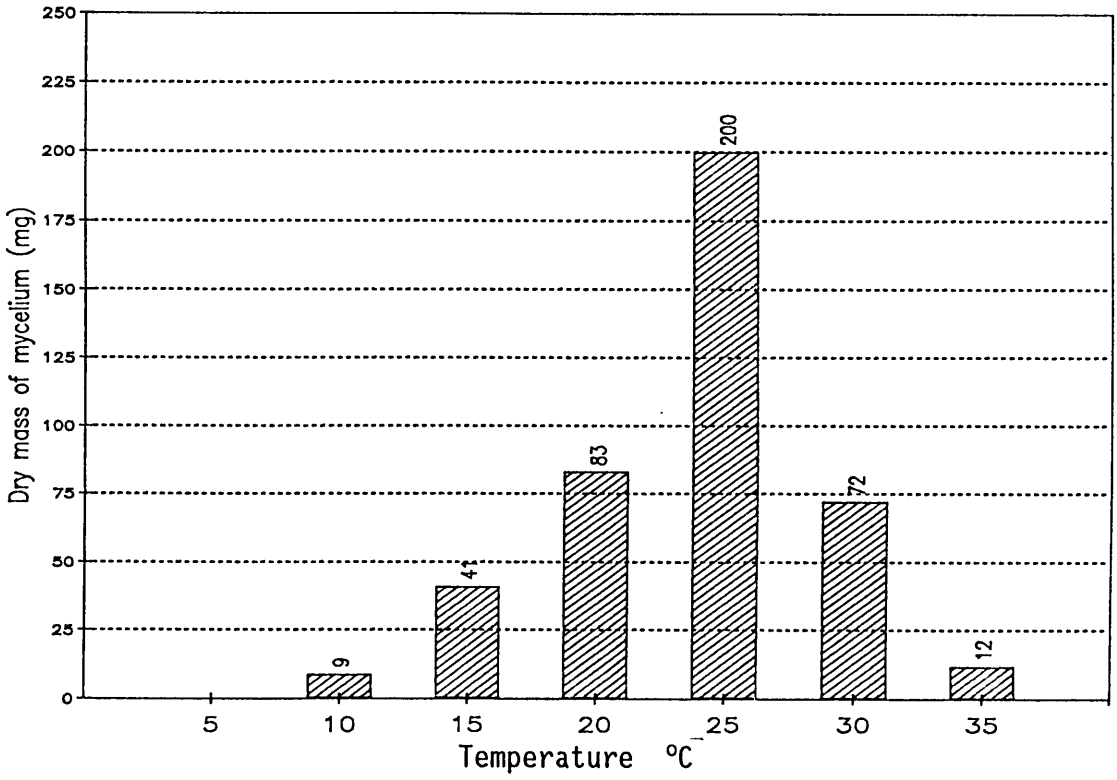


FIGURE 2(d). GROWTH OF *T. CLYPEATUS* AT DIFFERENT TEMPERATURES.

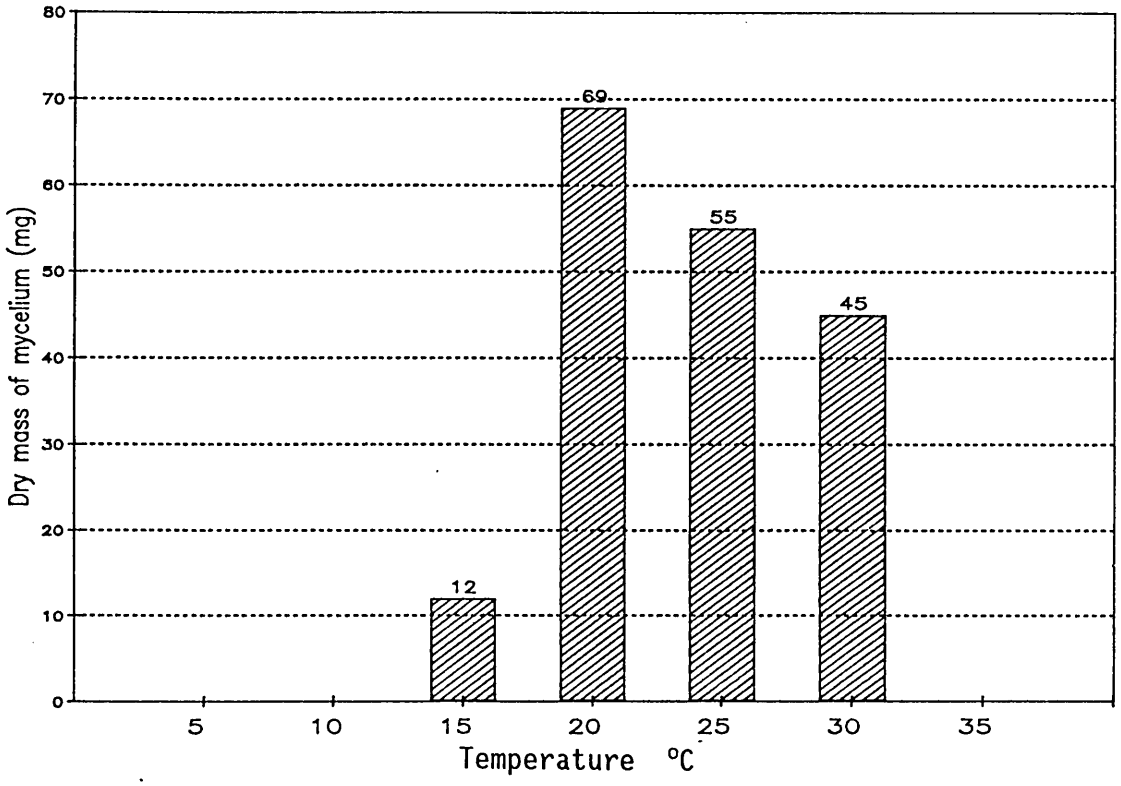



FIGURE 2(e). GROWTH OF *T. UMKOWAANI* AT DIFFERENT TEMPERATURES.

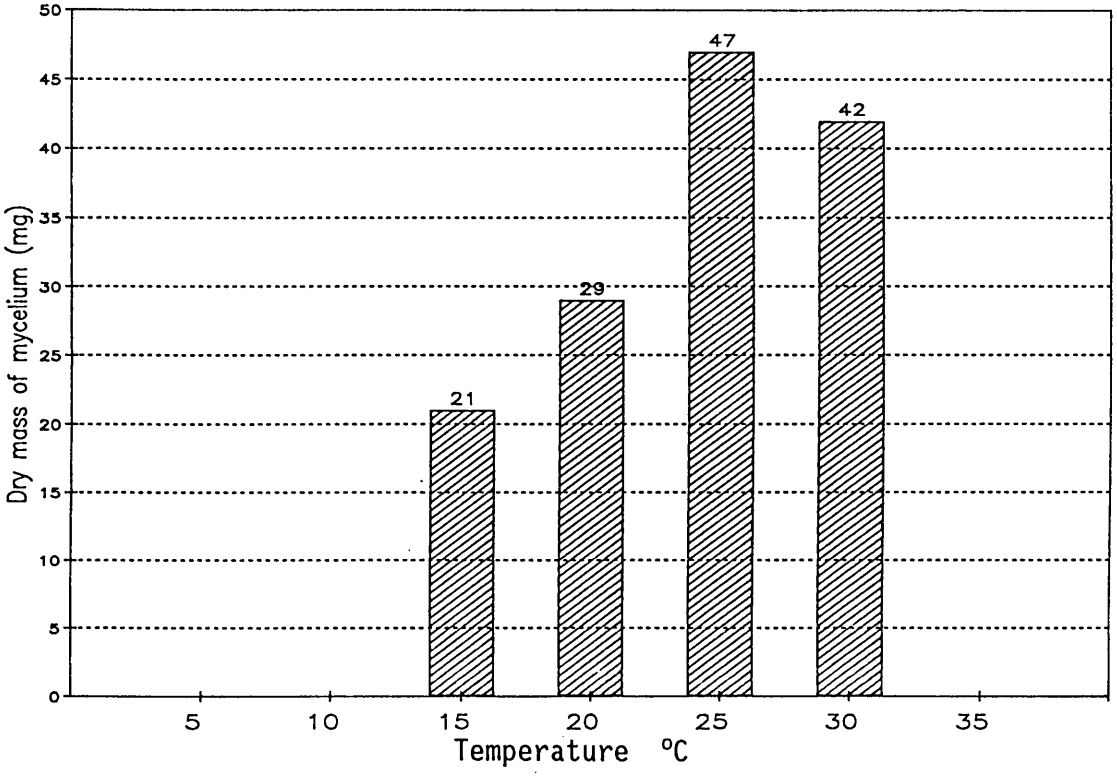


**TABLE 3.** GROWTH OF *TERMITOMYCES* UNDER DIFFERENT LIGHT CONDITIONS.

| Species tested | Light conditions | Mycelium dry mass (mg) |
|---|---|---|
| 1.*T. microcarpus* | CL | 122 |
| F(2,9)=2,75 | L/D | 115 |
| | CD | 164 |
| 2.*T. sagittaeformis* | CL | 129 |
| F(2,9)=1,39 | L/D | 109 |
| | CD | 146 |
| 3.*T. reticulatus* | CL | 116 |
| F(2,9)=0.15 | L/D | 135 |
| | CD | 147 |
| 4.*T. clypeatus* | CL | 17 |
| F(2,9)=1,14 | L/D | 20 |
| | CD | 23 |
| 5.*T. umkowaani* | CL | 58 |
| F(2,9)=2,88 | L/D | 46 |
| | CD | 116 |
| 6.Anamorph | CL | 21 |
| F(2,9)=1,45 | L/D | 24 |
| | CD | 25 |

CL=continuous light; L/D=alternating light and dark periods of 12 hours duration each; CD=continuous darkness.
Mycelium dry mass, n.s. at $P \leq 0.05$.

FIGURE 3. GROWTH OF *TERMITOMYCES* SPECIES UNDER DIFFERENT LIGHT CONDITIONS.

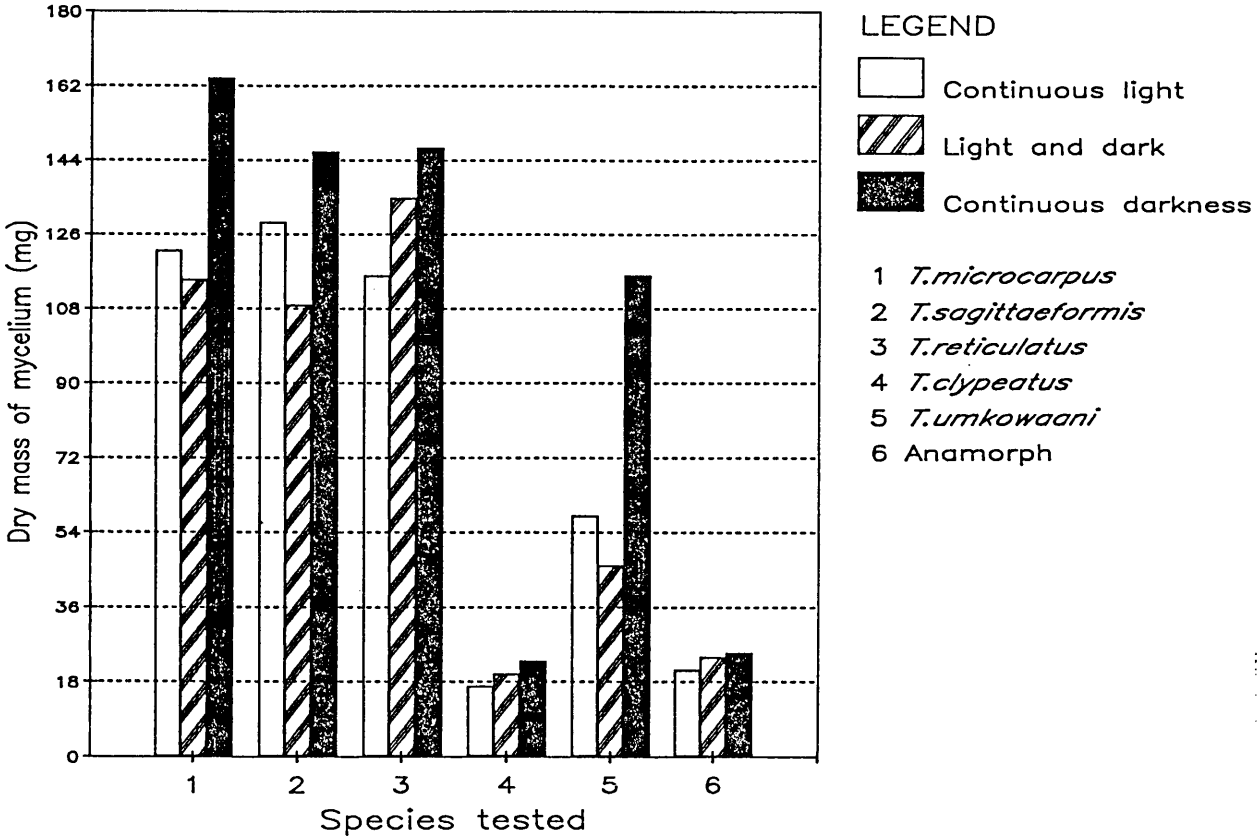
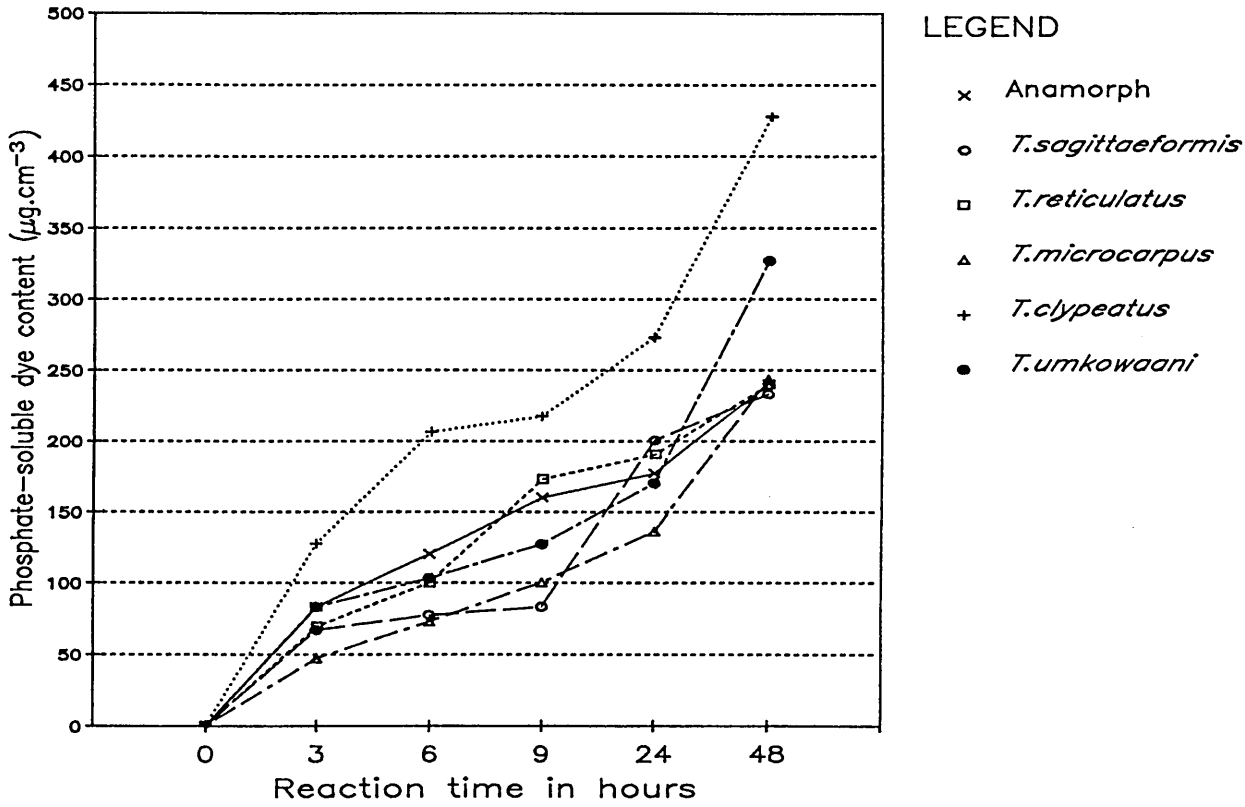



FIGURE 4(a). C1—ACTIVITY OF *TERMITOMYCES* CULTURE FILTRATES GROWN ON CRYSTALLINE CELLULOSE CULTURE MEDIUM.



Quimio (1977) conducted similar experiments with *T. cartilagineus* and he also reported slightly better growth under dark conditions. However details of the experimental conditions under which his experiments were conducted are not precise and the measurements recorded in this article do not differ significantly. De (1983) investigated the influence of light on basidiocarp formation by *T. microcarpus* and he established that basidiocarp formation was stimulated only when a period of six hours darkness was preceded by eighteen hours of light. Any different light treatment caused either no or incomplete sporocarp development. These results indicate that light exerts a more pronounced influence on basidiocarp formation than on the vegetative mycelium of *Termitomyces*. To ascertain the influence of light on fruit body development of *Termitomyces* species in general would require a thorough investigation into the conditions conducive for fruit body development.

## 2. Effect of cellulose (natural and synthetic) on the growth of mycelium and determination of activity of cellulolytic enzymes

The ability of species of *Termitomyces* to degrade crystalline cellulose fibres is presented in Table 4 and portrayed in Figs 4 a,b. It is clear from the results in Table 4 that all the species investigated have the ability to degrade crystalline cellulose fibres. This is indicated by the amount of mycelium produced and secretion of 1,4-ß-D-glucan cellobiohydrolase or $C_1$-enzyme which is responsible for the primary hydrolysis of crystalline cellulose fibres. The ability of the different species to utilize crystalline cellulose as a source of carbon differs very little. The mycelium dry mass differs significantly at all three levels, therefore the mycelium yield attained by the anamorph will differ significantly from those of *T. clypeatus* and *T. umkowaani*, while the latter species attained the highest mycelium yield. Culture filtrates of the species investigated display relatively high $C_1$-enzyme activity (Fig. 4 a) in particular those of *T. clypeatus* and *T. umkowaani* but do not differ significantly at the 5 % level. Thus mycelium yield and enzyme activity of the different species do not differ greatly and this observation will support the

statement of near equality of cellulose utilization. No significant correlation (r = + 0,32) exists between mycelium produced (x) and enzyme activity (y).

Mycelium of *Termitomyces* degrades the synthetic or cellulose derivative, carboxymethyl cellulose (CMC) and results are shown in Table 5 and Figs 5 a,b. Results presented in Table 5 indicate that the species investigated have the ability to hydrolyze CMC. When this carbon source is effectively utilized, it will support reasonable mycelium growth and stimulate the production of (1,4-ß-D-glucan 4-glucanohydrolase) or $C_X$-enzyme in sufficient quantities. The species examined differ in their ability to degrade CMC and this is reflected in the mycelium yield and the enzyme activity of the culture filtrates. Both these parameters of growth differ significantly between the species. *T. microcarpus* attain the highest mycelium yield followed by *T. umkowaani*, but their culture filtrates display lower $C_X$-activity than the filtrates of *T. reticulatus* and *T. clypeatus* which differ significantly from the $C_X$-activity of the other species. There is no significant correlation (r = - 0,38) between the mycelium yield and the enzyme activity in the filtrate. Consequently a high mycelium yield will not necessarily coincide with high enzyme activity or a low mycelium yield with low enzyme activity. The third enzyme in the trio of cellulolytic enzymes ß-glucosidase (EC 3.2.1.21) could not be detected in the culture filtrates either as a result of being secreted in very low quantities into the liquid medium or the enzyme is incapable of moving across the cell membrane. This may indicate that this enzyme is only active in the cytoplasm.

Osore & Okech (1983) detected cellulase ($C_X$) and ß-glucosidase activity in extracts of freeze-dried conidiophores (sporodochia) and fruit bodies of a *Termitomyces* sp. They obtained high activities of both enzymes in the conidiophores and fruit bodies. Abo-Khatwa (1978) investigated the cellulolytic enzymes of sporodochia from the combs of *Macrotermes subhyalinus* and detected all three enzymes necessary for the complete degradation of crystalline cellulose. Martin & Martin (1978, 1979) conducted similar research on sporodochia isolated from the combs of *Macrotermes natalensis* and they also detected high activities of all three cellulolytic enzymes in extracts of sporo-

dochia. Mishra & Sen-Sarma (1986) detected a comprehensive range of enzymes active in the sporodochia of *T. albuminosus* which included ß-glucosidase and filter paper $C_1$-cellulase. $C_x$-cellulase activity was detected by Skelton & Matanganyidze (1981) in extracts prepared from the fruit bodies of *T. microcarpus*. Thus it would seem that both sporodochia and fruit bodies of *Termitomyces* species so far investigated, have the complete multiple-component enzyme system necessary for the complete hydrolysis of crystalline cellulose. Research conducted by Wood (1969), Reese & Mandels (1971) and Wood & Mc Crae (1975) proved beyond any doubt that this enzyme complex consists of : (i) a $C_1$-enzyme which is active against crystalline cellulose (ii) $C_x$-enzyme which hydrolyses non-crystalline cellulose, soluble derivatives and low molecular weight oligosaccharides and (iv) ß-glucosidase which attacks mainly cellobiose. Reese, Sui & Levinson (1950) suggested in their original hypothesis that '$C_1$-enzymes' were responsible for the modification of crystalline cellulose before hydrolysis by the '$C_x$-enzymes' could commence. More recent research results contradict this mechanism of cellulose hydrolysis. Several authors ( Berghem & Pettersson, 1973; Berghem, Pettersson & Axio-Fredukssson, 1975; Eriksson & Pettersson, 1975) suggested that it was rather the $C_x$-enzymes which attacked the crystalline cellulose chains at random to open up chain ends before the $C_1$-enzymes can act. Eriksson & Pettersson (1975) provided evidence that the '$C_1$-enzyme' is actually a cellobiohydrolase which produce mainly cellobiose by attacking the non-reducing end of the cellulose chain in filtrates of crystalline cellulose culture media on which mycelium was grown and work synergistically to degrade crystalline cellulose to cellotriose and cellobiose units. To measure $C_1$-activity in filtrates with a mixture of $C_x$ and $C_1$-enzymes, the use of a substrate specific for $C_1$-enzymes was employed (cellulose-azure covalently bonded with a reactive dyestuff Remazol Brilliant Violet 5 R) which proved to be a much more sensitive method to assay $C_1$-activity than the measurement of reducing sugars. To measure $C_x$-activity in CMC filtrates posed no problem since only $C_x$-enzymes were active in the culture filtrates and therefore the measurement of reducing sugars was adequate. The

**TABLE 4.** CELLULOLYTIC ($C_1$) ACTIVITY OF CULTURE FILTRATES AND GROWTH OF *TERMITOMYCES* ON A NATURAL CELLULOSE CULTURE MEDIUM.

| | | | 1,4-β-D-Glucan cellobiohydrolase activity (expressed as original dyestuff equivalent of $PO_4$-soluble dyed cellodextrins produced in $\mu g.cm^{-3}$ of the reaction mixture) | | | | |
|---|---|---|---|---|---|---|---|
| Species tested | Growth (Activity) | | Reaction time in hours | | | | |
| | (x) | (y) | 3 | 6 | 9 | 24 | 48 |
| Anamorph | 122 | 156 | 83 | 120 | 160 | 177 | 240 |
| *T.sagittaeformis* | 175 | 132 | 67 | 77 | 83 | 200 | 233 |
| *T.reticulatus* | 186 | 155 | 70 | 100 | 173 | 190 | 240 |
| *T.microcarpus* | 187 | 120 | 47 | 73 | 100 | 136 | 243 |
| *T.clypeatus* | 210 | 250 | 127 | 207 | 217 | 273 | 427 |
| *T.umkowaani* | 233 | 162 | 83 | 103 | 127 | 170 | 327 |

Growth=mycelium dry mass (mg). $F(5,18)=28,04^{***}$

| $P \leq 0.05$ | 0.01 | 0.001 |
|---|---|---|
| LSD 21,01 | 28,78 | 39,22 |

Enzyme activity for total reaction period, n.s. at $P \leq 0.05$.
$F(5,24)=1,49$.
$r = + 0,32$ between x and y, n.s. at $P \leq 0.05$.

**TABLE 5.** CELLULOLYTIC ($C_x$) ACTIVITY OF CULTURE FILTRATES AND GROWTH OF *TERMITOMYCES* ON CMC CULTURE MEDIUM.

| | | | 1,4-β-D-Glucan 4-glucanohydrolase activity (expressed as cellobiose equivalent of reducing groups produced in $mg.cm^{-3}$ of the reaction mixture) | | | | | |
|---|---|---|---|---|---|---|---|---|
| Species tested | Growth | Activity | Reaction time in hours | | | | | |
| | (x) | (y) | 1 | 2 | 3 | 4 | 5 | 6 |
| Anamorph | 4 | 1,32 | 0,76 | 1,04 | 1,30 | 1,47 | 1,63 | 1,76 |
| *T.reticulatus* | 10 | 2,21 | 1,30 | 1,87 | 2,20 | 2,40 | 2,70 | 2,80 |
| *T.sagittaeformis* | 25 | 1,18 | 0,73 | 0,96 | 1,11 | 1,28 | 1,47 | 1,56 |
| *T.clypeatus* | 31 | 2,04 | 1,47 | 1,74 | 1,89 | 2,20 | 2,42 | 2,53 |
| *T.umkowaani* | 36 | 1,22 | 0,65 | 0,98 | 1,18 | 1,36 | 1,51 | 1,64 |
| *T.microcarpus* | 48 | 1,14 | 0,62 | 0,82 | 1,02 | 1,28 | 1,47 | 1,63 |

Growth=mycelium dry mass (mg). $F(5,18)=37,07^{***}$

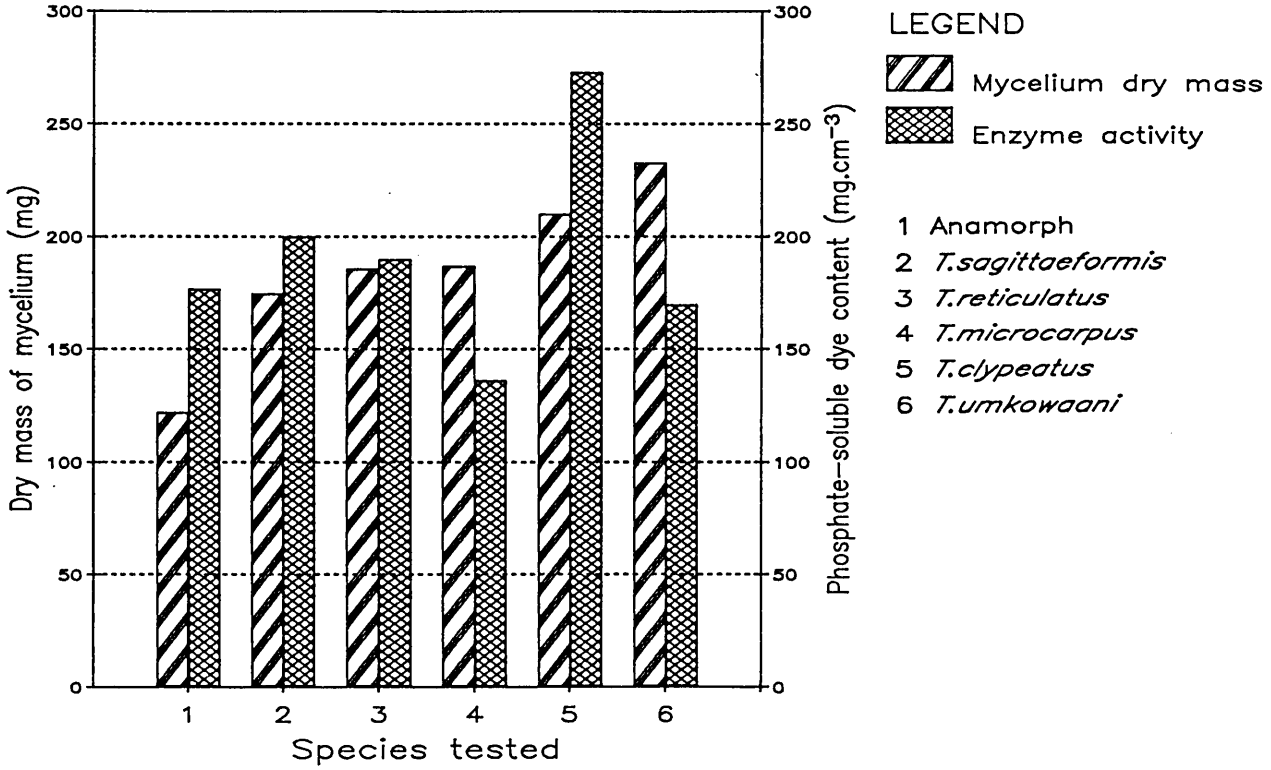
| $P \leq 0.05$ | 0.01 | 0.001 |
|---|---|---|
| LSD 7,99 | 10,94 | 14,91 |

Enzyme activity for total reaction period. $F(5,30)=8,19^{***}$

| $P \leq 0.05$ | 0.01 | 0.001 |
|---|---|---|
| LSD 0,48 | 0,64 | 0,85 |

$r = - 0,38$ between x and y, n.s. at $P \leq 0.05$.

FIGURE 4(b). C1—ACTIVITY OF CULTURE FILTRATES AND GROWTH OF *TERMITOMYCES* ON CRYSTALLINE CELLULOSE CULTURE MEDIUM.




Reaction time of enzyme assay, 24 hours.

FIGURE 5(a). Cx—ACTIVITY OF *TERMITOMYCES* CULTURE FILTRATES GROWN ON CMC CULTURE MEDIUM.

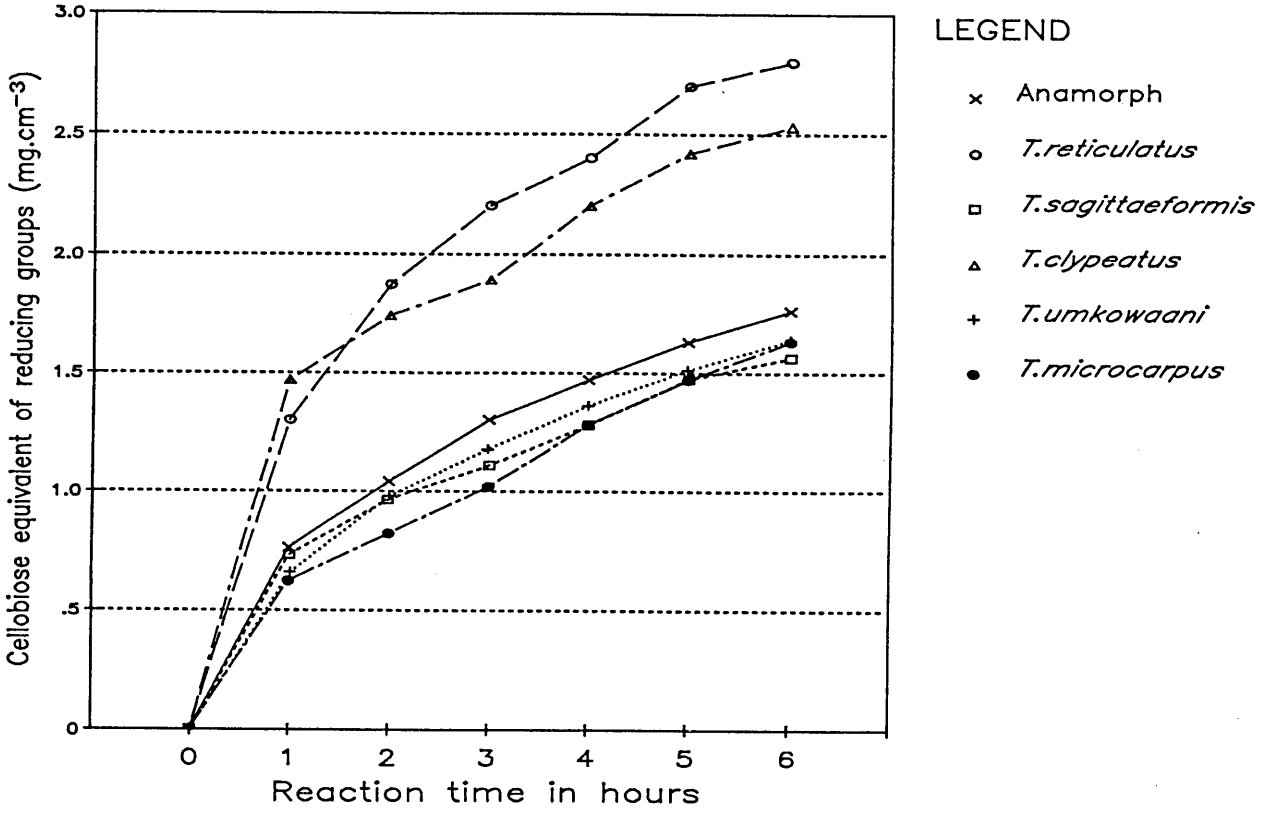
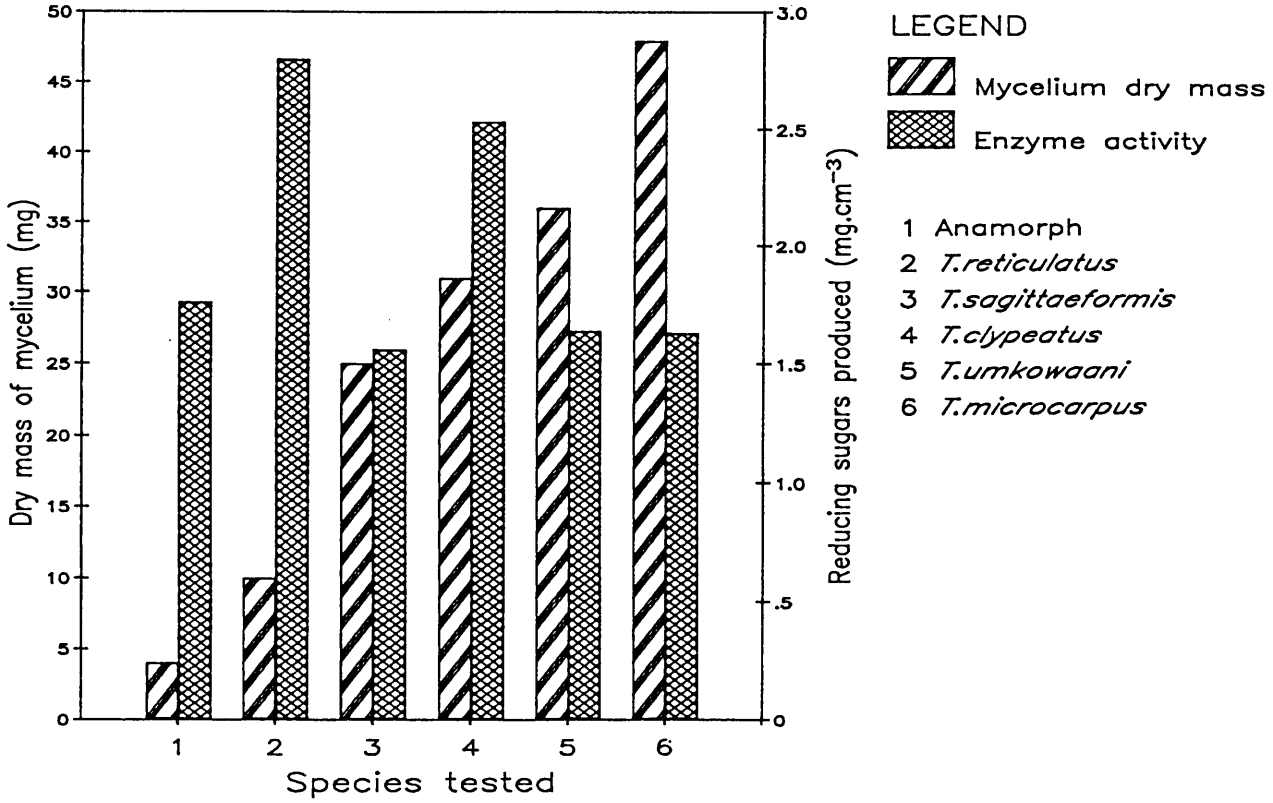
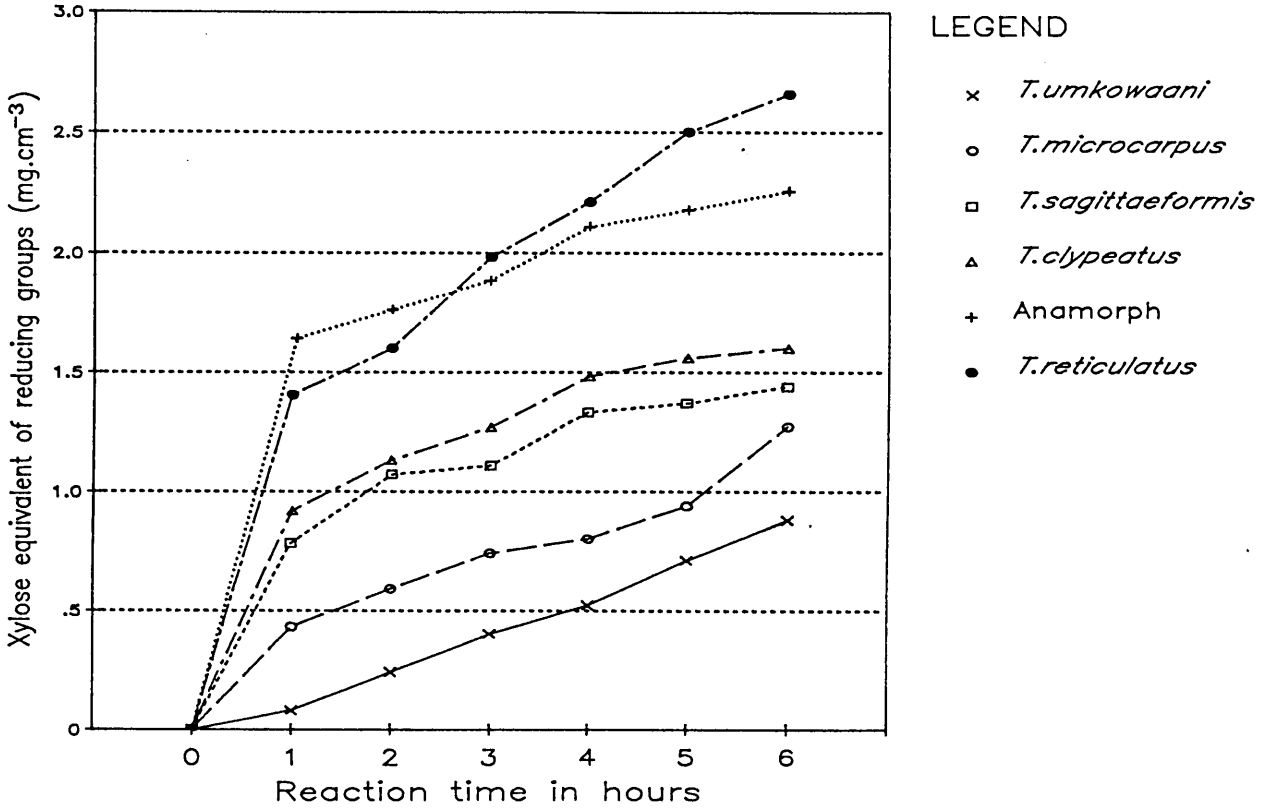


FIGURE 5(b). Cx—ACTIVITY OF CULTURE FILTRATES AND GROWTH OF *TERMITOMYCES* ON CMC MEDIUM.




Reaction time of enzyme assay,6 hours.

FIGURE 6(a). 1,4—BETA—XYLANASE ACTIVITY OF *TERMITOMYCES* CULTURE FILTRATES GROWN ON XYLAN CULTURE MEDIUM.



cellulolytic abilities of the *Termitomyces* species is presented in Table 4 and confirm the observations of above-mentioned authors who had conducted similar research. Both $C_1$ and $C_x$-enzyme activity was detected in culture filtrates on which mycelium from comb sporodochia and the context of fruit bodies have grown. The absence of ß-glucosidase in the filtrates can most probably be ascribed to the intracellular nature of this enzyme.

### 3. Effect of xylan on the growth of mycelium and determination of activity of hemicellulolytic enzymes

Utilization of the hemicellulose xylan as source of carbon by the species of *Termitomyces*, is presented in Tables 6 a,b and illustrated Figs 6 a,b and c. The different species clearly differ in their ability to hydrolyze xylan to the component monomeric subunits. This is evident from the mycelium yield and enzyme activity during a reaction time of six hours (Fig.6 c). Significant differences exist between the mycelium yields attained. The highest mycelium yields are attained by the anamorph and *T. reticulatus*. Both species also display the highest xylanase and xylobiase activity. *T. umkowaani* has poorly developed hemicellulolytic capabilities with the lowest mycelium yield and enzyme activity measurements (Table 6 a and Fig.6 c). A definite relation therefore exists between mycelium yield (x) and enzyme activity (y) in the case of the above-mentioned species. When all the species are compared a correlation coefficient of r = + 0,70 is obtained which does not differ significantly at $P \leq 0.05$ and might indicate a positive relationship between the variables, x and y if more measurements were available.

An endoxylanase (1,4-ß-D-xylan xylanohydrolase) was isolated, purified and characterized from the culture filtrates of *T. clypeatus* and all the properties of this enzyme were extensively investigated by several workers (Ghosh, 1981; Ghosh, *et al.*,1980; Khowala, *et al.*, 1988; Mukherjee & Sengupta, 1985). Mishra & Sen-Sarma (1986) detected xylanase activity in their extracts of *T. albuminosus* sporodochia. The *Termitomyces* species under investigation therefore has the ability to produce both enzymes necessary for the complete degradation of xylan,

**TABLE 6(a).** HEMICELLULOLYTIC (XYLANASE ACTIVITY) OF CULTURE FILTRATES AND GROWTH OF *TERMITOMYCES* ON XYLAN CULTURE MEDIUM.

| | | | 1,4-β-D-Xylan xylanohydrolase activity (expressed as xylose equivalent of reducing groups produced in mg.cm$^{-3}$ of the reaction mixture) | | | | | |
|---|---|---|---|---|---|---|---|---|
| Species tested | Growth Activity | | Reaction time in hours | | | | | |
| | (x) | (y) | 1 | 2 | 3 | 4 | 5 | 6 |
| *T.sagittaeformis* | 21 | 1,18 | 0,78 | 1,07 | 1,11 | 1,33 | 1,37 | 1,44 |
| *T.umkowaani* | 26 | 0,47 | 0,08 | 0,24 | 0,40 | 0,52 | 0,71 | 0,88 |
| *T.clypeatus* | 42 | 1,32 | 0,92 | 1,13 | 1,27 | 1,48 | 1,56 | 1,60 |
| *T.microcarpus* | 107 | 0,79 | 0,43 | 0,59 | 0,74 | 0,80 | 0,94 | 1,27 |
| *T.reticulatus* | 141 | 2,06 | 1,40 | 1,60 | 1,98 | 2,21 | 2,50 | 2,66 |
| Anamorph | 146 | 1,97 | 1,63 | 1,76 | 1,88 | 2,11 | 2,18 | 2,26 |

Growth=mycelium dry mass (mg). $F(5,24)=144,00^{***}$

| $P \leq 0.05$ | 0.01 | 0.001 |
|---|---|---|
| LSD 14,05 | 19,04 | 25,50 |

Enzyme activity for total reaction period. $F(5,30)=23,50^{***}$

| $P \leq 0.05$ | 0.01 | 0.001 |
|---|---|---|
| LSD 0,37 | 0,50 | 0,66 |

r = + 0,70 between x and y, n.s. at $P \leq 0.05$.

**TABLE 6(b).** HEMICELLULOLYTIC (XYLOBIASE ACTIVITY) OF *TERMITOMYCES* CULTURE FILTRATES GROWN ON XYLAN CULTURE MEDIUM.

| | | 1,4-β-D-Xylan xylohydrolase activity (expressed as o-nitrophenol concentration in mmol.cm$^{-3}$ of the reaction mixture) | | | | | |
|---|---|---|---|---|---|---|---|
| Species tested | Mean | Reaction time in hours | | | | | |
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| *T.umkowaani* | 0,15 | 0,10 | 0,12 | 0,13 | 0,14 | 0,19 | 0,25 |
| *T.sagittaeformis* | 0,22 | 0,10 | 0,12 | 0,21 | 0,25 | 0,31 | 0,36 |
| *T.clypeatus* | 0,61 | 0,26 | 0,40 | 0,54 | 0,70 | 0,84 | 0,95 |
| Anamorph | 0,68 | 0,30 | 0,45 | 0,65 | 0,76 | 0,90 | 1,05 |
| *T.reticulatus* | 0,79 | 0,35 | 0,54 | 0,68 | 0,90 | 1,05 | 1,23 |
| *T.microcarpus* | 1,05 | 0,62 | 0,83 | 0,93 | 1,13 | 1,30 | 1,50 |

Enzyme activity for total reaction period. $F(5,30)=11,7^{***}$

| $P \leq 0.05$ | 0.01 | 0.001 |
|---|---|---|
| LSD 0,28 | 0,38 | 0,51 |

FIGURE 6(b). XYLOBIASE ACTIVITY OF *TERMITOMYCES* CULTURE FILTRATES GROWN ON XYLAN CULTURE MEDIUM.

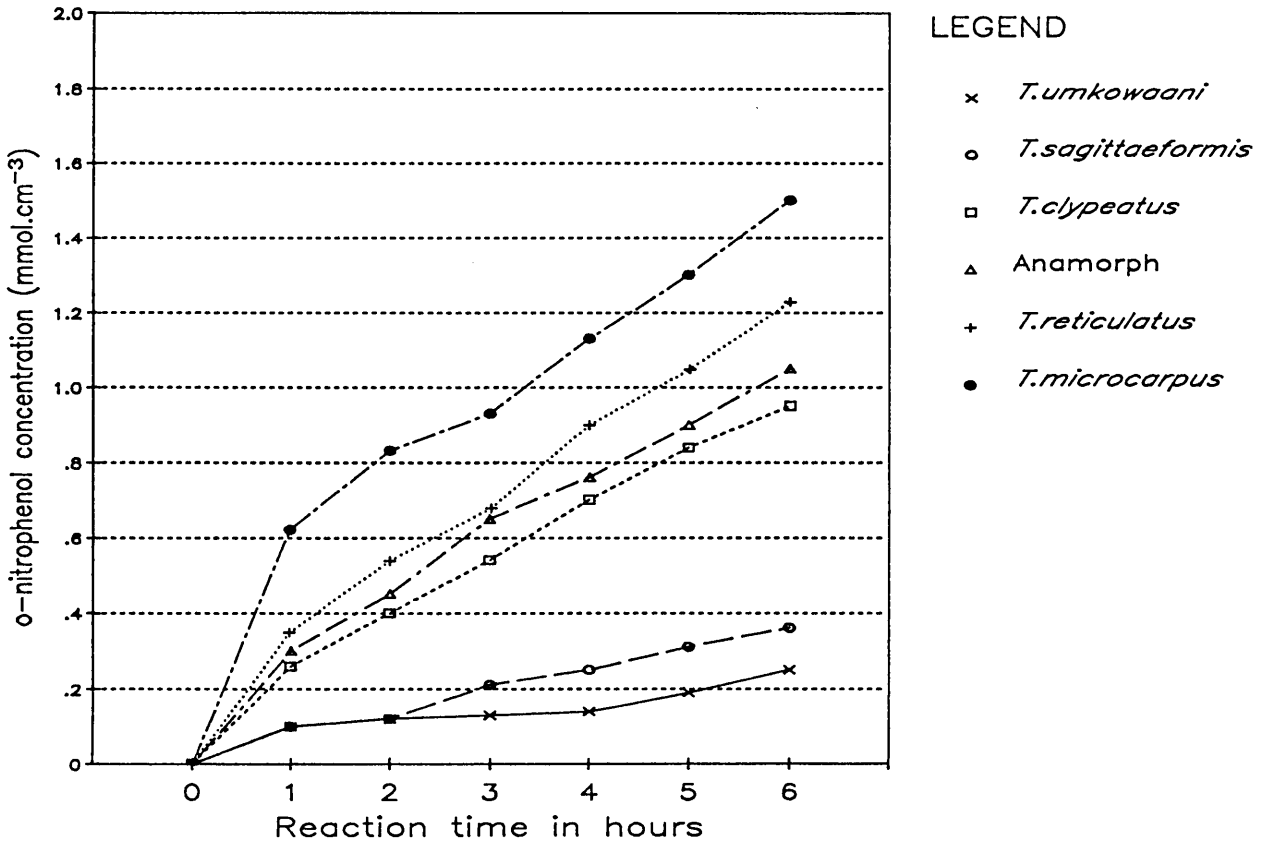
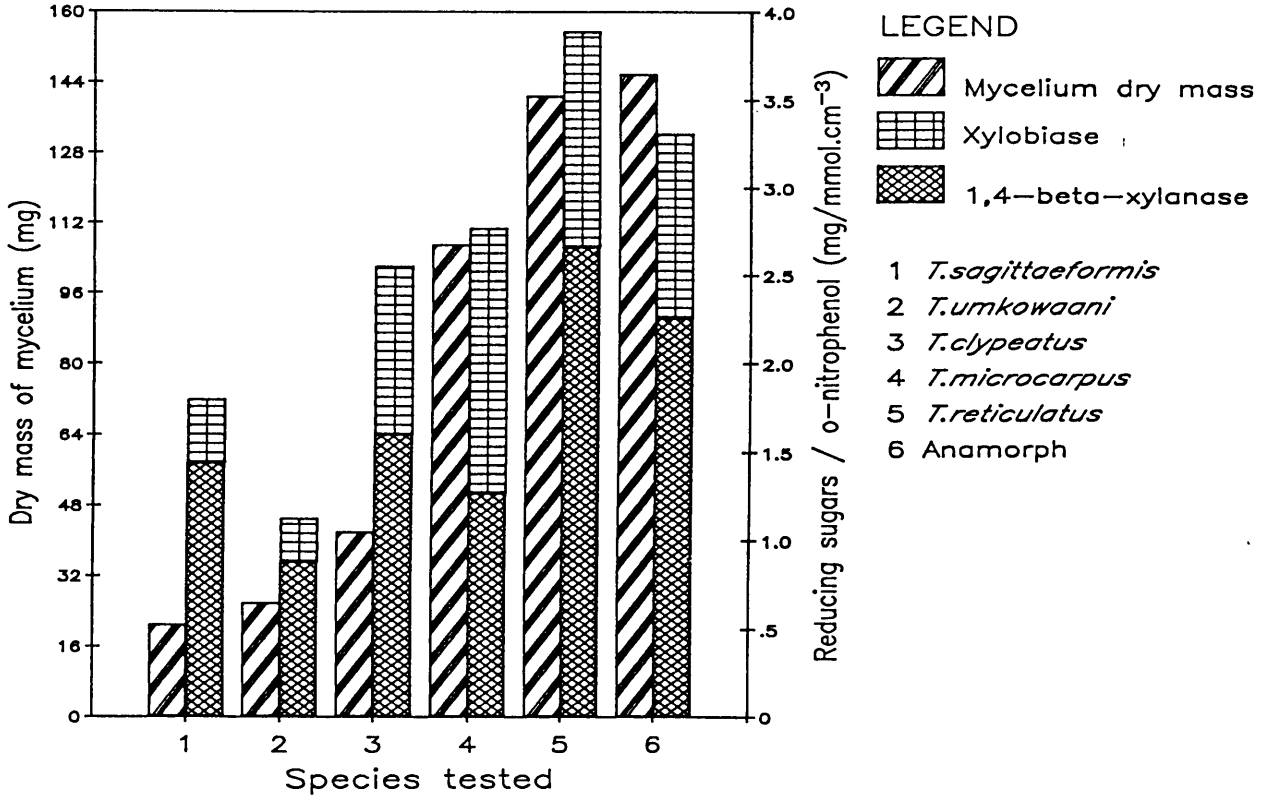



FIGURE 6(c). HEMICELLULOLYTIC ACTIVITY OF CULTURE FILTRATES AND GROWTH OF *TERMITOMYCES* ON XYLAN CULTURE MEDIUM.




Reaction time of enzyme assay ,6 hours.

viz. : 1,4- ß-D-Xylan xylanohydrolase which attacks 1,4-ß-linked glycosidic bonds of D-xylopyranoside units of xylan and 1,4-ß-D-Xylan xylanohydrolase which attacks lower oligosaccharides of D-xylopyranoside units and therefore xylobiose is the main end product. This enzyme complex display multisubstrate specific characteristics and is capable of hydrolyzing different types of hemicelluloses as well as starch-like compounds (Ghosh, 1981; Ghosh, Banerjee & Sengupta, 1980; Ghosh & Sengupta, 1987; Khowala, Mukherjee & Sengupta, 1988; Mukherjee & Sengupta, 1985). Comtat (1983) reported the production of several endoxylanases with very similar physical-chemical properties by a basidiomycete. The research results of these workers indicate that endoxylanases in general are active against a wide range of substrates and that *Termitomyces* may be able to degrade cellulose, hemicellulose and starch-like compounds in the natural substrate irrespective of the form in which these organic biopolymers occur by merely utilizing the endoxylanase enzyme complex.

## 4. Effect of starch on the growth of mycelium and determination of α-amylase activity

Hydrolysis of starch by the different species of *Termitomyces* is listed in Table 7 and represented in Fig.7a. The results in Table 7 indicate that the different species have the ability to degrade starch and produce α-amylase. The amylolytic capabilities of the different species are unequal and are reflected in the mycelium yield and enzyme activity. Significant differences exist between the mycelium yield attained by the anamorph and *T. clypeatus* and high yields attained by *T. microcarpus* and *T. reticulatus*. A similar observation can be made with regard to α-amylase activity in the culture filtrates. Low activity occurs in the filtrates of the first three species in contrast with the high filtrate activity of the last three species tested (Table 7 and Fig. 7 a). Although the anamorph displays the lowest mycelium yield and enzyme activity values, the cultures of *T. umkowaani* needed twenty days to attain similar growth and activity values and even fifty days to reach the second highest levels of growth and activity. This phenomenon can be ascribed to the slower growth rate of *T.umko-*

*waani* and may be coupled to a lower production (secretion) of α-amylase which is only released in small quantities into the medium extended over a long period. Culture filtrates of this mushroom did not display any α-amylase activity after twelve days incubation and therefore incubation was extended to twenty days before activity could be detected. A significant difference exists at the 1 % level between the filtrate activity of twenty and fifty days old cultures and confirms the observations of Ezmat El-Zalaki & Hamza (1979), Shu & Blackwood (1951) and Barnett & Fergus (1971) who reported a relationship between the age of the culture and enzyme activity of the filtrate. *T. reticulatus* has a strong amylolytic ability and displays maximum mycelium growth and α-amylase activity. Filtrate activity of this mushroom differs significantly at the 0,1 % level with the second highest enzyme activity measurement obtained from *T. umkowaani* filtrates (Fig. 7 a). A correlation coefficient of r = + 0,71 exists between the variables x and y but does not differ significantly at $P \leq 0.05$. When more measurements are available for x and y a more positive relation may emerge.

Ghosh & Sengupta (1987) purified a multisubstrate specific amylase from the culture filtrate of *T. clypeatus* and the enzyme was extensively purified and characterized by these authors. They observed that this enzyme could hydrolyze amylose, xylan, amylopectin, glycogen, arabinogalactan and arabinoxylan. The multisubstrate property of this enzyme would enable *Termitomyces* mycelium to utilize several carbon sources simultaneously with the aid of only one enzyme. Thus amylases and xylanases display multisubstrate specific properties and are capable of degrading a common range of polysaccharides. This ability will enhance the efficiency with which *Termitomyces* species will degrade the carbon sources present in the natural substrate (fungus comb) irrespective of the form in which the polysaccharides occur in the comb. Mishra & Sen-Sarma (1986) detected amylase and maltase activity in their extracts of *T. albuminosus* sporodochia. Although α-amylase enzymes occur almost universally in filamentous fungi including members of the Basidiomycotina (Cochrane, 1958), the ability to degrade starch-like substrates certainly differ. Ezmat El-Zalaki & Hamza (1979) determined the ability of five edible mushrooms

to hydrolyze starch in extracts of food wastes and they established that *Lentinus edodes* had the strongest amylolytic ability.

## 5. Effect of pectic acid substrates on the growth of mycelium and determination of activity of pectinolytic enzymes

Results listed in Table 8 and illustrated in Fig. 8 show that only *T. microcarpus* has the ability to degrade pectin and pectic acid efficiently. The results also indicate that *T. microcarpus* can hydrolyze pectic acid and pectin which require two different enzymes. Pectin, a partially methoxylated polygalacturonic acid is hydrolyzed by poly (1,4-α-D-galacturonide glucanohydrolase) and sodium polypectate which is a water soluble unmethoxylated pectic acid is hydrolyzed by polygalacturonase. Both enzymes are produced in sufficient quantities and attain high activities which do not differ significantly at the 1 % level. In the case of the anamorph mycelium growth is poor on both substrates and consequently pectic enzymes activity is to low to detect. None of the other species tested are able to grow on the pectic acid substrates (Table 19).

Martin & Martin (1979) reported high pectinase activity in extracts which they prepared from sporodochia on the combs of *Macrotermes natalensis*. Apparently only comb sporodochia which grow in direct contact with the fungus combs are able to produce pectinolytic enzymes and degrade pectic acid related compounds in the comb. When sporodochia are cultivated on synthetic media containing pectin and pectic acid substrates the ability to effectively degrade this polysaccharide is apparently lost. This would explain the poor growth of the anamorph in culture.

## 6. Effect of lipids (natural and synthetic) on the growth of mycelium and determination of activity of lipolytic enzymes

The utilization of *Termitomyces* mycelium of one natural and two synthetic lipids is presented in Table 9 and graphically illustrated in Fig. 9. With the exception of *T. clypeatus* all the species are capable of hydrolyzing olive oil and secrete lipase into the culture

**TABLE 7.** AMYLOLYTIC ACTIVITY OF CULTURE FILTRATES AND GROWTH OF *TERMITOMYCES* ON STARCH CULTURE MEDIUM.

| Species tested | Growth (x) | α-amylase activity (expressed as maltose equivalent of reducing groups produced in $mg.cm^{-3}$ of reaction mixture in six hours) (y) |
|---|---|---|
| Anamorph | 10 | 0,22 |
| *T.clypeatus* | 37 | 0,38 |
| *T.umkowaani* | 45 | 0,39 (20 days culture) |
| *T.microcarpus* | 96 | 0,69 |
| *T.sagittaeformis* | 41 | 1,24 |
| *T.umkowaani* | - | 1,60 (50 days culture) |
| *T.reticulatus* | 107 | 3,60 |

Growth=mycelium dry mass (mg). $F(5,18)=35,15^{***}$

| $P \leq$ | 0.05 | 0.01 | 0.001 |
|---|---|---|---|
| LSD | 18,78 | 25,73 | 35,06 |

Enzyme activity for total reaction period. $F(6,35)=50,75^{***}$

| $P \leq$ | 0.05 | 0.01 | 0.001 |
|---|---|---|---|
| LSD | 0,89 | 1,09 | 2,28 |

r = + 0,71 between x and y, n.s. at $P \leq 0.05$.

**TABLE 8.** PECTINOLYTIC ACTIVITY OF CULTURE FILTRATES AND GROWTH OF *T. MICROCARPUS* ON PECTIN AND Na-POLYPECTATE CULTURE MEDIA.

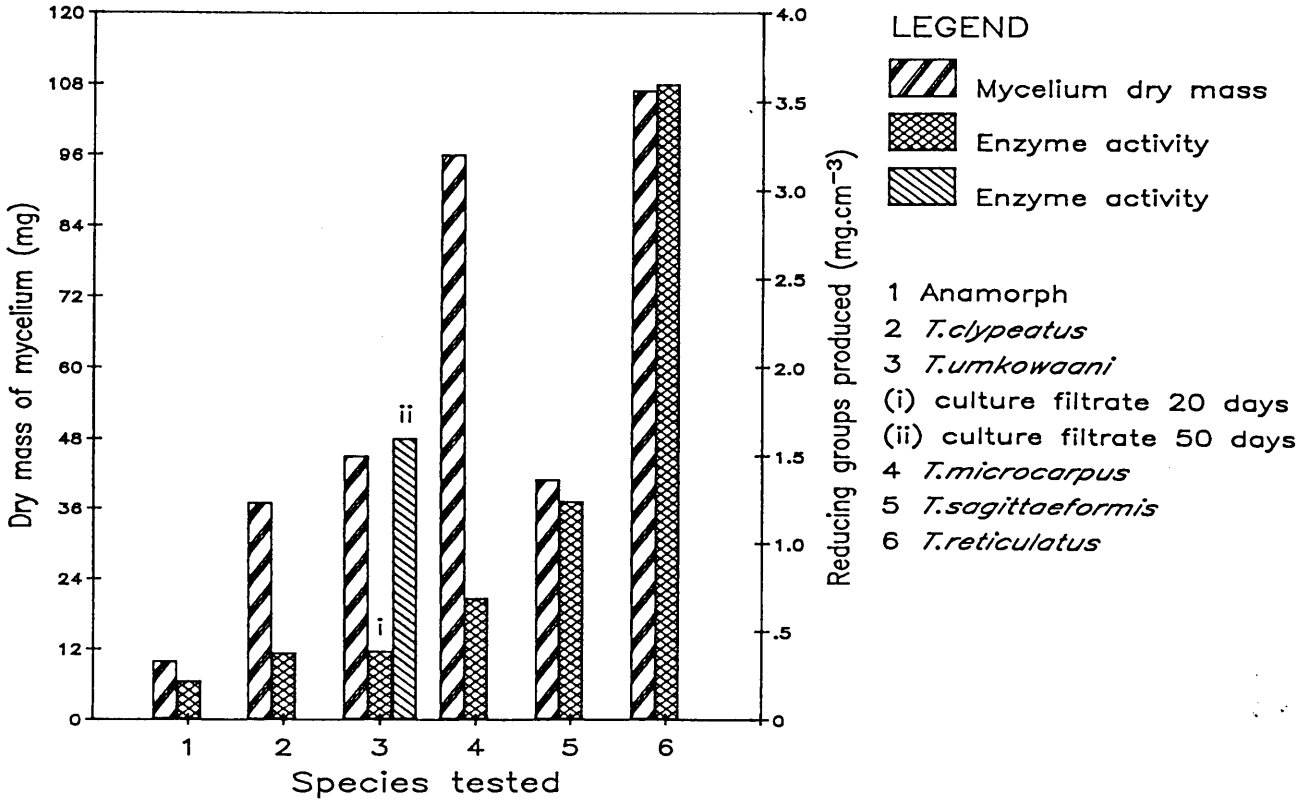
| Substrate hydrolysed | Growth | Activity | Pectinolytic activity (expressed as galacturonic acid equivalent of reducing groups produced in $mg.cm^{-3}$ of the reaction mixture) Reaction time in hours | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 |
| Pectin | 106 | 0,71 | 0,44 | 0,61 | 0,64 | 0,76 | 0,88 | 0,98 |
| Na-polypectate | 71 | 0,53 | 0,24 | 0,36 | 0,47 | 0,58 | 0,74 | 0,80 |

Growth=mycelium dry mass (mg). $F(1,6)=19,49^{**}$

| $P \leq$ | 0.05 | 0.01 |
|---|---|---|
| LSD | 19,90 | 29,38 |

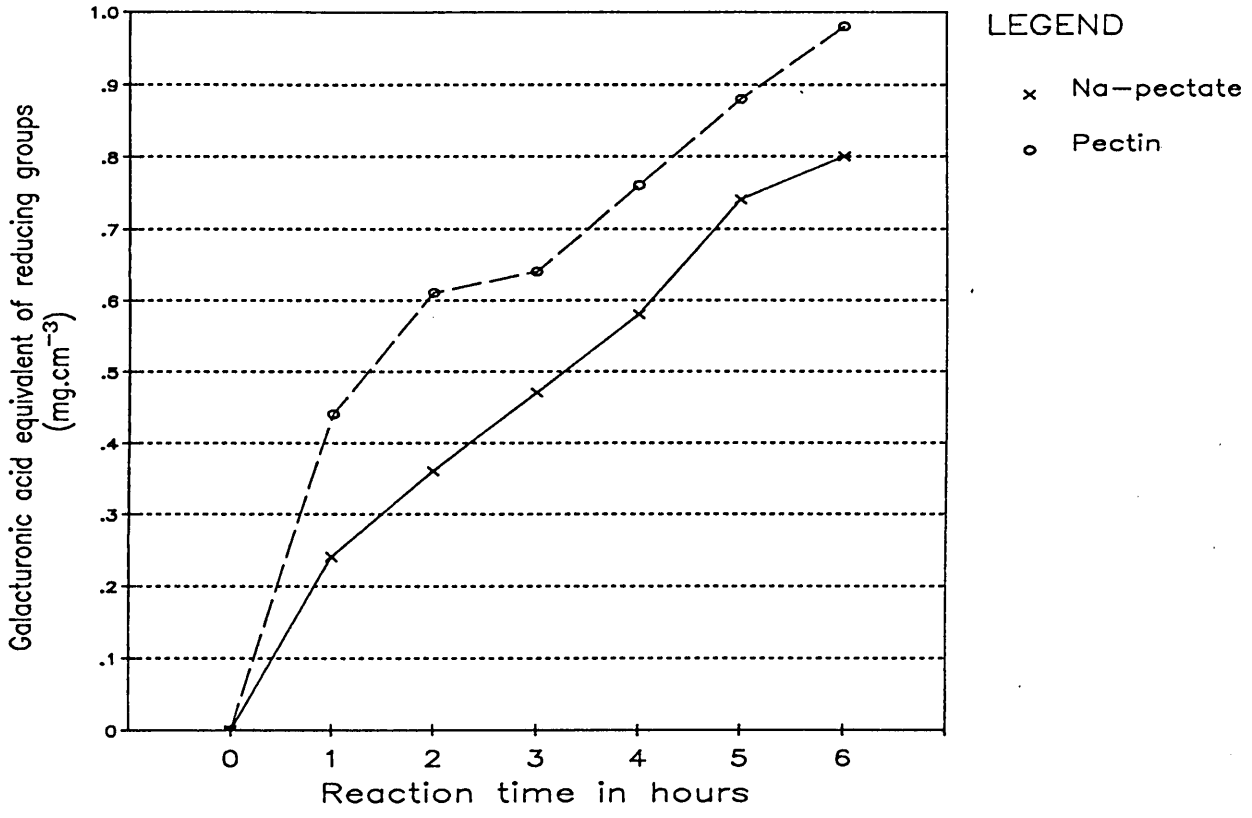
Enzyme activity for total reaction period. $F(1,10)=2,53$ n.s. at $P \leq 0.05$; 0.01; 0.001.

FIGURE 7(a). ALPHA–AMYLASE ACTIVITY OF CULTURE FILTRATES AND GROWTH OF *TERMITOMYCES* ON STARCH CULTURE MEDIUM.




Reaction time of enzyme assay,6 hours.

FIGURE 8. PECTINOLYTIC ACTIVITY OF *T. MICROCARPUS* CULTURE FILTRATES GROWN ON PECTIN AND Na–PECTATE CULTURE MEDIA.



fluid. Although hydrolysis proceeds at a slow rate over twenty-four hours, lipase like the $C_1$-enzyme requires extended reaction times to produce sufficient end products suitable for measurement. These enzymes are also present in low concentrations in the culture fluids. Nevertheless relatively high lipase activity was detected in the culture filtrates active against the natural lipid, olive oil. Only lipase produced in the presence of a natural lipid can be considered a proper lipase (Bergmeyer, 1974) and therefore olive oil and not the synthetic trioleate (Tween 85) was employed as substrate for lipase production. Both the water-soluble fatty acid esters, Tween 80 and Tween 85 support mycelium growth although the yield on Tween 80 is generally higher. All the species are capable of producing esterase and relatively high esterase activity occurs in the culture fluids (Table 9). The results indicate that *T. microcarpus* and the anamorph have the best developed lipolytic abilities when the average mycelium yield and the amount of oleic acid produced by the two are measured, although the difference between these species with respect to these parameters are small. Apparently *T. clypeatus* does not have the ability to hydrolyze triglycerides, caused by its inability to produce lipase, but this mushroom is capable of splitting monoglycerides such as Tween 80 with the aid of esterase. This would imply that *T. clypeatus* will be able to utilize fatty substrates only when sufficient monoglycerides are available in the comb. With the exception of *T. microcarpus* the species under investigation could not utilize dicarboxylic acids (see par.7) but the mycelium is certainly capable of assimilating monocarboxylic acids such as oleic acid. During the period of incubation enzyme hydrolysis liberated fatty acids into the culture medium and caused a drop in the pH value from 7,0 to approximately 3,5.

Emulsifying of the lipids (oils) proved to be essential for the following reasons : (i) to stimulate the secretion of lipase (ii) to enlarge the surface area of the numerous small lipid droplets (iii) to ensure proper contact between the enzyme in the culture medium and the lipid substrate as well as a more intimate contact with the mycelium and (iv) to keep the water-soluble lipid droplets in a fine homogenous suspension, thus preventing the individual lipid droplets

**TABLE 9.** LIPOLYTIC ACTIVITY OF CULTURE FILTRATES AND GROWTH OF *TERMITOMYCES* ON NUTRIENT MEDIA CONTAINING SYNTHETIC FATS.

| Species tested | Mycelium dry mass (mg) Tween 80 | Tween 85 | Enzyme activity (expressed) as oleic acid concentration in $\mu mol.cm^{-3}$ of assay mixture) Esterase | Lipase |
|---|---|---|---|---|
| *T.clypeatus* | 18 | 10 | 110 | - |
| *T.sagittaeformis* | 22 | 20 | 59 | 193 |
| *T.reticulatus* | 31 | 19 | 88 | 67 |
| *T.umkowaani* | 42 | 7 | 82 | 121 |
| *T.microcarpus* | 49 | 15 | 64 | 120 |
| Anamorph | 74 | 32 | 90 | 100 |

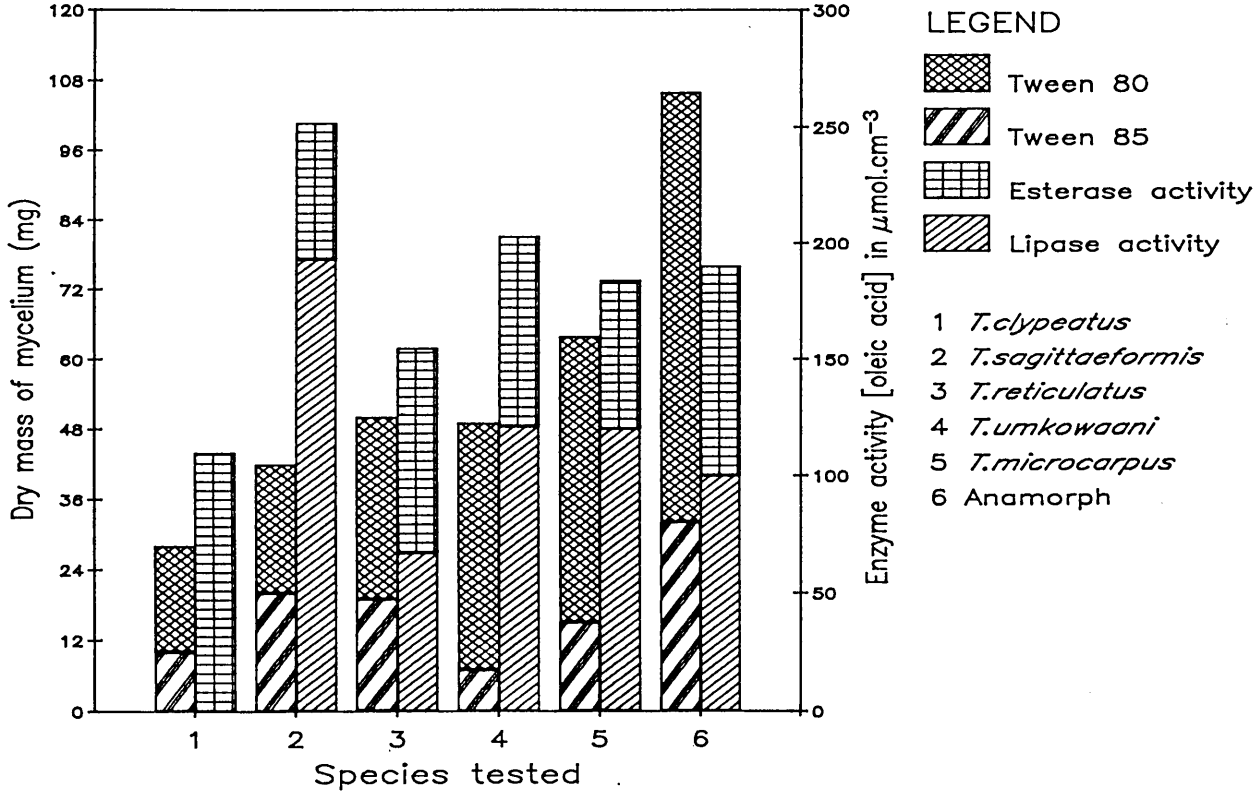
| | | | | |
|---|---|---|---|---|
| Tween 80, $F(5,18)=43,31^{***}$ | $P \leq 0.05$ | 0.01 | 0.001 | |
| | LSD 9,24 | 12,65 | 17,25 | |
| Tween 85, $F(5,18)=195,60^{***}$ | $P \leq 0.05$ | 0.01 | 0.001 | |
| | LSD 1,91 | 2,62 | 3,57 | |

**TABLE 10.** GROWTH OF *T. MICROCARPUS* ON DIFFERENT CARBON SOURCES.

| Carbon source | Mycelium dry mass (mg) | | |
|---|---|---|---|
| Tween 85 | 15 | D-Fructose | 56 |
| *mio*-Inositol | 26 | Sucrose | 59 |
| α-Ketoglutaric acid | 27 | Vitamins+fructose | 63 |
| Dextrin | 32 | Na-polypectate | 71 |
| D-Galactose | 36 | D-Mannose | 74 |
| β-D-Lactose | 36 | D-Galacturonic acid | 75 |
| D-Mannitol | 38 | Malic acid | 94 |
| Citric acid | 41 | Starch | 96 |
| D-Sorbitol | 46 | Pectinic acid | 106 |
| CMC | 48 | Xylan | 107 |
| D-Glucose | 48 | L-Sorbose | 135 |
| Tween 80 | 49 | Fumaric acid | 178 |
| α-D-Melibiose | 52 | Melezitose | 182 |
| D-Trehalose | 53 | Natural cellulose | 187 |

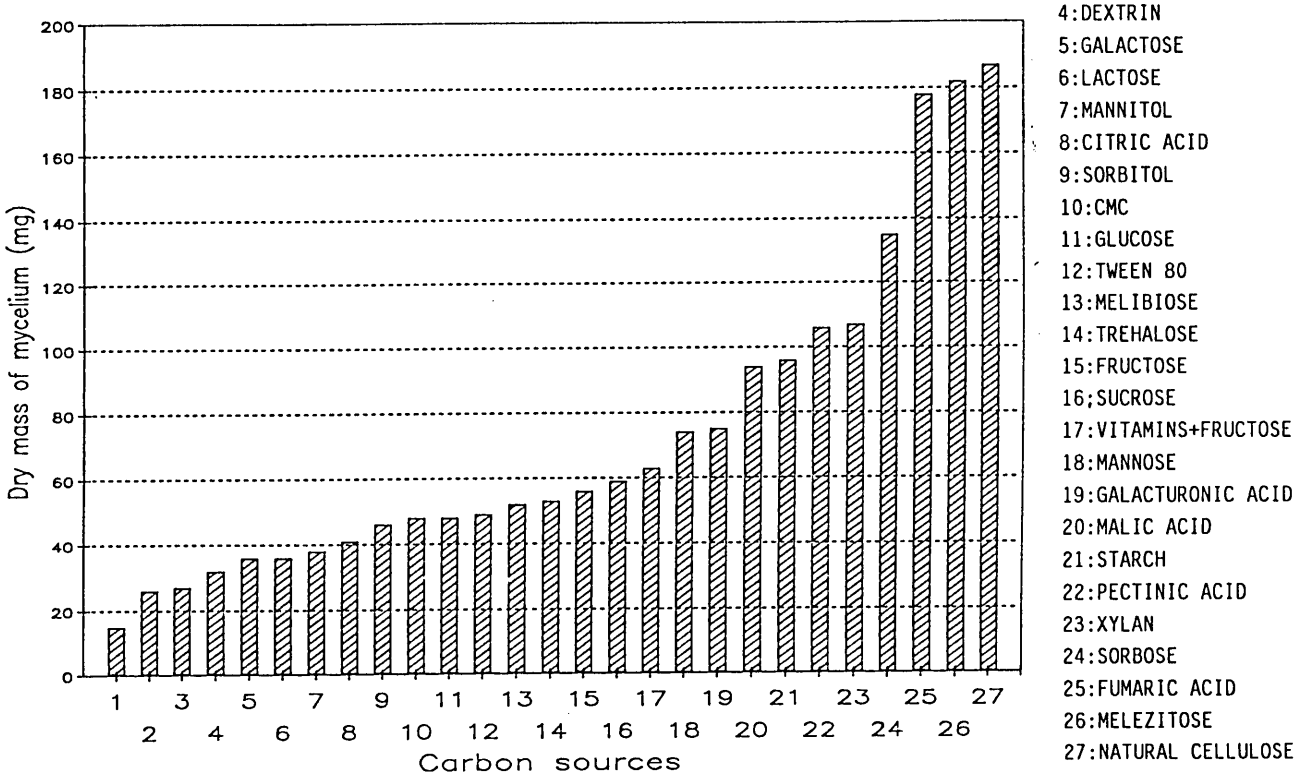
| | | | |
|---|---|---|---|
| $F(27,83)=8,31^{***}$ | $P \leq 0.05$ | 0.01 | 0.001 |
| | LSD 46,46 | 61,41 | 79,15 |

FIGURE 9. LIPOLYTIC ACTIVITY OF CULTURE FILTRATES AND GROWTH OF *TERMITOMYCES* ON MEDIA CONTAINING SYNTHETIC FATS.




Reaction time of enzyme assay,24 hours.

FIGURE 10. GROWTH OF *T.MICROCARPUS* ON DIFFERENT CARBON SOURCES.



from aggregating into huge floating drops. The water-soluble sorbitol esters, Tween 80 and Tween 85 proved to be ideal substrates for lipolytic enzyme studies and did not present any of the problems which were encountered with natural oils. It also proved unnecessary to shake these water-soluble lipid cultures during the incubation period but agitation on a reciprocating shaker was essential when olive oil cultures were incubated. When oils (olive oil, soya oil, sunflower oil etc.) were added to the basal medium without emulsifying it beforehand, no mycelium growth was observed.

Information with regard to the lipid utilization of *Termitomyces* species is at present limited. Skelton & Matanganyidze (1981) detected esterase activity in crude extracts of *T. microcarpus* basidiocarps while Mishra & Sen-Sarma (1986) detected lipase activity in extracts which they prepared from *T. albuminosus* sporodochia.

## 7. **Effect of other carbon sources and vitamins on the growth of mycelium**

Growth of *T. microcarpus* on different carbon sources is shown in Table 10 and illustrated in Fig. 10. The results indicate that this mushroom is able to grow on a broad range of carbon sources viz. : sugar alcohols, sugar acids, organic acids, mono, di, tri, and polysaccharides and two water-soluble synthetic fats. No growth was observed when two amino sugars, glucosamine HCI and galactosamine HCI were included in the medium which may be caused by the toxicity of these compounds. It is evident from the results in Table 10 that *T. microcarpus* shows a preference for certain carbon sources and the degree of utilization depends on the molecular configuration of the specific carbon source. Sugar alcohols are best utilized in the form of D-sorbitol, sugar acids in the form of D-galacturonic acid, organic acids in the form of fumaric acid, monoses in the form of L-sorbose, disaccharides in the form of sucrose and polysaccharides in the form of crystalline cellulose. The mycelium yield on the trisaccharide melezitose is almost as high as the maximum yield when cultivated on crystalline cellulose. Growth on the three polysaccharides, starch, pectic acid and xylan is almost equal and does not differ

significantly but compared to the growth on crystalline cellulose the difference is significant (see also Table 19). These findings confirm the strong cellulolytic ability of this mushroom ( Table 4 and Figs 4 a,b). *T. microcarpus* was the only species examined capable of producing pectinolytic enzymes (Table 8 and Fig. 8) and of utilizing the monomeric subunit, D-galacturonic acid. No stimulative effect on the growth of the mycelium was observed with a solution of multi-vitamins. This is evident from the results in Table 10 where the difference in mycelium yields between D-fructose and multi-vitamins + D-fructose is not significant. Therefore *T. microcarpus* does not require an external source of vitamins and seems capable of synthesizing all the necessary vitamins to supply its growth requirements.

Results obtained from the growth of *T. sagittaeformis* on different carbon sources is listed in Table 11 and illustrated in Fig. 11. Results indicate that this mushroom is able to grow on a broad range of carbon sources which include : sugar alcohols, sugar phosphates, mono, di, tri and polysaccharides and two synthetic water-soluble fats. As in the case of *T. microcarpus* this mushroom displays certain preferences with regard to the form in which the carbon source can be utilized. For example relatively good growth occurs on D-mannose while the alcohol derivative D-mannitol supports poor growth (Table 11). Likewise significantly better growth occurs on D-glucose than on D-cellobiose, a disaccharide which consists of two glucose molecules bonded by an ß-glucoside linkage and in the same manner good growth occurs on D-glucose and D-fructose which differ significantly from the growth on melezitose, a trisaccharide which consist of two glucose molecules and one fructose molecule. No growth occurs on L-sorbose while the alcohol derivative of D-glucose, D-sorbitol is utilized. The growth on D-glucose differs significantly at the 1 % level from the growth on D-trehalose, a disaccharide which consist of two glucose molecules bonded by an α-glucoside linkage. Maximum mycelium growth occur on crystalline cellulose which differs significantly at the 1 % level with all the carbon sources tested and accentuates the strong cellulolytic ability of this mushroom (Table 4 and Figs 4 a,b). Starch is more effectively hydrolyzed than xylan (Tables 7,6 and Figs 7a,6a) and next to cellulose, represents a more tenable polysaccharide than xylan. The monomeric subunit of both starch and cellulose (D-glucose),

supports better growth than the monomeric subunit of xylan, D-xylose (Table 11). Vitamins do not exert a stimulative effect on mycelium growth and the yield on this medium is significantly lower than the yield on D-fructose. This may be caused by certain vitamins which are present in too high concentrations as a result of the natural synthesis by the mycelium of all the vitamins required for normal growth. *T. sagittaeformis* is incapable of utilizing amino sugars due to the toxic effect of these compounds on growth. The disaccharides α-D-melibiose (an α-glucoside) support no growth due to the absence of α-galactosidase which hydrolyses this sugar and likewise D-lactose is not utilized due to the absence of the enzyme that attacks the glycoside linkage. The sugar alcohols, *mio*-inositol as well as *meso*-erythritol are not utilized. The four dicarboxylic acids of the Tricarboxylic Acid Cycle and the repeating unit of pectic acid, D-galacturonic acid support no growth. Two pentoses, L-arabinose and D-ribose as well as the methyldeoxyhexsoses, D-fucose and L-rhamnose are not utilized.

Utilization by *T. clypeatus* of different carbon sources is shown in Table 12 and graphically depicted in Fig. 12. This species is able to utilize the same range of carbon sources as *T. sagittaeformis*. The strong cellulolytic ability of this mushroom is reflected in the maximal mycelium yield that is attained on crystalline cellulose which differ significantly at the 0,1 % level with the rest of the sources (see also Tables 4,5 and Figs 5 a,4a). Good growth occurs on the repeating subunit of cellulose, D-glucose as well as the repeating disaccharide of cellulose, D-cellobiose. Sugars which resemble the configuration of D-glucose : D-fructose, D-trehalose, fructose 1,6-diphosphate and D-cellobiose support good growth and the mycelium yield does not differ significantly (Table 12). The results indicate that *T. clypeatus* does not require supplementary vitamins. This can be seen clearly as growth on the multi-vitamins + D-fructose medium and as growth on D-fructose medium but do not differ significantly. Xylan represent an excellent source of carbon and is effectively hydrolyzed by *T. clypeatus* (Tables 6 a,b and Figs 5 a,b). *T. clypeatus* is incapable of growth on the range of carbon sources which do not support the growth of *T. sagittaeformis* (Table 11). Ghosh & Sengupta (1978) inves-

tigated the carbon and nitrogen nutrition of *T. clypeatus* and they established that dextrin and starch stimulated mycelium growth greatly in comparison with D-glucose. They also determined the optimal carbon to nitrogen ratio for this mushroom. Sengupta, Naskar & Jana (1984) cultivated *T. clypeatus* mycelium on several natural substrates and detected amylase, xylanase and cellulase activity in extracts which they prepared from these substrates. From the results in Table 12 it is evident that *T. clypeatus* has the ability to hydrolyze starch, xylan and cellulose (see also Table 19) and therefore these polysaccharides are the main sources of carbon and respiratory energy for this mushroom.

The effect of different carbon sources on the growth of *T. umkowaani* is presented in Table 13 and illustrated in Fig. 13. *T. umkowaani* is capable of utilizing a broad range of carbon sources similar to the range utilized by *T. sagittaeformis* and *T. clypeatus*. *T. umkowaani* displays strong cellulolytic abilities with a high mycelium yield and enzyme activity when grown on CMC and crystalline cellulose (Table 4,5 and Figs 4a,5a). The mycelium yields attained on crystalline cellulose differs significantly from the yields attained on the rest of the carbon sources (Table 13 and Fig. 13). Starch is also effectively hydrolyzed with high $\alpha$-amylase activity in older cultures (Table 7 and Fig. 7a) and in combination with cellulose supplies all the necessary carbon and respiratory energy for growth. It is therefore not surprising that good growth occurs on D-glucose and D-cellobiose, the repeating subunits of starch and cellulose. Sugars which resemble the configuration of D-glucose, D-fructose and D-trehalose are also effectively utilized. The pentosan xylan is not effectively degraded by *T. umkowaani* and only moderate growth and enzyme activity are attained (Table 6 a,b and Figs 6 a,b) on this carbon source. The monomeric subunit of xylan, D-xylose supports poor growth and mycelium yield on this pentose differs significantly from the yields attained on hexose sugars resembling D-glucose (Table 13). The mycelium yield on the multi-vitamins + D-fructose medium differs significantly from the mycelium yield on D-fructose at the 1 % level. It would therefore seem that vitamins do exert a stimulative influence on the growth of the mycelium of *T. umkowaani*. This stimulative

**TABLE 11.** GROWTH OF *T.SAGITTAEFORMIS* ON DIFFERENT CARBON SOURCES.

| Carbon source | Mycelium dry mass (mg) | | |
|---|---|---|---|
| D-Galactose | 5 | Tween 85 | 26 |
| D-Mannitol | 6 | D-Sorbitol | 28 |
| Melezitose | 10 | Glycerol | 29 |
| Xylan | 21 | Dextrin | 33 |
| D-Trehalose | 22 | Vitamins+fructose | 33 |
| Tween 80 | 22 | Fruc-1,6-diphosphate | 39 |
| D-Xylose | 22 | D-Glucose | 40 |
| CMC | 25 | Starch | 41 |
| D-Mannose | 26 | D-Fructose | 50 |
| D-Cellobiose | 26 | Natural cellulose | 175 |

$F(19,60)=58,67^{***}$ $P \leq 0.05$ 0.01 0.001
LSD 13,30 17,80 23,15

Carbon sources not utilized: D-Galactosamine; D-Glucosamine; D-Lactose; *mio*-Inositol; *meso*-Erythritol; α-D-Melibiose; Citric acid; Malic acid; Pectinic acid; α-Ketoglutaric acid; Fumaric acid; D-Galacturonic acid; L-Sorbose; D-Fucose; L-Rhamnose; L-Arabinose; D-Ribose.

**TABLE 12.** GROWTH OF *T. CLYPEATUS* ON DIFFERENT CARBON SOURCES.

| Carbon source | Mycelium dry mass (mg) | | |
|---|---|---|---|
| D-Mannitol | 9 | Vitamins+fructose | 38 |
| Melizitose | 13 | Xylan | 42 |
| Tween 85 | 16 | D-Fructose | 43 |
| Tween 80 | 18 | D-Trehalose | 46 |
| Dextrin | 18 | Fruc-1,6-diphosphate | 50 |
| D-Sorbitol | 21 | D-Glucose | 63 |
| CMC | 31 | D-Cellobiose | 69 |
| D-Xylose | 32 | Glycerol | 85 |
| D-Mannose | 34 | Natural cellulose | 210 |
| Starch | 37 | | |

$F(18,57)=9,05^{***}$ $P \leq 0.05$ 0.01 0.001
LSD 42,43 56,40 73,40

Carbon sources not utilized: D-Galactosamine; D-Glucosamine; D-Lactose; D-Galactose; *mio*-Inositol; *meso*-Erythritol; α-D-Melibiose; Malic acid; Citric acid; α-Ketoglutaric acid; Pectinic acid; Fumaric acid; D-Galacturonic acid; L-Sorbose; D-Fucose; L-Rhamnose; L-Arabinose; D-Ribose.

**TABLE 13.** GROWTH OF *T. UMKOWAANI* ON DIFFERENT CARBON SOURCES.

| Carbon source | Mycelium dry mass (mg) | | |
|---|---|---|---|
| Melezitose | 11 | Dextrin | 41 |
| Tween 85 | 13 | Tween 80 | 42 |
| D-Xylose | 16 | D-Glucose | 45 |
| Glycerol | 16 | Starch | 45 |
| D-Mannitol | 25 | D-Cellobiose | 49 |
| Xylan | 26 | D-Fructose | 53 |
| D-Sorbitol | 31 | D-Trehalose | 56 |
| D-Mannose | 32 | Vitamins+fructose | 99 |
| Fruc-1,6-diphosphate | 33 | Natural cellulose | 233 |
| CMC | 36 | | |

$F(18,57)=31,04^{***}$ $P \leq 0.05$ 0.01 0.001
LSD 24,73 32,90 42,79

Carbon sources not utilized: D-Galactosamine; D-Glucosamine; D-Lactose; D-Galactose; *mio*-Inositol; *meso*-Erythritol; α-D-Melibiose; Citric acid; Malic acid; α-Ketoglutaric acid; Fumaric acid; Pectinic acid; D-Galacturonic acid; L-Sorbose; D-Fucose; L-Rhamnose; L-Arabinose; D-Ribose.

**TABLE 14.** GROWTH OF *T. MICROCARPUS* ON DIFFERENT NITROGEN SOURCES.

| Nitrogen source | Mycelium dry mass (mg) | | |
|---|---|---|---|
| $(NH_4)_2HPO_4$ | 39 | Urea | 85 |
| $(NH_4)_2SO_4$ | 41 | Casein | 102 |
| $NaNO_3$ | 45 | Soytone | 117 |
| $NH_4NO_3$ | 51 | Peptone | 127 |
| $KNO_3$ | 53 | Yeast extract | 171 |
| $Ca(NO_3)_2.3H_2O$ | 53 | | |

$F(10,33)=16,80^{***}$ $P \leq 0.05$ 0.01 0.001
LSD 30,36 40,89 54,21

FIGURE 11. GROWTH OF *T. SAGITTAEFORMIS* ON DIFFERENT CARBON SOURCES.

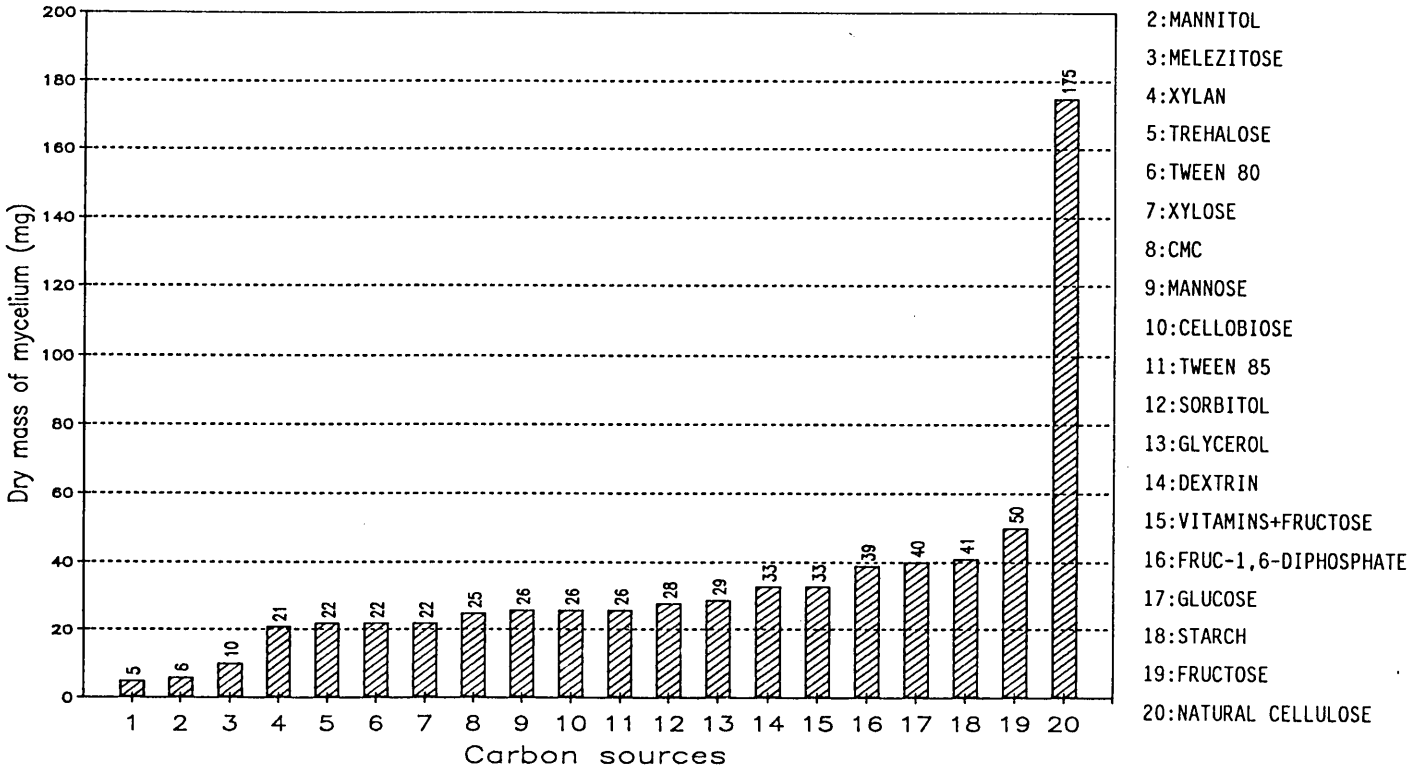



FIGURE 12. GROWTH OF *T. CLYPEATUS* ON DIFFERENT CARBON SOURCES.

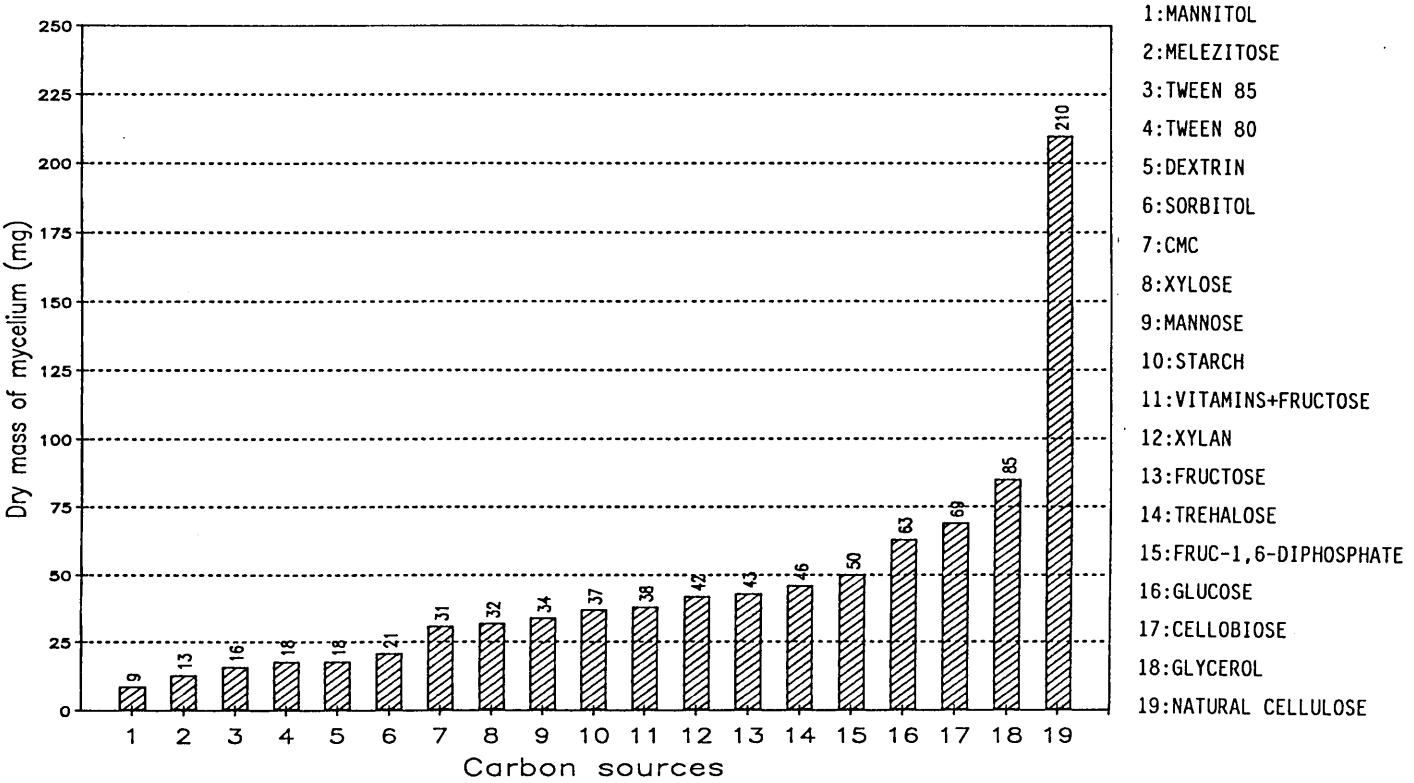


FIGURE 13. GROWTH OF *T. UMKOWAANI* ON DIFFERENT CARBON SOURCES.

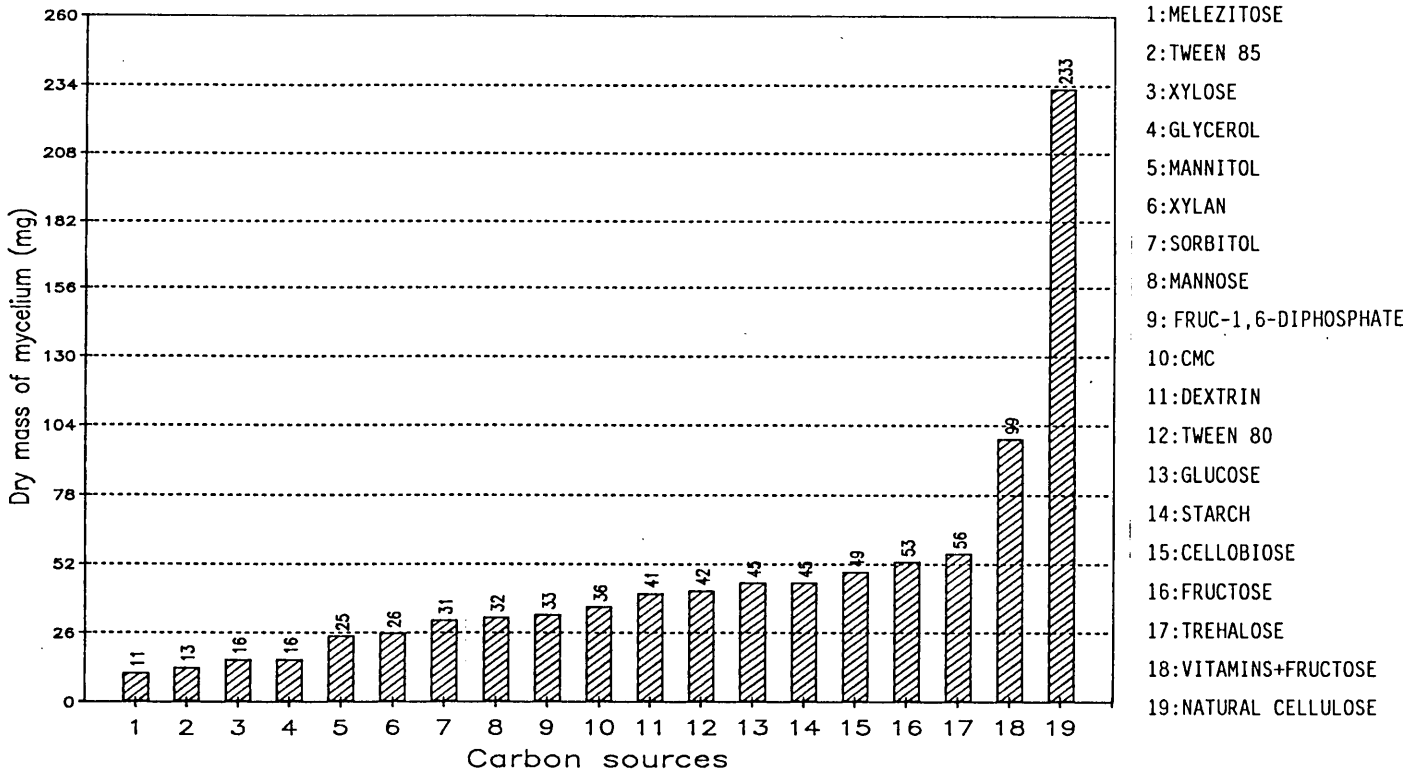
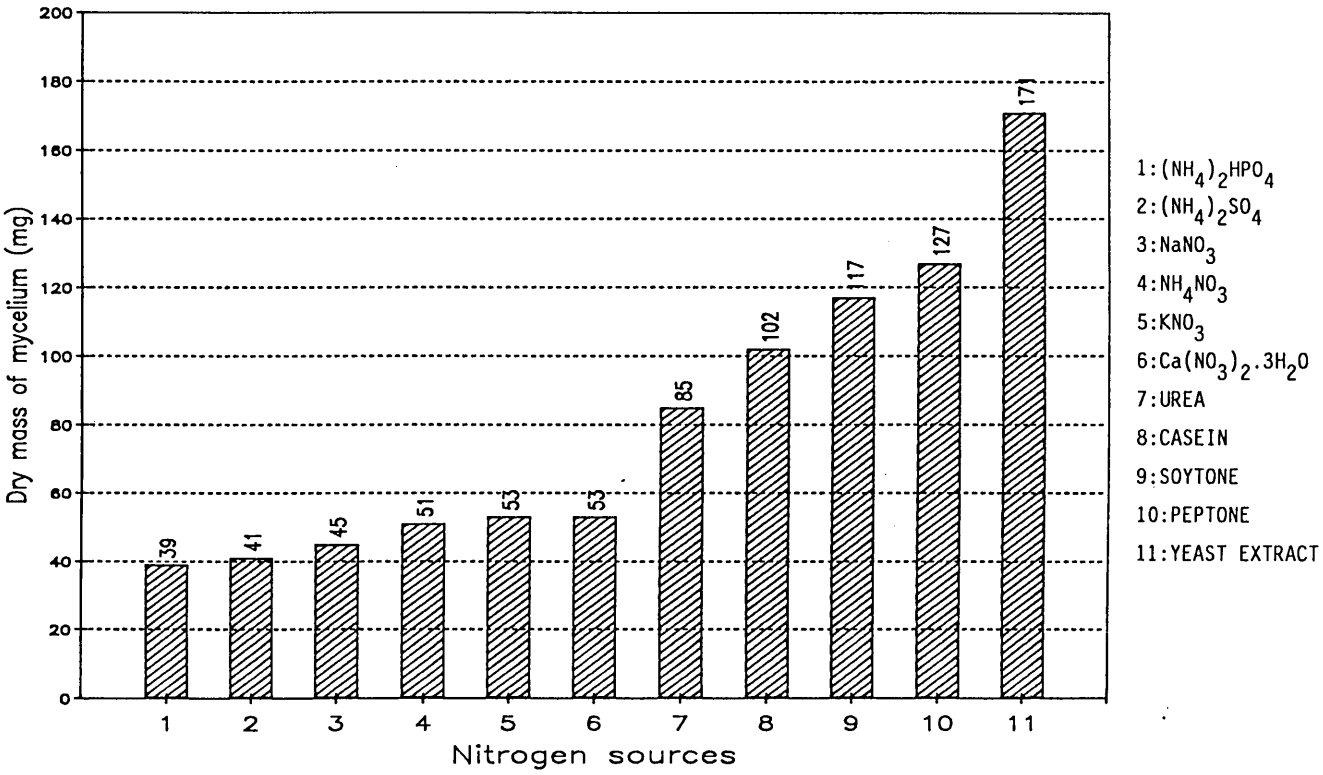



FIGURE 14. GROWTH OF *T. MICROCARPUS* ON DIFFERENT NITROGEN SOURCES.



influence must be seen in perspective when compared to the superior growth on crystalline cellulose which lack vitamins and differs significantly (0,1 % level) when compared to the growth on the vitamin medium. Vitamins are therefore not a compulsory requirement for the normal growth of *T. umkowaani* and when available exert only a partially or restricted stimulative influence on mycelium growth. This seem to be the case when purely vegetative mycelium is cultivated on synthetic media and may not reflect the vitamin requirements of the mycelium where it grows in contact with the comb. The vitamin requirements may also increase when basidiocarps are produced during the rainy season. The growth of the basidiocarps result in a dramatic increase in energy consumption and the reproductive growth phase is also coupled with the depletion of nutrients in the combs. It would therefore seem that *T. umkowaani* is no exception and like the other species investigated does not require supplementary vitamins when cultivated on a synthetic medium.

Batra & Batra (1979) determined the influence of different vitamins on the growth of *T. albuminosus* mycelium in a qualitative manner. They observed a strong positive growth reaction when a vitamin mixture was added to the basal medium and that no growth occurred when thiamine was excluded from the medium. However they did not describe their sterilization methods of the vitamin solutions and no quantitative measurements of mycelium growth were recorded. Chandra & Purkayastha (1977) evaluated the influence of five different vitamins on the growth of *T. eurhizus* mycelium. They observed that mycelium growth was significantly higher when ascorbic acid and thiamine were included in the basal medium in relation to the basal medium without vitamins. However they did not describe their sterilization methods of the vitamin solutions and no assessment was made of the cellulolytic and hemicellulolytic abilities of *T. eurhizus*. Without proper cold sterilization methods no experiments conducted to determine the effect of vitamins or any other heat liable compound on mycelium growth are of any value and the results are not relaible.

## 8. **Discussion**

The six species investigated display a specific carbon nutrition pattern with a preference for certain types of carbohydrates. Sources of carbon viz. : crystalline cellulose, starch, xylan, CMC, pectic acid and lipids are degraded and assimilated by the different species in variable degrees of efficiency (Table 19). The carbon sources listed in Table 19 are ordered according to the average growth of all six species on each carbon source, in order of decreasing magnitude. All the species have strong cellulolytic abilities and degrade crystalline cellulose. *T. umkowaani* and *T. clypeatus* are the most effective cellulose degraders while xylan is efficiently degraded by the anamorph and *T. reticulatus*. Species with strong amylolytic abilities are *T. reticulatus* and *T. microcarpus* (51 % and 58 % of the growth attained on natural cellulose respectively) while only the latter is capable of hydrolyzing pectic acid to a considerable degree. Other sources of carbon, either as mono or disaccharides are preferred in the form of D-glucose or certain other simple sugars resembling its configuration. Poor to moderate growth occur on sugar alcohols, D-xylose, melezitose and no visible growth was observed when amino sugars, D-lactose, α-D-melibiose (disaccharides), *mio*-inositol, *meso*-erythritol (sugar alcohols), D-fucose, L-rhamnose (deoxy methylhexoses), pectic acid, D-galacturonic acid and the dicarboxylic acids were included in the basal medium. The monoses D-galactose, L-arabinose, L-sorbose and D-ribose were also not utilized. The carbon nutrition requirements of *T. microcarpus* differs considerably from the carbon requirements of the other species. This is evident from the results in Table 9 which indicate the ability of this mushroom to utilize dicarboxylic acids, *mio*-inositol, D-galactose, D-lactose and α-D-melibiose. The mycelium yield attained on the trisaccharide melezitose does not differ significantly from the yield attained on crystalline cellulose and represents a very efficient form of carbon for *T. microcarpus*. This sugar is poorly utilized by the other species. It would seem that the peculiar carbon nutrition pattern of *T. microcarpus* confirms the placement of this species in the subgenus Praetermitomyces as effected by Heim (1977) or alternatively in the genus *Podabrella* Singer (Singer, 1949). The growth requirements of *T.*

*eurhizus* were extensively investigated by Chandra & Purkayastha (1977) and their results regarding carbon nutritional patterns confirm my observations of the carbon nutritional patterns of Eutermitomyces species. They established that *T. eurhizus* was not capable of utilizing dicarboxylic acids with poor to moderate growth on D-lactose and D-xylose. No growth occurred on L-sorbose while D-glucose and simple sugars resembling its configuration proved to be excellent sources of carbon.

The carbon nutrition requirements of *Termitomyces* mycelium cultivated on defined synthetic media seem to correspond with the chemical composition of the natural substrate and the form and abundance of the carbon containing compounds available to the mycelium in the fungus combs. Batra & Batra (1979) determined the chemical composition of fungus combs constructed by *Odontotermes* obesus and obtained a cellulose : lignin ratio of 1,50 in newly constructed combs. They observed that combs which had borne a flush of basidiocarps had a significantly lower cellulose content and a higher lignin content than comb material without basidiocarps. They cultivated sporodochial mycelium of *T. albuminosus* on CMC and Walseth cellulose media. Both cellulose culture media supported growth while a well defined zone was produced in the Walseth cellulose medium. Proof of the high cellulose contents was supplied by Arshad *et al.* (1987) who obtained a base and acid-insoluble humin fraction which accounted for 40 % of the dry mass of the comb and consisted mainly of crystalline cellulose. Mishra & Sen-Sarma (1979) established that fungus combs of *Odontotermes* and *Microtermes* spp. had a hemicellulose content of between 19,8 % and 32 % as well as a cellulose content of between 12,8 % and 20,0 %. The same authors investigated the starch content of fungus combs and obtained values of 23,2 % to 30,5 % while Rohrmann (1978) also detected starch in an acid-detergent soluble fraction which was prepared from comb extracts. Rohrmann & Rossman (1980) investigated the mycoflora of termitaria capable of degrading cellulose and they established that the sporodochial mycelium of *Termitomyces* was the major decomposer of cellulose in the comb. It therefore seems that natural plant cell wall cellulose occurs in sufficient quantities in termite constructed fungus combs and

represents the major source of carbon from which metabolic energy can be generated through cell respiration. The mycelium of *Termitomyces* synthesize abundant cellulolytic enzymes and therefore the cellulose in the comb is readily decomposed and assimilated before any of the other polysaccharides in the comb are degraded. Several authors determined the lignin content of fungus combs constructed by different termite species and they obtained values which ranged from 14,6 % to 55,6 % (Abo-Khatwa, 1976; Becker & Seifert, 1963; Cmelik & Douglas, 1970; Mishra & Sen-Sarma, 1979; Rohrmann & Rossman, 1980). According to Mishra & Sen-Sarma (1979, 1980) only a limited amount of lignin can be degraded by termites of the subfamily Macrotermitinae. This digestion process is accomplished by the intestinal microflora where the process of oxidation is intracellular. These authors established that the faecal and nest material of termitaria had a high lignin content (35,9 % to 55,6 %) in contrast with the low content of fungus combs 20,2 % to 29,2 %. Rohrmann & Rossman (1980) detected lignase enzyme activity using the syringaldazine test only in newly constructed fungus combs that were actively penetrated by *Termitomyces* mycelium. These authors consider *Termitomyces* a decomposer of lignin although the employment of the syringaldazine test may not be a very reliable test for the detection of lignase enzymes. It is highly improbable that the products of lignin degradation (vanillin, p-hydroxybenzaldehyde, varatralaldehyde, humic acids and phenolic compounds) can be utilized by *Termitomyces*. At present no conclusive evidence exists to prove beyond doubt that the mycelium of *Termitomyces* produces lignase enzymes either in a synthetic medium or on the natural substrate. The complexity of investigating lignin degradation is compounded by the unavailability of natural crystalline lignin. Preparations of lignin involve hydrolysis with strong base and acid solutions at high temperatures (Pearl, 1967; Schwalbe, 1925) which cause excessive degradation of the lignin molecule and are consequently unacceptable for degradation studies. More research is necessary to elucidate the true nature of the lignin molecule and to refine the methods of extraction. Skelton (1986) extracted a polyphenol oxidase from *T. microcarpus* basidiocarps, an enzyme involved in the degradation of lignin. He employed a synthetic substrate

(dihydroxyphenylalanine) to assay enzyme activity though more enzymes are involved in the degradation of lignin, a process which is still incompletely understood (Haider & Trojanowski, 1975; Kirk, Schultz, Conners, Lorenz & Zeikus, 1978; Kirk, 1971). There exists only two feasible extraction methods that will yield a moderately unaltered lignin preparation developed by Björkman (1956) and Brauns (1962). Brauns's (1962) preparation represents only a small part of the lignin in wood and is not strictly representative of the bulk of the native lignin (Kirk, 1971). Björkman's (1956) preparation yields a relatively unchanged lignin that is acceptable for growth requirement studies. Next to cellulose and hemicellulose, lignin is the most abundant organic biopolymer in nature and is continuously cycled and constitutes a major part of detritus and plant litter (Griffen, 1981). These materials are collected by foraging workers and used in the construction of fungus combs. It is therefore not surprising that the combs contain a substantial amount of lignin. Whether lignin can serve as the sole source of carbon for the growth of *Termitomyces* is still uncertain. It has been established that certain species of white-rot Basidiomycetes are incapable of utilizing lignin unless the nutrient medium is supplemented with cellulose, cellobiose, glucose or xylose (Kirk, 1971). In the natural state lignin is interwoven with cellulose, hemicellulose, protein and other carbohydrates and it is therefore highly improbable that lignin alone will be degraded but may be utilized simultaneously and in combination with other carbon sources. These sources of carbon may facilitate the utilization of lignin. Due to the well developed ability of *Termitomyces* to degrade cellulose, hemicellulose and starch it would seem that lignin where it is present in the natural state does not represent an important source of carbon. Lignin may be degraded only partially by the mycelium of *Termitomyces*, since alternative carbohydrate polymers occurs in sufficient quantities in newly constructed combs which will provide all the carbon and energy necessary for mycelium growth. It was therefore decided not to assess the capability of *Termitomyces* mycelium to degrade lignin because of (i) the difficulties involved in preparing a suitable natural lignin substrate (ii) the insolubility of such a preparation in synthetic media (iii) the extenuated adaption

phase necessary before oxidation can commence and (iv) the unknown enzyme complex responsible for the complete degradation of the lignin molecule.

The *Termitomyces* species evaluated are capable of utilizing a broad range of different mono, di and trisaccharides as well as the different sugar derivatives. Various authors have detected some of the sugars in fungus combs constructed by termites of the subfamily Macrotermitinae. Schnitzer & Preston (1987) and Arshad & Schnitzer (1987) employed sophisticated biochemical methods to determine the chemical composition of comb material. They detected high concentrations of carbohydrates in the whole termite fungus comb and in the base and acid-insoluble humin fractions. After acid hydrolysis of the comb material they obtained the following monosaccharides in decreasing order of concentration : glucose, xylose, arabinose, galactose, mannose, rhamnose and fucose. These data are in accord with the high cellulose, starch and hemicellulose contents of fungus combs because glucose is the repeating unit of cellulose and starch whereas xylose, arabinose, galactose and mannose are the monomeric subunits of hemicelluloses. Small quantities of glucosamine, rhamnose and fucose were detected by these authors in the combs and are not utilized by *Termitomyces* mycelium in culture. Mishra & Sen-Sarma (1979) detected the same simple sugars in their comb extracts obtained from different termite species. Both reducing sugars and D-mannitol were extracted from combs analyzed by Abo-Khatwa (1976). The sugar alcohol D-mannitol occur in high concentrations (5,5-6,9 %) in the comb material but does not represent an efficient source of carbon for *Termitomyces*. Mishra & Sen-Sarma (1986) collected sporodochia from the combs of *T. albuminosus* and they prepared a crude extract from this material which they assayed for various enzyme activities. They detected enzymes active against various disaccharides for example : sucrose, maltose, trehalose, cellobiose, melibiose, lactose and raffinose which is a trisaccharide. The authors stated that the presence of these enzymes in the comb sporodochia would indicate the presence of the corresponding substrate in the fungus comb.

Various authors analyzed the chemical composition of fungus combs by utilizing conventional as well as sophisticated methods. They also paid special attention to that fraction of the combs which contains

oils and lipids. Arshad & Schnitzer (1987) analyzed this fraction of the comb with the aid of infrared spectrophotometry and $^{13}C$ nuclear magnetic resonance and detected the presence of carbon arising from $CO_2H$ groups which could originate from amide and ester groups. They calculated that the $CO_2H$ groups contained 9,5 % of the carbon in this specific fraction and that approximately 30 % of the mass of the comb consisted of long chain and branched alkanoic acids of which a certain percentage may contain lipid molecules and fatty acids. Mishra & Sen-Sarma (1979) determined the alcohol : benzene soluble fraction of combs constructed by four different termite species and obtained values between 1,4 % and 5,0 %. Abo-Khatwa (1976) measured the lipid content of combs constructed by *Macrotermes subhyalinus* and obtained a value of 22 % to 27 %, based on dry mass. They ascertained that 74,2 % of the lipid fraction consisted of mono and triglycerides. Cmelik & Douglas (1970) analyzed the lipids extracted from the combs of *Macrotermes goliath* and *Odontotermes badius*. They established that 57 % of the lipids consisted of free fatty acids of which palmitic acid, oleic acid and linoleic acid were present in substantial quantities. The fatty acids consisted of saturated and mono-unsaturated acids with an odd number of carbon atoms. Mono-esters comprised 43 % of the total lipid fraction while substantial amounts of hydroxylated fatty acids were located in the combs of *O. badius*. It would therefore seem that the species investigated would be able to hydrolyze mono and triglycerides in the comb by secreting esterase and lipase respectively into the substrate as well as assimilating the main end products of enzyme hydrolysis, namely free fatty acids. Even *T. clypeatus* which lacks the enzyme lipase (Table 9) would be able to utilize monoglycerides and free fatty acids. These acids represent an instant source of energy and are capable of generating much more energy when metabolized than the more abundant reducing sugars when compared on a molecular basis. This physiological phenomenon may contribute to the high energy consumption of the rapidly growing sporocarps during the rainy season. When one considers though the strong cellulolytic, hemicellulolytic and amylolytic abilities of the species investigated (Tables 4-7 and Figs 4-7) as well as the high cellulose, hemicellulose and starch contents of the fungus combs

(Arshad & Schnitzer, 1987; Cmelik & Douglas, 1970; Mishra & Sen-Sarma, 1979) in comparison with the low lipid content of the combs, it would seem highly unlikely that lipids could contribute much to the energy requirements of *Termitomyces* mycelium when growing on the comb. This observation is further substantiated by the findings of Batra & Batra (1979) who observed a lowering of the cellulose content in combs which had borne a flush of basidiocarps. The results in Table 19 indicate that the major organic biopolymers in the fungus combs viz. : cellulose, hemicellulose, starch and proteins support better growth than the two synthetic fats, Tween 80 and Tween 85.

Execution of enzyme tests (hydrolases secreted into the medium) proved to be a valuable aid in determining the degrading abilities of the *Termitomyces* species under investigation. Results obtained by utilizing standard methods (mycelium dry mass, loss in dry mass of the substrate utilized and measurements of the radial growth of the colony) are insufficient if used alone and the results obtained do not reflect the true capability of each species to degrade a specific organic polymer. However when these methods are supplemented by properly executed enzyme assays utilizing modern biochemical techniques, the results obtained would provide more information with respect to the ability of the specific species to decompose an organic biopolymer. Enzyme studies will indicate whether the mycelium can produce and secrete the necessary enzyme(s) responsible for the hydrolysis of a specific organic biopolymer. Enzyme activity will provide a indication of the relative quantity in which the enzyme is produced and the actual end products of enzyme hydrolysis can be accurately determined. Although the results in this investigation indicate that no distinct correlation exists between mycelium yield and enzyme activity, secretion of hydrolases into the liquid medium by rapidly growing mycelium is a clear indication that the specific substrate is being degraded.

## 9. Effect of nitrogen sources on the growth of mycelium and determination of trypsin activity

Results in Fig. 14 and Table 14 represent the growth of *T. microcarpus* on different nitrogen sources. With respect to the inorganic sources

no specific preference for either ammonium or nitrate can be observed while urea prove to be the best inorganic nitrogen source. Mycelium yield on this source differs significantly at the 0,1 % level from the yield attained on the other inorganic sources. Growth on the complex organic sources differs significantly at the 1 % and 0,1 % levels (Table 14) from the growth on urea. Results show that optimal growth is attained on complex organic sources such as casein, soytone, peptone and yeast extract which consist primarily of a mixture of amino acids. Although soytone and yeast extract in addition also contain carbon sources and growth factors which will stimulate growth, the nitrogen sources casein and peptone consist mainly of a mixture of amino acids. *T. microcarpus* has the ability to produce various proteases that catalyze the hydrolysis of the four organic substrates. Casein is hydrolyzed by trypsin (Table 18). Skelton & Matanganyidze (1981) obtained a high proteolytic activity in extracts which they prepared from *T. microcarpus* fruit bodies. They regarded this proteinase as a thiol proteinase which hydrolyzed casein and keratin. Parent & Skelton (1977) also detected high activities of this proteinase in their crude extracts of this mushroom.

Influence of different nitrogen sources on the growth of *T. sagittaeformis* is shown in Table 15 and illustrated in Fig. 15. As in the case of *T. microcarpus* urea proved to be the best source of inorganic nitrogen while good growth occurred on ammonium chloride and ammonium tartrate. In the case of *T. sagittaeformis* a distinct preference is displayed for ammonium containing nitrogen compounds. Nitrogen in the form of complex organic sources, soytone and casein ensures maximal mycelium growth and a higher yield is attained on these sources than on the amino acids : L-arginine, L-methionine, L-alanine and L-glutamine which differ at the 0,1 % level from the growth on soytone and casein. With the exception of L-glutamic acid which is an excellent source of nitrogen (growth on this source does not differ significantly from the growth on casein and soytone), results indicate that *T. sagittaeformis* prefers nitrogen in the form of a mixture of amino acids to individual amino acids. Significantly better growth (at the 1 % level) occurs on L-glutamic acid in relation to the growth on L-glutamine. *T. sagittaeformis* prefers amino acids with acidic side chains

to the amide form L-glutamine, with an uncharged polar side chain. This observation is in accord with the view of Griffen (1981) that amino acids with acidic side chains produce better growth than amino acids with uncharged polar or nonpolar side chains.

Results in Fig. 16 and Table 16 represent the influence of different nitrogen sources on the growth of *T. clypeatus*. Results show that both inorganic and organic sources are utilized and good growth occurs on $NH_4CI$, urea and ammonium tartrate. As in the case of *T. sagittaeformis* inorganic nitrogen in the form of ammonium salts supports better growth than nitrate-containing sources. Nitrogen sources which contain a mixture of amino acids, yeast extract, casein and soytone support superior growth in relation to individual inorganic sources. Ghosh & Sengupta (1978) investigated the nitrogen nutrition of *T. clypeatus* and established that growth of this mushroom was well supported by $NH_4NO_3$, $(NH_4)_2SO_4$ and $NH_4H_2PO_4$. In contrast with the results presented in Table 16, they obtained poor growth on urea and $NH_4CI$. Organic nitrogen sources were not included in their investigation.

Utilization of different nitrogen sources by *T. umkowaani* is presented in Table 17 and illustrated in Fig. 17. It is evident that both inorganic and organic sources are utilized although no specific preference for either organic or inorganic sources are apparent. With respect to complex organic sources only casein supports good growth while modest growth occurs on peptone and soytone. Maximal growth is attained on L-glutamine, the amide of L-glutamic acid which differs significantly from the growth on all the nitrogen sources tested. Ammonium chloride represents the best inorganic source. With respect to inorganic nitrogen, the ammonium salts seem to support better growth than the nitrate salts and organic nitrogen in the form of L-alanine and L-glutamine. Chandra & Purkayastha (1977) investigated the nitrogen nutrition of *T. eurhizus* and evaluated the influence of various inorganic and organic nitrogen sources. Their results showed a distinct preference for certain amino acids and complex organic sources, for example high yields were attained on DL-alanine, DL-aspartic acid, casein hydrolysate, peptone and yeast extract.

Casein was chosen to represent the protein-containing compounds

**TABLE 15.** GROWTH OF *T. SAGITTAEFORMIS* ON DIFFERENT NITROGEN SOURCES.

| Nitrogen source | Mycelium dry mass (mg) | | |
|---|---|---|---|
| $NaNO_3$ | 11 | L-Methionine | 30 |
| $Ca(NO_3)_2$ | 12 | Urea | 31 |
| $(NH_4)_2SO_4$ | 13 | L-Alanine | 33 |
| $NH_4NO_3$ | 18 | L-Glutamine | 33 |
| Peptone | 24 | Yeast extract | 39 |
| $NH_4CI$ | 25 | L-Glutamic acid | 47 |
| L-Arginine | 27 | Soytone | 50 |
| $NH_4$ tartrate | 27 | Casein | 52 |

$F(15,48)=4,18^{***}$ $P \leq 0.05$ 0.01 0.001
LSD 18,01 24,10 31,66
Nitrogen sources not utilized: $(NH_4)_2HPO_4$; $KNO_3$.

**TABLE 16.** GROWTH OF *T. CLYPEATUS* ON DIFFERENT NITROGEN SOURCES.

| Nitrogen source | Mycelium dry mass (mg) | | |
|---|---|---|---|
| $(NH_4)SO_4$ | 10 | L-Glutamine | 32 |
| $Ca(NO_3)_2.3H_2O$ | 12 | L-Arginine | 34 |
| $NaNO_3$ | 14 | L-Methionine | 34 |
| $(NH_4)_2HPO_4$ | 17 | Peptone | 47 |
| $NH_4NO_3$ | 19 | L-Glutamic acid | 51 |
| $NH_4CI$ | 20 | L-Alanine | 56 |
| Urea | 25 | Casein | 56 |
| $NH_4$ tartrate | 26 | Yeast extract | 58 |
| | | Soytone | 62 |

$F(16,51)=50,19^{***}$ $P \leq 0.05$ 0.01 0.001
LSD 9,97 13,26 17,24
Nitrogen source not utilized: $KNO_3$.

**TABLE 17.** GROWTH OF *T. UMKOWAANI* ON DIFFERENT NITROGEN SOURCES.

| Nitrogen source | Mycelium dry mass (mg) | | |
|---|---|---|---|
| $KNO_3$ | 13 | L-Glutamic acid | 31 |
| Peptone | 14 | $(NH_4)_2SO_4$ | 31 |
| $(NH_4)_2HPO_4$ | 14 | $NH_4NO_3$ | 31 |
| $NaNO_3$ | 15 | L-Arginine | 32 |
| Yeast extract | 15 | Urea | 33 |
| Soytone | 15 | Casein | 36 |
| $Ca(NO_3)_2.3H_2O$ | 20 | L-Alanine | 37 |
| $NH_4$ tartrate | 23 | $NH_4CI$ | 50 |
| L-Methionine | 23 | L-Glutamine | 72 |

$F(17,54)=7,62^{***}$ $P \leq 0.05$ 0.01 0.001
LSD 15,49 20,60 26,79

**TABLE 18.** TRYPSIN ACTIVITY OF CULTURE FILTRATES AND GROWTH OF *TERMITOMYCES* ON CASEIN CULTURE MEDIUM.

| Species tested | Mycelium dry mass (mg) | Trypsin activity (expressed as tryptic units $x10^{-3}$) |
|---|---|---|
| Anamorph | 6 | 12 |
| *T.reticulatus* | 23 | 15 |
| *T.umkowaani* | 36 | 11 |
| *T.sagittaeformis* | 52 | 16 |
| *T.clypeatus* | 56 | 14 |
| *T.microcarpus* | 102 | 22 |

Mycelium dry mass, $F(5,18)=123,30^{***}$ $P \leq 0.05$ 0.01 0.001
LSD 8,87 12,15 16,56
Enzyme activity, $F(5,12)=1,12$ n.s. at $P \leq 0.05$.

TABLE 19. THE ABILITY OF TERMITOMYCES TO UTILISE CERTAIN NITROGEN AND CARBON SOURCES.

| | Mycelium dry mass (mg) | | | | | |
|---|---|---|---|---|---|---|
| | Species tested | | | | | |
| Substrate utilised | 1 | 2 | 3 | 4 | 5 | 6 |
| Natural cellulose | 187 | 175 | 186 | 210 | 233 | 122 (84) |
| Xylan | 107 (57[a]) | 21 (12) | 141 (76) | 42 (20) | 26 (11) | 146 |
| Starch | 96 (51) | 41 (23) | 107 (58) | 37 (18) | 45 (19) | 10 (7) |
| Casein | 102 (55) | 52 (30) | 23 (12) | 56 (27) | 36 (15) | 6 (4) |
| Tween 80 | 49 (29) | 22 (13) | 31 (17) | 18 (9) | 42 (18) | 74 (51) |
| CMC | 48 (26) | 25 (14) | 10 (5) | 31 (15) | 36 (15) | 4 (3) |
| Tween 85 | 15 (8) | 20 (11) | 19 (10) | 10 (5) | 7 (3) | 32 (22) |
| Pectin | 106 (57) | - | - | - | - | 13 (9) |
| Na-polypectate | 71 (37) | - | - | - | - | 2 (1) |

| | | | | |
|---|---|---|---|---|
| 1. T.microcarpus | $F(8,27)=95,36^{***}$ | $^{*}P<0.05$ | $^{**}P<0.01$ | $^{***}P<0.001$ |
| | | LSD 14,85 | 20,05 | 26,70 |
| 2. T.sagittaeformis | $F(6,21)=79,58^{***}$ | $^{*}P<0.05$ | $^{**}P<0.01$ | $^{***}P<0.001$ |
| | | LSD 18,51 | 25,20 | 34,00 |
| 3. T.reticulatus | $F(6,21)=95,64^{***}$ | $^{*}P<0.05$ | $^{**}P<0.01$ | $^{***}P<0.001$ |
| | | LSD 21,18 | 28,75 | 38,78 |
| 4. T.clypeatus | $F(6,21)=14,91^{***}$ | $^{*}P<0.05$ | $^{**}P<0.01$ | $^{***}P<0.001$ |
| | | LSD 51,94 | 70,70 | 95,38 |
| 5. T.umkowaani | $F(6,21)=80,13^{***}$ | $^{*}P<0.05$ | $^{**}P<0.01$ | $^{***}P<0.001$ |
| | | LSD 25,35 | 34,51 | 46,55 |
| 6. Anamorph | $F(8,27)=65,61^{***}$ | $^{*}P<0.05$ | $^{**}P<0.01$ | $^{***}P<0.001$ |
| | | LSD 18,60 | 25,12 | 33,46 |

[a] Percentage mycelium dry mass attained on each carbon source in relation with the maximal dry mass attained on natural cellulose or xylan.

FIGURE 15. GROWTH OF *T. SAGITTAEFORMIS* ON DIFFERENT NITROGEN SOURCES.

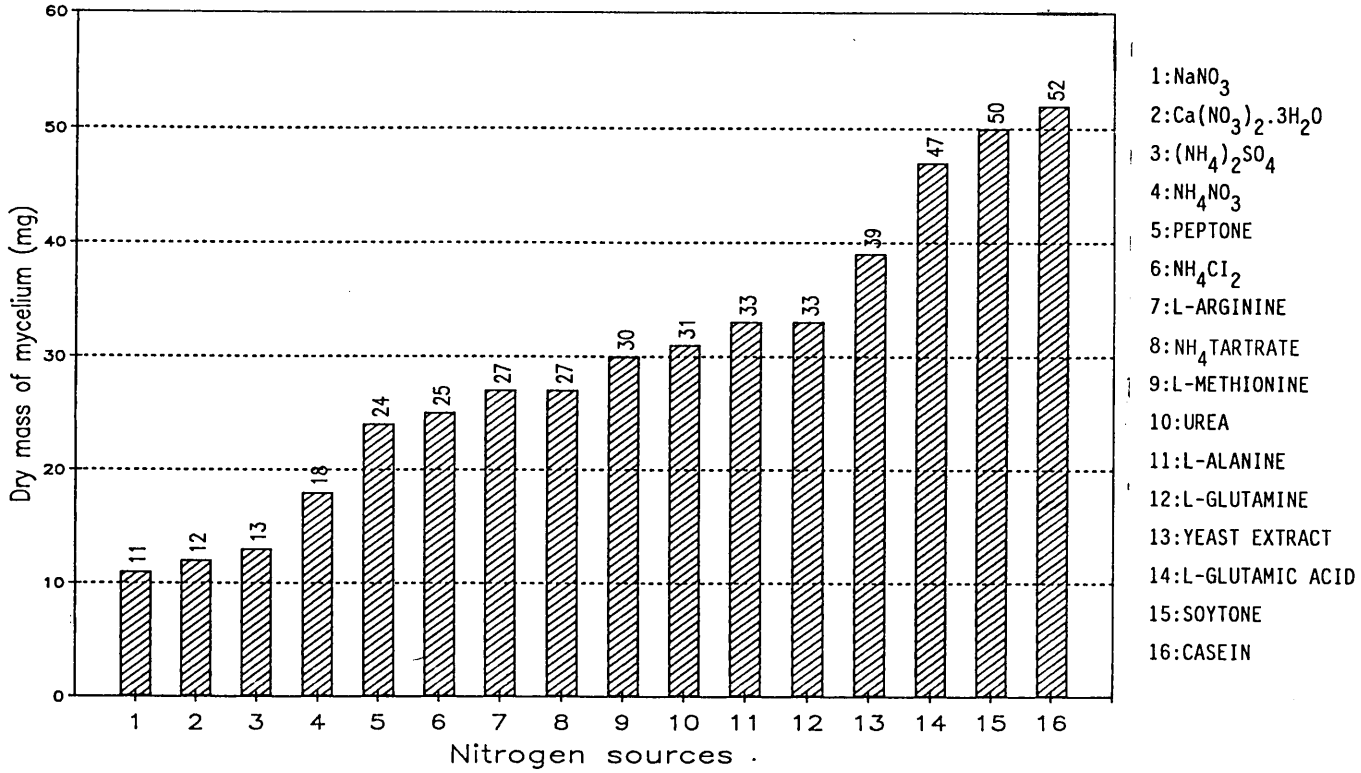



FIGURE 16. GROWTH OF *T. CLYPEATUS* ON DIFFERENT NITROGEN SOURCES.

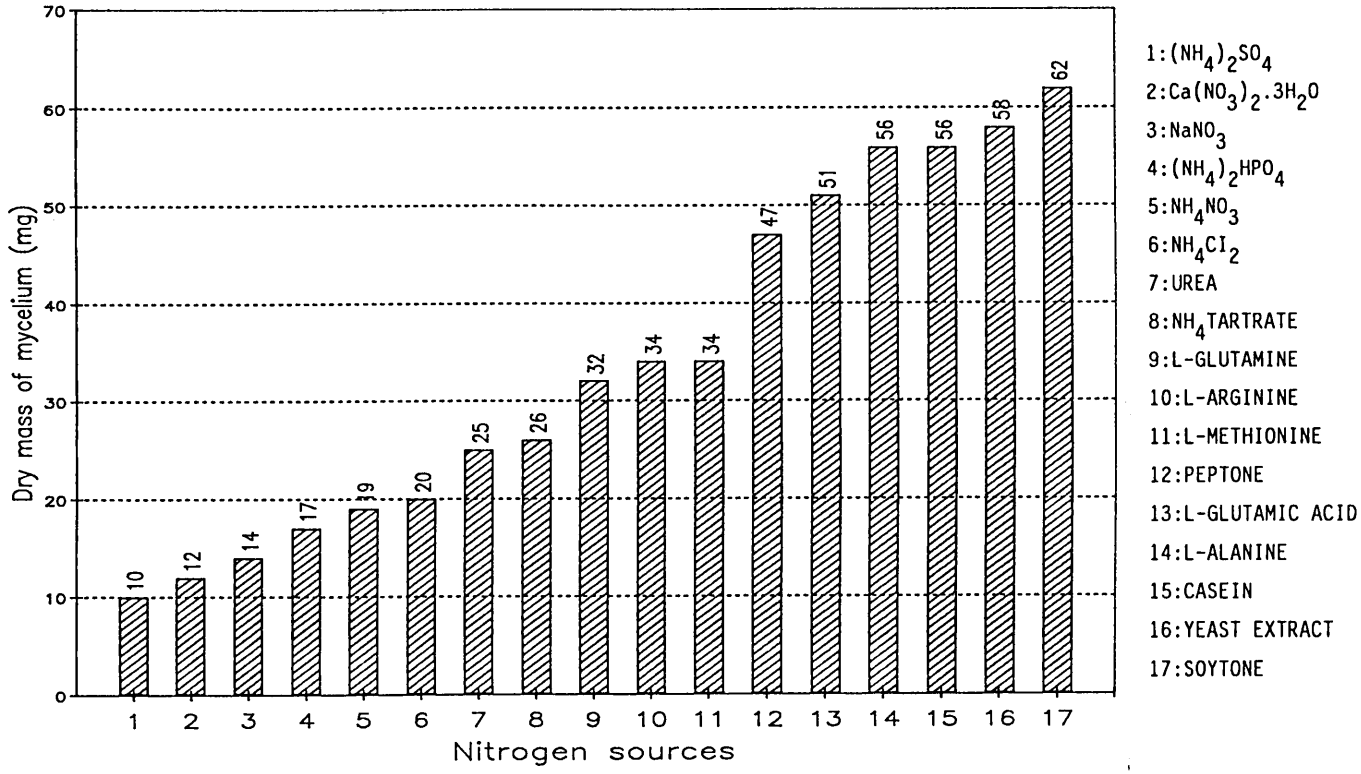


FIGURE 17. GROWTH OF *T. UMKOWAANI* ON DIFFERENT NITROGEN SOURCES.

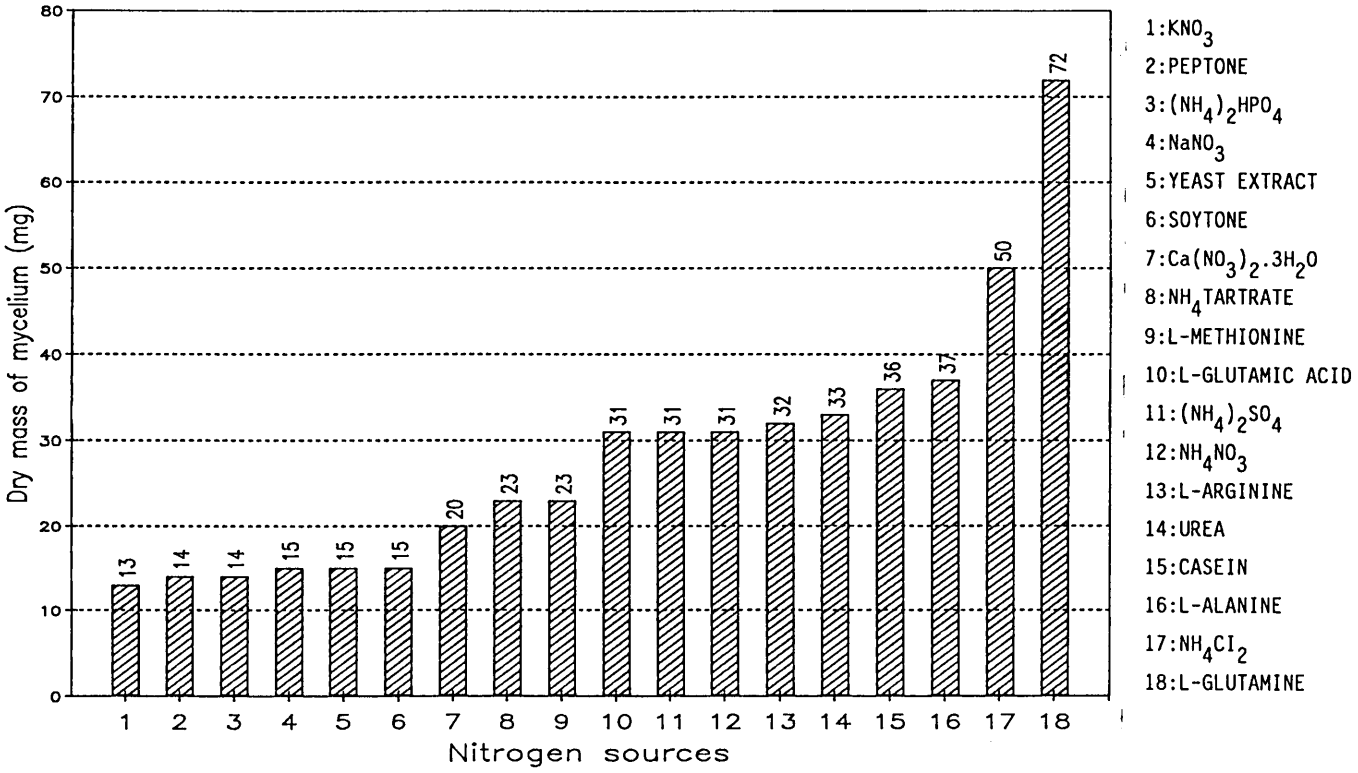
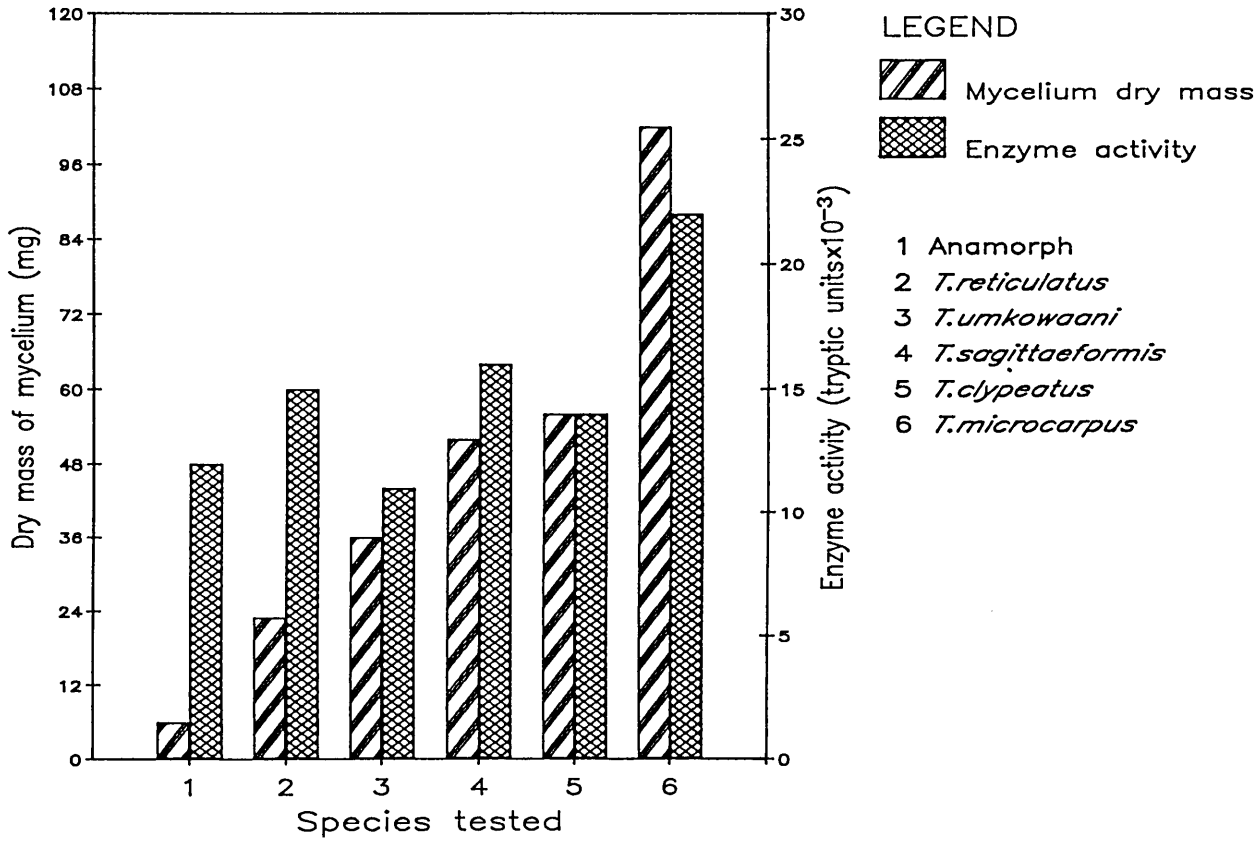



FIGURE 18. TRYPSIN ACTIVITY OF CULTURE FILTRATES AND GROWTH OF *TERMITOMYCES* ON CASEIN CULTURE MEDIUM.



in the fungus combs. Ability of the six species to hydrolyze casein is given in Table 18 and graphically illustrated in Fig. 18. It is apparent that all the species are capable of producing extracellular trypsin and of absorbing amino acids which are released into the medium during enzymatic hydrolysis. There is no correlation between the mycelium yield and enzyme activity except in the case of *T. microcarpus* which displays the highest mycelium yield and trypsin activity. Culture filtrate trypsin activity does not differ significantly at the 5 % level while mycelium yields do differ significantly at the 5 %, 1 % and 0,1 % levels.

## 10. **Discussion**

Generally speaking the *Termitomyces* species tested prefer nitrogen in a complex form. Amino acids also present an efficient source of nitrogen when supplied as single amino acids while inorganic sources support only moderate growth. Batra & Batra (1979) conducted a preliminary investigation on the nutritional requirements of *T. albuminosus* and they recorded that ammonium nitrate as the sole inorganic source was efficiently utilized, but organic sources proved to be superior. Although the nitrogen content of fungus combs is low, 1,2 % to 2,2 % (Mishra & Sen-Sarma, 1979) and 1,11 % to 1,52 % (Rohrmann, 1978), Arshad & Schnitzer (1978) obtained several acidic, basic and neutral amino acids in the combs after hot acid hydrolysis which accounted for 68,3 % of the total nitrogen in the comb. They could not detect any free amino acids prior to acid hydrolysis and therefore the amino acids originated from peptides, polypeptides and proteins. According to Arshad & Schnitzer (1987) proteinaceous materials were the major nitrogen components in the fungus combs with a protein content of 8,9 %. Mishra & Sen-Sarma (1986) detected two proteases in a crude extract of *T. albuminosus* comb sporodochia active against casein and peptone. Mishra & Sen-Sarma (1979) detected nine different free amino acids in the combs of *Odontotermes obesus* but in very low quantities. Their results are in agreement with the results presented in Tables 14 to 17 and indicate that the *Termitomyces* species investigated utilize mainly the proteinaceous materials in the comb

and not the free amino acids, which occur in almost undetectable quantities. *Termitomyces* mycelium has the ability to produce proteases necessary for degradation of protein in the comb and the liberated amino acid mixture is then absorbed by the growing mycelium. Absorbed amino acids will fulfil the bulk of the nitrogen requirements of the actively growing mycelium and inorganic nitrogen sources will only be utilized to a limited extent or not at all as long as sufficient amino acids are available in the comb. End result of the protein digestion process is to increase the amount of nitrogenous compounds from the low nitrogen contents of the composted material (1,7 %) to the high nitrogen contents of the comb sporodochia (7,3 %) and to lower the high C : N ratio of the comb (25,5) to a C : N ratio value of 6,2 in the sporodochia (Matsumoto, 1976; Rohrmann, 1978). Apparently this transformation process is essential to supplement the nitrogen content of the worker termite diet (Batra, 1975) because it has been observed that nymphs and workers of fungus-growing termites would consume *Termitomyces* sporodochia along with the comb material.

## 11. **Growth of *T. umkowaani* on different natural substrates**

The following natural substrates are efficiently colonized and penetrated by mycelium of *T. umkowaani* : sorghum, wheat and rye combined with wheat bran and sawdust respectively. No conspicuous difference in the growth or density of mycelium could be observed between the natural substrates hydrated with basal medium with or without vitamins and the yeast extract, soytone, starch solution. Moderately good growth occurs on wheat bran and wheat straw but mycelium growth is not as dense as on the combined substrates (Fig. 1 j-l). The tobacco leaves and sawdust substrates do not support any growth.

Although only a limited number of substrates are evaluated when many different natural substrates and in different combinations could have been investigated, this preliminary investigation indicated certain growth requirements. The substrates wheat bran, wheat straw and sawdust consist mainly of cellulose, hemicellulose and lignin which will require a longer adaptation period for production of cellulolytic and hemicellulolytic enzymes before growth can commence. Addition of wheat, rye and sorghum to wheat bran and sawdust

stimulated mycelium growth to a marked degree. Wheat, rye and sorghum consist mainly of starch and will stimulate the production of amylolytic enzymes. Hydrolysis of starch will provide sufficient quantities of soluble sugars viz. : maltose, glucose and low molecular weight oligosaccharides which will provide sufficient carbon and energy to support active growth of the mycelium. After starch hydrolysis has commenced, degradation of the cellulose, hemicellulose and perhaps the lignin constituents may follow to support additional mycelium growth. Thus the initial hydrolysis of starch may facilitate the subsequent degradation of other organic polymers which are more resistant to microbial degradation. The texture of the natural substrates proved to be an important factor in obtaining a luxurious and dense mycelium growth. It was therefore necessary to pulverize the substrates with the aid of a Wiley mill and to pass the particles through a no. 20 wire mesh to obtain the right particle size. After the substrates were thoroughly mixed and hydrated the fine compact texture of the prepared substrates resembled the texture and structure of the natural comb. This method of substrate preparation ensured dense mycelium growth with proper penetration of the substrate.

Similar investigations were conducted by Quimio (1977) on *T. cartilagineus*. Quimio (1977) obtained excellent vegetative mycelium growth on rice bran, corn meal, coconut meat pulp and mud press from sugar cane. His efforts to induce basidiocarp formation failed. Sengupta, Naskar & Jana (1984) evaluated the mycelium growth of *T.clypeatus* on sawdust, wheat bran, green coconut coir and sugar cane bagasse. Substrates were hydrated with a basal medium and they observed that all the agrowastes supported mycelium growth although poor growth occurred on sawdust. The other agrowastes supported better growth and crude extracts of mycelium yielded high amylase, xylanase and cellulase activities especially extracts prepared from mycelium grown on wheat bran. It is therefore possible to grow mycelium of *Termitomyces* on natural substrates.

The nutritional requirements of the different *Termitomyces* species investigated in this study, do not seem to be comprehensive and specific. The chemical composition of the fungus combs closely resem-

ble those of the natural substrates utilized in this investigation. All the necessary carbon sources e.g., cellulose, hemicellulose, starch, lipids and nitrogen sources both organic and inorganic, as well as several vitamins are present in the natural substrates and the fungus combs. Optimal incubation conditions at optimal temperature and relative humidity in combination with all the nutrients required, ensured at least maximal vegetative mycelium growth. *In vitro* cultivation under aseptical conditions mimics the *in vivo* cultivation of *Termitomyces* mycelium on the combs. The gastrointestinal and or salivary excretions of the worker termites exert a fungistatic effect on alien fungal growth as well as a stimulative effect on the growth of *Termitomyces* mycelium (Sannasi & Sundara Rajulu, 1976; Batra & Batra, 1966). It seems improbable that the workers are responsible for the transformation from vegetative to reproductive growth because of the rapid depletion of the nutrients in the comb by the growing sporocarps on which the termite colony depends for continued survival. This statement is confirmed by observations at termitaria of renewed termite activity during sporocarp production. The long pseudorhizae and pilei are rapidly consumed by workers from below and many young developing primordia are consumed on the combs as soon as they are formed. The combs are therefore not abandoned by the workers during sporocarp production. Only changes in environmental conditions e.g. carbon dioxide content, temperature, relative humidity or atmospheric pressure may be responsible for reproductive growth. Sporocarps are frequently encountered during the rainy season from November to April during which thunderstorms, high relative humidity, drop in atmospheric pressure, increased termite activity and higher ground temperatures are prevalent. One would therefore expect that higher ground temperatures and sufficient rainfall would be sufficient to induce sporocarp production, but sporocarps have been collected at the end of April during which much lower ground temperatures, relative humidity and rainfall prevail and specimens have even been collected in the middle of the winter season, July to August, with almost no perceptible rain, low temperatures and very arid conditions coupled with retarded termite activity. Therefore at present no specific pattern of environmental conditions conducive for sporocarp development can be ascertained. Apparently both subtropical and semi-

arid environmental conditions can stimulate sporocarp development in the southern African region. It is therefore extremely difficult to distinguish a definite set of environmental conditions which stimulate reproductive growth of this genus. Long term research on the environmental conditions prevailing in the fungus comb chambers during the formation of primordia are essential to pinpoint the exact composition of atmospheric and other physical-chemical factors. Only after this information has been acquired would it be possible to simulate the specific combination of environmental conditions under laboratory conditions and attempt basidiocarp production.

## 12. **Nutritional and biological value of mycelium protein**

In an attempt to determine the nutritional value of the four mushrooms under investigation it was necessary to assess the protein quality rather than simply the crude protein content. To achieve this goal the amino acid composition and in particular the essential amino acid content was determined. No attempt was made to assess the fat, carbohydrate, fiber, vitamin and mineral content of the mushrooms because the protein fraction is rated as the most important component contributing to the nutritional value of a food (Crisan & Sands, 1978). The amino acid composition of the *Termitomyces* species evaluated is presented in Table 20 and is compared with the amino acid composition of *Agaricus bisporus* and a specially formulated essential amino acid pattern recommended by the Protein Advisory Group of the Food and Agricultural Organization of the United Nations (FAO 1973), which is based upon the essential amino acid (EAA) dietary requirements of normal adults. In Table 20 ten EAA and eight non-essential amino acids are compared and the total EAA content of each mushroom is calculated. *T. sagittaeformis* has the highest total amino acid content followed by *A. bisporus*, *T. umkowaani* and *T. reticulatus* while the percentage total EAA in relation to the total amino acid content is highest in *T. umkowaani* (43,76 %).

These results are further evaluated in the form of FAO ratios in Table 21. The FAO ratio is the content of each EAA in the test protein relative to the content of the particular EAA in the FAO reference

pattern expressed as a percentage. EAA content of *T. umkowaani* protein compares favourably with the EAA content of the FAO reference pattern with only leucine, phenylalanine, tyrosine and isoleucine as limiting amino acids while the other EAA exceeds the EAA content of the reference pattern in particular the sulfur-containing amino acids (methionine, cysteine) *T. umkowaani* contain 69,7 % of the leucine and 79,2 % of the aromatic amino acid content of the reference pattern while the methionine and cysteine content exceeds that of the reference pattern by 132 %. EAA content of *T. sagittaeformis* protein exceeds the EAA content of the reference pattern with respect to all the EAA and in the case of *A. bisporus* only the sulfur-containing amino acids are lower. EAA content of *T. reticulatus* protein compares less favourably with five EAA present in lower quantities than in the reference protein. Phenylalanine, tyrosine, threonine, and tryptophan are present in quantities approximately equal to their respective amounts in the reference pattern. The EAA content of the *Termitomyces* species compares less favourably with that of *A. bisporus* than with the FAO reference pattern because of the higher EAA content of *A. bisporus* protein. The methionine and cysteine content of *T. umkowaani* exceeds that of *A. bisporus* by 277,7 % while all the EAA of *T. sagittaeformis* with the exception of threonine and tryptophan exceed the respective amounts contained by *A. bisporus*. EAA content of *T. reticulatus* protein is lower with respect to all ten EAA.

Oser (1951,1959) suggested the application of an Essential Amino Acid Index (EAA Index) to evaluate dietary protein. The EAA Index is equal to the geometric mean of the FAO ratios and therefore the minimum ratio of test protein EAA content relative to that of the reference protein is 1% and the maximum 100% (Oser 1959). According to Oser (1951) any EAA which is present in higher amounts than in the reference protein is considered to be equal and not superior to the reference protein, therefore no credit is granted if any EAA in the test protein exceeds the amount contained in the reference protein. Consequently some of the FAO ratios and EAA Indexes presented in Table 21 should not exceed 100 % when the test protein contains more of a particular EAA than the reference protein according to Oser (1951). However in this investigation the true values were calculated to il-

lustrate the margin by which the particular EAA in the test protein exceeds the amount contained in the reference protein. EAA Indexes in Table 21 indicate that *Termitomyces* protein compares more favourable with the FAO reference pattern than with *A. bisporus* protein. The EAA Index calculated for *T. umkowaani* exceeds the EAA content of the reference pattern by 12,2 % and *T. sagittaeformis* exceeds the EAA content of the reference pattern by 57,1 %. *T. reticulatus* contain only 54 % of the EAA content of the reference pattern. EAA content of *A. bisporus* protein is 27,2 % higher than that of the reference pattern. When compared to *A. bisporus* the EAA Index of *T. umkowaani* drops from 112,2 to 87,9 % and that of *T. sagittaeformis* from 157,1 to 123,2 % and the EAA Index of *T. reticulatus* decreases from 54,0 to 44,3 %. A similar trend is apparent when the Biological Value (BV) and Nutritional Index (NI) are considered.

According to Oser (1951) the EAA Index is closely correlated with the BV and the EAA Index can be employed to predict the BV of a test protein (Crisan & Sands, 1978). According to Oser (1951) the BV may be considered as a coefficient of the utilization of EAA and is a function of the absorbed content of EAA which the animal organism is not capable of synthesizing. The BV would therefore give an indication of the percentage of nitrogen retained by the animal after consuming the test protein. Biological Values presented in Table 21 are most probably too high due to the fact that mushroom protein is not 100 % digestible. Digestibility of mushroom protein is closer to 60 % to 70 % because of the presence of a significant amount of non-protein nitrogen in the chitinous cell walls (Gilbert & Robinson, 1957). Nevertheless these values were calculated using the following equation : BV = 1,09(EAA Index) - 11,7 which would imply a higher degree of EAA utilization when protein of *A. bisporus*, *T. umkowaani* and *T. sagittaeformis* are consumed whereas a smaller amount of nitrogen would be retained when protein of *T. reticulatus* is consumed. The NI assists in comparing mushrooms with small amounts of high quality protein with species containing larger quantities of protein of lesser nutritional value (Crisan & Sands, 1978). When the formula :

$$N = \frac{(\text{EAA Index} \times \% \text{ crude test protein})}{100}$$

TABLE 20. COMPARATIVE AMINO ACID COMPOSITION OF THREE TERMITOMYCES SPECIES, AGARICUS BISPORUS AND A HIGHLY NUTRITIVE REFERENCE PROTEIN.

| Amino acid | T.umkowaani %(N=16)[b] | T.umkowaani gmAA[c] gmN | T.sagittaeformis %(N=16) | T.sagittaeformis gmAA gmN | T.reticulatus %(N=16) | T.reticulatus gmAA gmN | A.bisporus %(N=16) | A.bisporus gmAA gmN | FAO reference protein[a] %(N=16) | FAO reference protein gmAA gmN |
|---|---|---|---|---|---|---|---|---|---|---|
| Essential: | | | | | | | | | | |
| Isoleucine | 3,44 | 0,215 | 6,51 | 0,406 | 0,73 | 0,045 | 5,06 | 0,316 | 4,00 | 0,250 |
| Leucine | 4,91 | 0,306 | 10,90 | 0,681 | 2,07 | 0,129 | 7,42 | 0,463 | 7,04 | 0,440 |
| Lysine | 5,54 | 0,346 | 6,60 | 0,412 | 3,16 | 0,197 | 6,22 | 0,388 | 5,44 | 0,340 |
| Met+ Cys | 8,17 | 0,510 | 5,08 | 0,317 | 1,16 | 0,072 | 2,16 | 0,135 | 3,52 | 0,220 |
| Phe+Tyr | 4,82 | 0,301 | 8,48 | 0,530 | 6,20 | 0,387 | 7,62 | 0,476 | 6,08 | 0,380 |
| Threonine | 4,22 | 0,263 | 7,23 | 0,451 | 3,82 | 0,238 | 7,92 | 0,495 | 4,00 | 0,250 |
| Tryptophane | 2,14 | 0,133 | 1,98 | 0,123 | 0,97 | 0,060 | 2,00 | 0,125 | 0,96 | 0,060 |
| Valine | 4,80 | 0,300 | 8,22 | 0,513 | 3,69 | 0,230 | 7,18 | 0,448 | 4,96 | 0,310 |
| Total essential amino acids: | | (43,76%) | | (42,82%) | | (39,07%) | | (30,51%) | | |
| | | 2,374 | | 3,433 | | 1,358 | | 2,846 | | 2,250 |
| Non-essential: | | | | | | | | | | |
| Aspartic acid | 5,74 | 0,358 | 9,57 | 0,598 | 5,18 | 0,136 | 16,96 | 1,060 | no data available | |
| Glutamic acid | 9,06 | 0,566 | 13,60 | 0,580 | 7,46 | 0,466 | 24,80 | 1,550 | | |
| Serine | 5,31 | 0,331 | 8,62 | 0,601 | 4,50 | 0,281 | 12,12 | 0,757 | | |
| Glycine | 9,69 | 0,605 | 13,28 | 0,830 | 6,38 | 0,398 | 13,20 | 0,825 | | |
| Histidine | 2,39 | 0,149 | 3,12 | 0,195 | 2,28 | 0,142 | 6,02 | 0,376 | | |
| Alanine | 6,72 | 0,420 | 11,36 | 0,710 | 5,79 | 0,361 | 13,52 | 0,845 | | |
| Arginine | 6,12 | 0,382 | 6,20 | 0,387 | 2,23 | 0,139 | 6,84 | 0,427 | | |
| Proline | 3,84 | 0,240 | 6,62 | 0,413 | 3,11 | 0,194 | 10,28 | 0,642 | | |
| Total amino acids: | | 4,782 | | 7,577 | | 3,343 | | 6,482 | | |

[a] Food and Agriculture Organisation (1970; 1973).
[b] Amino acid contents expressed on a nitrogen = 16 basis.
[c] Data presented as grams of amino acid per gram of corrected crude protein nitrogen.

TABLE 21. COMPARATIVE NUTRITIVE VALUE OF THREE TERMITOMYCES SPECIES and AGARICUS BISPORUS.

| Species: | Essential Amino Acid Index Limiting amino acids A.bisporus | | FAO | | Biological Value $BV_{A.bisporus}$ | $BV_{FAO}$ | Amino Acid Score (FAO) | Crude Protein (Nx4,38) | Nutritional Index A.bisporus | FAO |
|---|---|---|---|---|---|---|---|---|---|---|
| A.bisporus | --- | --- | $100^{127.2*}$ | sc | --- | 97,3 | 61,4 | 30,2 | --- | 30,2 |
| T.umkowaani | 87,9 | Thr,ar,Leu | $100^{112.2}$ | Leu,ar,Ile | 84,1 | 97,3 | 69,7 | 26,3 | 23,1 | 26,3 |
| T.sagittaeformis | $100^{123.2}$ | Thr,Trp | $100^{157,1}$ | none | 97,3 | 97,3 | 100> | 35,0 | 35,0 | 35,0 |
| T.reticulatus | 44,3 | Ile,Leu,Thr | 54,0 | Ile,Leu,sc | 36,7 | 47,1 | 18,2 | 25,4 | 11,2 | 13,7 |

| Essential Amino acid: | FAO ratio 1 | 2 | 3 | 4 | A. bisporus ratio 2 | 3 | 4 |
|---|---|---|---|---|---|---|---|
| Isoleucine | 126,4 | 86,0 | 162,7 | 18,2 | 68,0 | 128,4 | 14,2 |
| Leucine | 105,1 | 69,7 | 154,8 | 29,4 | 66,0 | 147,0 | 40,8 |
| Lysine | 114,0 | 101,8 | 121,3 | 58,0 | 89,1 | 106,1 | 50,7 |
| Met+Cys | 61,4 | 232,0 | 144,2 | 32,7 | 377,7 | 234,8 | 53,3 |
| Phe+Tyr | 125,1 | 79,2 | 139,4 | 101,9 | 63,2 | 111,3 | 81,2 |
| Threonine | 198,0 | 105,5 | 180,7 | 95,5 | 53,1 | 91,1 | 48,0 |
| Tryptophane | 208,3 | 221,7 | 205,0 | 100,0 | 106,4 | 98,4 | 48,0 |
| Valine | 144,7 | 96,7 | 165,7 | 74,3 | 66,9 | 114,5 | 51,3 |

*Total Essential Amino Acid Index value.
Crude protein content expressed on a dry mass basis.
The limiting amino acids are listed in order of their decreasing importance in each case with: sc, sulfur-containing amino acids (methionine + cysteine); ar, aromatic amino acids (phenylalanine + tyrosine).
Species tested: 1 = A.bisporus ; 2 = T.umkowaani ; 3 = T.sagittaeformis ; 4 = T.reticulatus.

is applied, *T. umkowaani* protein will attain a higher NI value of 26,3 in comparison with a lower NI value of 13,7 for *T. reticulatus* because of the lower EAA content of the latter. Only the three most limiting EAA are recorded for each mushroom in order of increasing magnitude (from the most limiting to the less limiting). No limiting EAA were recorded for *T. sagittaeformis* when compared with the FAO reference pattern because all the EAA of this mushroom exceed the EAA content of the reference pattern by a considerable margin. Oser (1959) suggested that all the EAA should be evaluated when a test protein is assessed for its nutritional quality and not only the most limiting EAA when compared to a reference protein (Amino Acid Score).

## 13. DISCUSSION

A few factors should be taken into consideration before the implications of the results are discussed. These are the variability of the crude protein content of mushrooms and the amino acid content which is in a constant state of flux. Maggioni, Passera, Renosto & Benetti (1968) established that specimens of *A. bisporus* contained a crude protein content that varied from 21 % to 35 % and even as high as 44 % depending on the specific strain used and the particular production phase under investigation. Composition of the substrate will influence the amino acid composition substantially (Le Roux & Danglot, 1972; Maggioni, *et al.*, 1968). According to Crisan & Sands (1978) the availability of each EAA may alter the nutritional value of mushroom protein drastically. Lasota, Mlodecki & Wlodarczyk (1968) established that only 10 % to 62 % of the lysine present in several mushrooms was available for utilization. This factor will reduce the calculated EAA Index, BV and NI of each mushroom. The *in vivo* availability of free and EAA both in quality and quality at the site of protein synthesis will even further complicate the evaluation of mushroom protein nutritional quality (Bano & Rajarathnam, 1982). Consequently the conditions under which this analysis were executed were standarised as much as possible in an effort to reduce these variables. This comprised the use of only one defined medium and identical incubation conditions. The variable factors mentioned above

necessitated the use of artificially grown mycelium and not fully mature fruit bodies for amino acid analysis. Fruit bodies of *Termitomyces* are extremely scarce and are produced under extremely variable environmental conditions in nature which will influence the amino acid composition and protein content substantially. Nevertheless the results in Tables 20 and 21 should give an indication of the nutritional value of mushroom protein contained by the four different mushrooms. Protein quality of *T. umkowaani* and *T. sagittaeformis* mycelium compares favourably with that of the FAO reference pattern and *A. bisporus* while the protein of *T. reticulatus* mycelium seems to be of a lesser nutritional quality. The observations are apparent when the different EAA Indexes, Biological Values, Amino Acid Scores and Nutritional Indexes are compared. Both *T. umkowaani* and *T. sagittaeformis* mycelium should supply all the necessary amino acids required by a normal adult and in sufficient quantities whereas the protein of *T. reticulatus* would only partially comply with the amino acid requirements of a normal adult due to the presence of four limiting amino acids, isoleucine, methionine, leucine and cysteine. An interesting observation which has emerged from these results is the exceptionally high nutritional value of *T. sagittaeformis* protein which exceed that of the other mushrooms. This mushroom has the smallest fruit bodies (Fig.35 c) in comparison with the huge fruit bodies of *T. umkowaani* and *T. reticulatus* (Fig.35 a,b). Whether the edibility of this species can compare with that of *T. umkowaani* and *T. reticulatus* is a matter of personal taste. A distinct quality of mycelial protein of *T. umkowaani* is the exceptionally high content of methionine and cysteine which exceeds that of egg protein by 67 %. The sulfur-containing amino acids are usually limiting in mushroom protein and the high methionine and cysteine content of *T. umkowaani* protein will at least equal the methionine and cysteine content of meat protein. Apart from the exceptional edibility of this mushroom the quality of the mycelium protein certainly warrants any attempt to cultivate this species. Although only a few articles have been published on the nutritional value of *Termitomyces* mycelium protein most of the published results indicate that the nutritional value of *Termitomyces* species in general is superior to that of the commonly cultivated mushrooms with respect

to protein quality, carbohydrate, fat, vitamin and mineral content (Ogundana & Fagade, 1981). Results listed in Tables 20 and 21 provide additional proof that some species of *Termitomyces* contain protein of high nutritional value.

The most comprehensive amino acid analysis of *Termitomyces* protein was executed by Crisan & Sands (1978), although they did not properly identify all the species investigated. They detected all the essential and non-essential amino acids in protein of five different species of *Termitomyces* and EAA Indexes, Biological Values and Nutritional Indexes were calculated for these species. They recorded EAA Indexes which ranged from 23,3 to 31,8 and crude protein content which ranged from 27,4 % to 33,0 %. Their measurements compare favourably with the results in Tables 20 and 21 and are well within the range of measurements recorded in Tables 20 and 21. Bano, Ahmed and Shrivastava (1964) determined the amino acid composition of a *Termitomyces* species and they detected seventeen amino acids of which ten were essential. High values were recorded for histidine and arginine. A similar analysis was performed by Purkayastha & Chandra (1975) on protein obtained from the sporocarps of *T. eurhizus* but as a result of poor analytical procedures they detected only ten amino acids. Mukiibi (1973) evaluated ten amino acids obtained from five *Termitomyces* spp. and calculated Biological Values for these mushrooms which ranged from 31 to 50. According to Mukiibi (1973) *Termitomyces* protein was inferior to cereal and legume protein which attained Biological Values between 55 and 73. However the calculated Biological Values are incorrect because he included non-essential amino acids in his calculation. EAA Indexes from which Biological Values can be derived were also not calculated. The results are therefore of little value. Ogundana & Fagade (1981) determined the nitrogen and crude protein content of *T. robustus* and *T. clypeatus* fruit bodies and obtained values of 5,83 % and 5,02 % for nitrogen and 36,4 % and 31,4 % for crude protein respectively using the conversion factor (N x 6,25). Chandra & Purkayastha (1976) conducted feeding experiments with laboratory mice and included dry mycelium powder in their diets. They recorded a significant gain in body mass of mice fed with the dried mycelium powder *T. eurhizus* in

**Fig. 19** (a - l). Growth habit of *Termitomyces* mycelium in pure culture. Fig.19(a). Culture of *T. umkowaani* illustrating the tough, raised cerebriform mycelium and the light and darker brown patches in the mat. Fig.19(b). Culture of *T. reticulatus* illustrating the soft, raised, creamy white stroma resembling callus growth. Fig.19(c). Culture of *T. sagittaeformis* illustrating the soft, raised, light brown, cerebriform mycelium. Fig.19(d). Culture of *T. clypeatus* illustrating the tough, raised, creamy white, cerebriform mycelium. Fig.19(e). Culture of *T. microcarpus* illustrating the cottony-woolly mycelium (See also Fig.22 g). Fig.19(f). Culture of *M. natalensis* comb sporodochia illustrating the creamy white farinaceous mycelium. Fig.19(g). Primary isolates from the stipe context of *T. umkowaani* fruit bodies after six weeks growth. Fig.19(h). Culture prepared from the previous culture (four weeks growth). Fig.19(i). Fungus comb of *Macrotermes natalensis* covered with white spherical sporodochia. Fig.19(j-l). Growth habit of *T. umkowaani* mycelium on a natural substrate, illustrating the development of tough cerebriform, mycelium.

comparison with mice fed on mycelium powder of four commonly cultivated mushrooms.

## B. MORPHOLOGY

### 1. Microscopic characters of sporodochia from the combs of:

#### 1.1 *Macrotermes natalensis*

**Thallic-arthric conidia:** conidia holoarthric, monilioid, thin-walled, smooth, ellipsoid-allantoid to broadly-cylindrical with truncate to slightly obtuse ends, and conidial chains repeatedly branch sympodially. Conidial chains develop from undifferentiated conidiogenous hyphae with the same diameter as the conidia. Deeply-staining granular protoplasm present but polar vacuoles not so well defined in young and mature conidia as in culture produced conidia, 12-25 x 7-10 µm. Holoarthric conidia are produced in basipetal succession. Conidia secede after separation of the transverse septa. Conidiogenous hyphae terminal and determinate and apical growth ceases before septation commences.

**Globular cysts:** thick-walled, inflated cells situated at the periphery of sporodochia consisting of (i) a basal obovate-ellipsoid cell with truncate ends, deeply-staining contents and a slightly thickened cell wall, 31-60 x 14-36 µm and (ii) an apical globose to subglobose cell with a thick wall (1.8-3.0 µm wide), and a yellowish granular protoplasm, 20-50 x 10-31 µm (Fig.20 a-h).

#### 1.2 *Odontotermes badius*

**Thallic-arthric conidia:** conidia holoarthric, monilioid, thin-walled, narrowly ellipsoid to broadly-cylindrical with truncate to slightly obtuse ends, conidial chains repeatedly branch sympodially. Conidial chains are borne on distal, broadly-fusiform conidiophore cells. Granular protoplasm of conidia stains deeply with one or two polar vacuoles present in older seceded conidia, 12-33 x 7-9 µm.

**Conidiophore cells:** conidiophores consist of aggregates of

repeatedly dichotomously branched, monilioid, thin-walled, inflated conidiophore cells which consist of deeply-staining, fusiform, pedicellate cells, 66-70 x 7-8 µm and thin-walled, broadly-fusiform primary and secondary cells with granular protoplasm and wrinkled (collapsed) cell walls. Rapidly growing conidiogenous hyphae stain deeply while conidiophore cells in mature sporodochia stain lightly, almost hyaline. Primary cells, 46-79 x 21-33 µm are attached to the pedicellate cells and the secondary cells, 25-37 x 13-19 µm bear the conidial chains.

**Globular cysts:** monilioid, thick-walled, obovate to subglobose inflated cells, 13-33 µm diam, and are situated at the periphery of the sporodochia, intermingled with conidiospores and seem to increase in length by budding of the most distal cell (Fig.21 a-h).

2. **Cultural description of:**

1 *Termitomyces* sp. (sporodochia from the comb of *M. natalensis* in culture)

**Growth characters.** Growth is slow, colony diameter reaching 15 mm in three weeks. Advancing zone bayed, appressed for 1-2 mm then raised. Mycelial mat coarse, granular due to the presence of numerous spherical, downy sporodochia (± 0.1-0.3 mm diam) covering entire surface of the mat. Mat is tough, raised (2-3 mm) forming a stroma,not readily separable from the agar. Sporodochial mycelium develops directly on the stroma and detaches readily. Mycelial mats white at first, changing to creamy white in older cultures with downy, farinaceous appearance Reverse unchanged. No growth on gallic and tannic acid. Small diffusion zone on gallic acid. Strong positive reaction with guaiacol reagent (Fig.19 f).

**Hyphal characters.**

**Advancing zone.** Hyphae thin-walled, branched with simple septa; deeply-staining granular protoplasm in young, rapidly growing hyphal tips (conidiogenous hyphae). Older hyphae translucent and hyaline, 3-5 µm wide.

**Hyphal characters.**

**Advancing zone.** Hyphae thin-walled, branched with simple septa; deeply-staining granular protoplasm in young, rapidly growing hyphal tips (conidiogenous hyphae). Older hyphae translucent and hyaline, 3-5 µm wide.

**Aerial mycelium.** (a) Hyphae as in the advancing zone, 3-10 µm wide.(b) Conidiophores and conidia: holoarthric conidia, monilioid, thin-walled, cylindrical to narrowly-ellipsoid, with truncate to slightly obtuse ends, 17-40 x 8-12 µm. Conidial chains with successive sympodial branches; conidia are produced in basipetal succession. Conidia secede after separation of the transverse septa. Young conidia stains deeply, with homogenous, granular protoplasm. In older conidia the granular protoplasm is condensed into a thin peripheral layer mainly at the cell poles and in a thin median transverse band. Usually two large polar vacuoles are situated on either side of the transverse band. Conidial chains develop from distinct long,narrow, undifferentiated, sympodially branched, conidiogenous hyphae with deeply-staining granular protoplasm. Conidiogenous hyphae are terminal and determinate. The rapidly growing conidiogenous hyphae (2-3 µm wide) enlarge enormously in width to form 8-12 µm wide cylindrical conidia which enlarge further after liberation. Conidiophores are loosely aggregated in the spherical sporodochia which cover the entire stroma surface and resemble comb sporodochia, but the conidiophores in the culture sporodochia are less compactly arranged and the culture sporodochia are much smaller.(c) Sphaerocysts: inflated, thin-walled, fully developed, ungerminated seceded conidia with obtuse ends. Cell contents, translucent, hyaline with a thin layer of granular protoplasm concentrated at the cell poles. Cell walls collapse upon the slightest contact and the sphaerocysts take on the appearance of empty cells with collapsed, wrinkled cell walls. Cell shape varies from globose to allantoid to broadly ellipsoid-ovoid, 17-70 x 13-40 µm. **Submerged mycelium.** Hyphae as in the

advancing zone, 2-5 μm wide (Fig.22 a-f ; Fig.23 a-e).

The most striking features of sporodochial cultures obtained from the combs of *M.natalensis* are the granular mycelial mat, the numerous white, spherical, downy sporodochia and the creamy white, farinaceous mat of older cultures.

2.2 *Termitomyces reticulatus* (Sporodochia from the combs of *O. badius* in culture)

**Growth characters.** Growth is slow, colony diameter reaching 10 mm after three weeks growth. Advancing zone bayed, raised right to the colony edge. Creamy white mycelial mat, stromatic, raised (5-6 mm), cerebriform, tuberculous, tough, not readily separable from the agar. Stroma covered by downy, farinaceous layer of creamy white, spherical sporodochia. Reverse unchanged. No growth on gallic and tannic acid media and no diffusion zones produced. Strong positive reaction with guaiacol reagent (Fig.19 b).

**Hyphal characters.**

**Advancing zone.** Hyphae thin-walled, branched with simple septa and deeply-staining distal hyphal segments (conidiogenous hyphae), 2-5 μm wide.

**Aerial mycelium.** (a) Hyphae as in advancing zone, 2-8 μm (b) Conidiophores, conidia and conidium ontogeny : identical to those described for *M. natalensis* comb sporodochia in culture with respect to the general shape of the conidiophores and conidium ontogeny. The two cultures differ though with respect to conidial shape which may be cylindrical or reniform, obovate, allantoid, broadly-ellipsoid or subglobose. Elongating tips of some conidiogenous hyphae are hooked (bent) and reniform conidia will form after delimitation of the hyphal tip. Conidiogenous hyphae which are slightly, undulated give rise to allantoid conidia after separation of the individual conidia. The shape of conidiogenous hyphae will

**Fig. 20** (a - h). Light micrographs of sporodochia from the comb of *Macrotermes natalensis*.
Fig.20(a). Young, developing conidiogenous hyphae (ch) at the periphery of the sporodochium. Fig.20(b). Sympodially branched conidiogenous hypha (sb) and the formation of the first apical conidium (ac). Fig.20(c). Fully developed conidiophores. Fig.20(d). Inflated, thick-walled cells at the periphery of the sporodochium. Fig.20(e,f). Thick-walled, subglobose, apical cell (ac) with granular cell contents and thick-walled, inflated basal cell (bc). Fig. 20(g). Developing holoarthric conidia illustrating conidiogenous hyphal segments of different lengths (scale bar = 20 $\mu$m).Fig.20(h). Young developing sporodochium (scale bar = 50 $\mu$m).

**Fig. 21** (a - h). Light micrographs of sporodochia from the comb of *Odontotermes badius*.
Fig.21(a - c). Young developing conidiogenous hyphae (ch) showing the sympodial branching pattern and the formation of transverse septa (ts). Fig.21(c,f). Monilioid, inflated, thick-walled cells (mc). Fig.21(d,f). Deeply-staining, pedicellate cells (pc). Fig.21(e,g). Fully developed conidiophores illustrating the huge, broadly-fusiform primary cells (pc) and the smaller secondary cells (sc) bearing conidial chains. Fig.21(h). Stem of fully developed pedicellate sporodochium (scale bar = 20 $\mu$m).

**Fig. 22** (a - f). Light micrographs illustrating conidiophore development of natural sporodochia in culture.
Fig.22(d). Young, deeply-staining conidiogenous hyphae in the advancing zone. Fig.22(a). Conidiogenous hypha delimited with a transverse septum (ts) and the inception of a new sympodially branched conidiogenous hypha (sb). Fig.22(b,c, & e). Delimitation of new holoarthric conidia from existing hyphae and the formation of new branches. Fig.22(f). Fully developed conidiophore with two to three conidia per chain. Fig.22(g,h). Aged, inflated conidia, illustrating the thin, polar, granular protoplasm layer (pl) and the huge collapsed conidia in the aerial mycelium (scale bar = 20 $\mu$m).

**Fig. 23** (a - e). Scanning electron micrographs illustrating conidiophore development of natural sporodochia in culture. Developing, sympodially branched (sb) conidiogenous hyphae (ch) and mature conidia (mc) (scale bar = 10 $\mu$m).

**Fig. 24** (a - g). Light micrographs illustrating germination mode of conidia.

Fig.24(a,b). Conidia produced by sporodochia on the combs of *Odontotermes badius* showing bipolar germination. Fig.24(c). Bipolar germination of a conidium in culture situated near an inflated, aged conidium or sphaerocyst (sc). Fig.24(d,e). Germination by means of a single polar germ-tube of a conidium with a thin, peripheral, granular protoplasm layer (gp). Fig.24(f). Bipolar germination of conidia illustrating the emergence of numerous new branches from developing germ-tubes. Fig.24(g). Conidium germinating with four germ-tubes of which three is slightly subpolar. Note the vacuolate appearance of the germ-tubes (scale bar = 20 $\mu$m).

therefore determine the shape of the delimited, liberated conidia which are 12-38 x 8-13 µm.(c) Sphaerocysts : identical in shape to those described for *M. natalensis* comb sporodochia in culture except more numerous, larger, 16-93 x 14-44 µm and may still occur in a chain.

**Submerged mycelium.** Hyphae as in advancing zone, may contain scattered ungerminated conidia (Fig.27 a-h ; Fig.28 a-h)

Marked morphological features of *T. reticulatus* comb sporodochial cultures are the creamy white, cerebriform, tuberculous, raised mycelium that resemble plant tissue callus growth and the white farinose sporodochial layer on the stroma.

## 3. Germination studies (conidia)

Conidiophores develop from mycelium which originates from germinating conidia. Conidia produced in culture and in the comb sporodochia germinate readily in the semi-solid medium after eighteen to twenty-four hours incubation and conidiophores required five to seven days to develop fully. The growing mycelium ramifies and branches extensively to form a dense stroma. Before liberation conidia germinate with a single polar or subpolar germ-tube, and liberated conidia germinate with one to four germ-tubes which emerge polar and or subpolar, usually two germ-tubes per pole. The distinct protoplasm pattern of old liberated conidia extends into the elongating germ-tubes and the ramifying, branched vegetative hyphae. Conidia produced in culture and on the comb do not require any supplementary nutrients to induce germination because conidial germination was possible in a semi-solid water agar medium (Fig.24 a-g).

## 4. General discussion and conclusions

Few but distinct differences exist with respect to macro and microscopic characters of sporodochia from the combs of

*Macrotermes natalensis* and *Odontotermes badius*. The following microscopic characters are similar : (i) conidial size (ii) branching pattern of conidial chains (conidiogenous hyphae) (iii) conidium ontogeny and (iv) the presence of large, inflated globular cysts with thickened refractive walls that are situated at the periphery of sporodochia. Comb sporodochia differ with respect to : (i) size and shape of the conidiophore hyphae (cells) and (ii) the particular arrangement of the globular cysts at the periphery of the sporodochia. Therefore differences between the sporodochia from the combs of *M. natalensis* and *O. badius* are more conspicuous than differences between the respective cultures.

Descriptions of *Termitomyces* comb sporodochia, obtained from the combs of different termite species of the subfamily Macrotermitinae throughout the Paleotropical Region, seem to be generally similar although not identical. Descriptions and observations by various authors over a long period of time lack precise and detailed information as well as proper illustrations of the investigated material. It is therefore difficult to compare descriptions. Only cultural descriptions recorded by Batra & Batra (1966,1979) of sporodochia isolated from the combs of *Odontotermes obesus* (Rambur) and *O. gurdaspurensis* (Holm. & Holm.) in India and those of Heim (1977) from Africa contained photographic illustrations. Batra & Batra (1979) also investigated the morphology of natural sporodochia isolated from the combs of *T. albuminosus* (Berk.) Heim. Their observations and measurements with regard to microscopic characters conform completely with my description of sporodochia from the combs of *O. badius*. However they did not observe the germination of natural conidia nor did they describe conidium ontogeny. Batra & Batra's (1966,1979) description as well as those of other authors (Cheo 1942,1948; Petch, 1913) indicate that the *O. badius* type of sporodochia seem to be associated with most species of *Termitomyces* worldwide and not only with *T. reticulatus* and *T. albuminosus*. Due to the lack of fruit bodies at the collection site

it is not known at present which species of *Termitomyces* is associated with the *M. natalensis* type comb sporodochia. Heim (1977) conducted an elaborate investigation of the culture characteristics of comb sporodochia of several African species of *Termitomyces* in particular those of *T. striatus*. He recorded the presence of sphaerocysts, conidia and globular cysts in his cultures and he described the method of germination displayed by conidia in culture, which is completely in accord with my observations except for the globular cysts which I have not observed in my cultures. Heim (1977) stated that he observed the initial stages of globular cyst germination in old cultures and that these cells may be restricted to a purely vegetative role rather than a reproductive one. The large, inflated, cytoplasm-rich globular cysts and the large thin-walled conidiophore cells of the *O. badius* type comb sporodochia may serve as a source of nutrition for feeding worker termites because of the high nutritive value of comb sporodochia (Matsumoto, 1976; Mishra & Sen-Sarma, 1986; Rohrmann, 1978; Rohrmann & Rossman, 1980). Batra (1975) recorded the consumption of sporodochia by workers and nymphs of fungus-growing termites. Thomas (1985) prepared conidial suspensions of *Termitomyces* sporodochia in sterile distilled water which were then plated onto nutrient media. She formulated a selective medium which would favour the germination of *Termitomyces* conidia. She observed conidial germination only macroscopically and subsequently no microscopic details regarding conidial germination or hyphal characters were recorded. Thomas (1987 a,b) repeatedly isolated colonies of *Termitomyces* from dilution plates taken from comb material and various samples of termite manipulated organic material. However no details with regard to growth characters or the nature of the hyphal elements of the colonies were recorded. Both Petch (1913) and Cheo (1942,1948) described micro and macroscopic characters of comb sporodochia from termitaria in Ceylon and China. Their descriptions are similar to those of Batra & Batra (1979) and Heim

(1977). Petch (1913) also observed monilioid, globular cysts on the sporodochial periphery which repeatedly branch dichotomously and elongate by budding of the apical cell. He recorded the presence of chains of oval cells (conidia) which germinated in water or nutrient broth, but the globular cells did not. According to Petch (1913) the chains of conidia produced new branches immediately below each septum, confirming the sympodial branching pattern. The sporodochia of *Termitomyces* have been described by various authors under different names. Savage (1850) considered it a *Trichia* while Berkely (1882) named it *Aegerita duthei* (Berk.) and Cheo (1942) preferred the name *Termitosphaeria duthei* (Berk.) Ciferri. Jumelle & Perrier de la Bathie (1910) thought that *A. duthei* was the anamorph of *Xylaria nigripes* Klotzsch. However Heim (1977) clarified the matter and proved conclusively that sporodochia on the combs of Macrotermitinae represent the anamorph of the genus *Termitomyces*. Bathellier (1927) and Heim (1977) observed the emergence of numerous pseudorhizal primordia from sporodochia on combs of different termite species which developed into fruit bodies. This evidence as well as the microscopical cultural similarity of sporodochial cultures as reported by Heim (1977) and Batra & Batra (1979) for African and Indian species, prove that the comb sporodochia represent the anamorph of *Termitomyces* and not a different fungus.

This study represents only a preliminary investigation of the macro and microscopic characters of comb and culture sporodochia of the genus *Termitomyces* in South Africa. Many more isolates of sporodochia would have to be made from the combs of all the fungus-growing termites in South Africa in order to ascertain whether other types of comb sporodochia exist apart from the *M. natalensis* and *O. badius* type. The *O.badius* type seems to be universally associated with most species of *Termitomyces*. The associated fruit bodies, together with the specific termite host must be collected and properly identified, before further deductions are possible.

Basidiome context cultures of fruit bodies would prove to be essential for comparison with sporodochial cultures. The aquisition of more isolates of comb sporodochia was hampered by (i) lack of fruit bodies which serve as a guide to the subterranean fungus combs (ii) numerous newly constructed combs which did not contain any sporodochia, or (iii) combs which have been cleared of all sporodochia by the feeding worker termites.

## 5. Description of basidiome context cultures of :

### 5.1 *Termitomyces umkowaani*

**Growth characters.** Growth is slow, colony diameter ± 15 mm after three weeks growth. Advancing zone bayed, farinaceous, appressed for 3-5 mm then raised. Mycelial mat forming a stroma, velvety, 'cream colour' to 'buff yellow', tough, cerebriform, raised, (4-5 mm), not readily separable from the agar, covered with numerous, white, spherical, downy sporodochia. The mat of aged cultures (four weeks growth), darkens to 'buckthorn brown' and 'wood brown' and become crustose. Reverse unchanged. Distinct mushroomy, sharp pepper-like odour is emitted by the mycelium. Moderately strong diffusion zones are produced on gallic and tannic acid agar but no growth. Guaiacol test positive (Figs 19 a,g,h ; 34 c,d).

**Hyphal characters.**

**Advancing zone.** Hyphae thin-walled, sympodially branched with simple septa; conidiogenous hyphae with deeply-staining granular protoplasm; older hyphae translucent, hyaline, 3-8 µm wide.

**Aerial mycelium.** (a) Hyphae as in advancing zone, 3-6 µm wide.(b) Conidiophores and conidia : ontogeny, morphology and germination as described in par.2.1 and par.3, except conidia slightly larger, 18-74 x 11-20 µm. Conidial shape may be cylindrical or reniform, obovate, allantoid, broadly-ellipsoid or

subglobose. As in the cultures of *O. badius* comb sporodochia the elongating tips of some conidiogenous hyphae are hooked (bent) and will form reniform conidia after the delimitation of the hyphal tip. Conidiogenous hyphae which are undulated give rise to allantoid conidia after separation of the transverse septa.(c) Sphaerocysts : the origin and morphology as described in par.2.1, 21-70 x 14-37 µm.

**Submerged mycelium.** (a) Hyphae as in advancing zone, 6-8 µm wide.(b) Large, ungerminated conidia scattered throughout the agar, of variable shape either single or monilioid with large vacuoles and thin peripheral, granular protoplasm (Fig.25 a-g; Fig.26 a-e).

The sharp, pepper-like mushroomy odour emitted by the mycelium the presence of ungerminated, vacuolate conidia in the submerged mycelium and the stroma with patches of dark brown, crustose mycelium are characteristic of *T. umkowaani* cultures.

## 5.2 *Termitomyces reticulatus*

**Growth characters.** Growth is slow, colony diameter ± 15 mm after three weeks growth. Advancing zone bayed, raised right to the limit of growth. Mycelial mat a stroma, creamy white at first changing to 'buff yellow' and is raised, (5-7 mm), moderate soft, cerebriform, tuberculous not readily separable from the agar. Stroma, farinaceous, granular covered with numerous globose, creamy white (± 0.1-0.3 mm diam) sporodochia. Reverse unchanged. No growth on gallic and tannic acid and no diffusion zones are produced. Guaiacol test positive (Fig.19 b, 34 a,b).

**Hyphal characters.**

**Advancing zone.** Hyphae thin-walled, sympodially branched with simple septa; conidiogenous hyphae, deeply-staining, 2-5 µm wide.

**Aerial mycelium.** (a) Hyphae as in advancing zone, 2-8 µm wide.(b) Conidiophores and conidia : morphology and ontogeny as

described in par. 2.1 , 12-38 x 8-13 µm. (c) Sphaerocysts : identical in shape to those described in par. 2.1 except larger and more numerous, 16-93 x 14-44 µm.

**Submerged mycelium.** Hyphae as in advancing zone, may contain ungerminated, scattered conidia (Fig.27 a-h ; Fig.28 a-h ; Fig.29 a-f).

Marked morphological features of *T. reticulatus* cultures are the creamy white, moderate soft, tuberculous, cerebriform, raised stroma, the white farinaceous sporodochial layer on the stroma surface and the numerous, large, monilioid sphaerocysts in the aerial mycelium.

### 5.3 *Termitomyces sagittaeformis*

**Growth characters.** Growth is slow, colony diameter ± 15 mm after three weeks growth. Advancing zone bayed, appressed for ± 2 mm then raised. Mycelial mat a smooth, velvety stroma, 'buffy brown' to 'cinnamon buff', raised (± 3 mm), cerebriform, soft and separate readily from the agar. Stroma covered with a white, farinaceous sporodochial layer. First ± 5 mm of advancing zone, creamy white, farinaceous and the rest of the stroma change to 'cinnamon buff'. Reverse unchanged. Distinct, sharp mushroomy odour is emitted by the culture. No growth on gallic and tannic acid with no diffusion zones. Guaiacol test negative (Fig.19 c).

**Hyphal characters.**

**Advancing zone.** Hyphae thin-walled, sympodially branched with simple septa; deeply-staining conidiogenous hyphae, older hyphae hyaline, 2-5 µm wide.

**Aerial mycelium.** (a) Hyphae as in advancing zone, 3-7 µm wide.(b) Conidiophores and conidia : morphology and ontogeny as described in par. 2.1, 18-37 x 10-22 µm. (c) Sphaerocysts: identical in shape to those described in par.2.1, 19-80 x 11-34 µm.

**Submerged mycelium.** Hyphae as in advancing zone (Fig.30 a-f; Fig.31 a-h).

Characteristic features of *T. sagittaeformis* cultures are the soft,'cinnamon buff', cerebriform stroma and the creamy white, farinaceous advancing zone.

## 5.4 *Termitomyces clypeatus*

**Growth characters.** Growth is slow, colony diameter ± 12 mm after three weeks growth. Advancing zone bayed, mat is raised right to the colony edge. Hyphae in the first 2-5 mm of the advancing zone are slightly fimbriate (developing synnemata). Mycelial mat a smooth, velvety stroma, creamy white with patches of tough, raised (± 3 mm), cerebriform mycelium, not readily separable from the agar. Numerous white synnematous structures develop in patches on the stroma. Reverse unchanged. Growth on tannic and gallic acid is limited with no diffusion zones. Guaiacol reaction negative (Fig.19 d ; Fig.34 e,f).

**Hyphal characters**

**Advancing zone.** Hyphae thin-walled, sympodially branched with simple septa; deeply-staining conidiogenous hyphae; older hyphae hyaline, 3-7 µm wide.

**Aerial mycelium.** (a) Hyphae as in advancing zone.(b). Conidiophores and conidia : morphology and ontogeny as described in par. 2.1 with the addition that conidiophores are closely compacted and are situated in, distinct, erect, mycelial tufts that resemble synnemata. Conidia, 18-36 x 16- 29 µm. (c) Sphaerocysts : identical in shape to those described in par. 2.1 , 17-32 x 16-29 µm.

**Submerged mycelium.** Hyphae as in advancing zone, 2-6 µm wide (Fig.32 a-h ; Fig.33 a-h).

*T. clypeatus* cultures are characterized by the tough, creamy white, raised, cerebriform stroma covered with patches of erect,

**Fig. 25** (a - g). Light micrographs illustrating conidiophore development in the cultures of *T. umkowaani*.
Fig.25(a,b). Developing, deeply-staining, conidiogenous hyphae (ch) illustrating the typical sympodial branching (sb) pattern. Fig.25(c - e). Delimitation of conidiogenous hyphal segments by the formation of transverse septa (st) and enlargement of conidiophore hyphae and conidia. Fig.25(f,g). Fully developed dentritic conidiophores and conidia with polar vacuoles (pv) (scale bar = 20 $\mu$m).

**Fig. 26** (a - e). Scanning electron micrographs illustrating conidiophore development in the cultures of *T. umkowaani*.
Fig.26(a,b). Young conidiogenous hyphae in the advancing zone followed by the formation of a transverse septum (ts) to delimit an apical conidium (ac). Fig.26(c - e). Developing conidiophores and conidia showing the sympodial branching pattern and mature conidia (mc) (scale bar = 10 $\mu$m).

**Fig. 27** (a - h). Light micrographs illustrating conidiophore development in the cultures of *T. reticulatus*.
Fig.27(a). Newly formed conidiogenous hypha (ch) at the tip of rapid growing vegetative hyphae and a young sympodial branch (sb) developing just behind a septum. Fig.27(b). Substantial enlargement of a conidiogenous hypha and the delimitation of a new conidium with polar vacuoles (pv). Fig.27 (c). Delimitation of a new apical conidium (ac) from a conidiogenous hypha and the formation of a new conidium from a sympodial branch (sb). Fig.27(d). Fully developed conidiophore illustrating three mature conidia (a,b and c) in the main axis with one conidium situated sideways which developed from a sympodial branch (d). Lower set of three conidia developed in the same way with the two conidia situated in the same axis delimited from an unbranched conidiogenous hypha, followed by substantial enlargement of the newly produced conidia. Fig.27(e - g). Deeply-staining conidiogenous hyphal segments delimited by transverse septa followed by enlargement of conidia and conidiophore hyphae. Fig.27(g). Fully developed dentritic conidiophore. Fig.27(h). Numerous germinating conidia illustrating the typical sympodial branching pattern of the growing germ-tubes (gt) (scale bar = 20 $\mu$m).

**Fig. 28** (a - h). Scanning electron micrographs illustrating conidiophore development in the cultures of *T. reticulatus*. Fig.28(a). Young conidiogenous hyphae growing at the limit of the advancing zone, producing bent sympodial branches (sb). Fig.28(b). Enlargement of newly produced conidia after the formation of transverse septa (ts). Fig.28(c). Creeping conidiogenous hyphae on the agar surface showing the formation of transverse septa (ts). Fig.28(d,e,f). Illustrating mature conidia (mc) and bent conidiogenous hyphae (ch). Fig.28(g). Conidiophores nearing completion and Fig. 28(h), a typical dendritic conidiophore (scale bar = 10 $\mu$m).

**Fig. 29** (a - f). Transmission electron micrographs, illustrating differentiation of conidial wall layers of adjacent, seceding, holoarthric conidia in the cultures of *T. reticulatus*. Fig. 29 (a). Formation of inner wall (iw) and outer wall (ow) layers. Fig. 29 (b,c). Rupturing outer wall layers (arrows) before liberation of conidia, dolipore (dp), lipid droplet (ld), vacuole (v). Fig. 29 (d). Formation of sympodial branch (sb) and differentiation of conidial wall layers, nuclei (n). Fig. 29 (e). Delimitation of an apical conidium (ap) and the formation of vacuoles (v) (scale bar = 1 $\mu$m). Fig. 29 (f). Thin section through a young, developing dolipore with typical septal swellings (scale bar = 500 nm).

**Fig. 30** (a - f). Light micrographs illustrating conidiophore development in the cultures of *T. sagittaeformis*.
Fig.30(a). Deeply-staining conidiogenous hypha with two sympodial branches (sb) and a polar vacuole (pv). Fig.30(b,c,d). Developing conidiophores illustrating conidiogenous hyphae (ch), the formation of transverse septa (ts), conidia with polar vacuoles (pv) and the median transverse protoplasm band (md). Fig.30(e,f). Fully developed conidiophores (scale bar = 20 $\mu$m).

**Fig. 31** (a - h). Scanning electron micrographs illustrating conidiophore development in the cultures of *T. sagittaeformis*.
Fig.31(a). Young, developing conidiogenous hyphae (ch) in the advancing zone. Fig.31(b - d,f). Closely compacted conidiogenous hyphae growing in and on the agar surface in the advancing zone, illustrating the formation of transverse septa (ts) in the conidiogenous hyphae, the development of new conidial chains and sympodial branches (sb). Fig.31(e). Emergence of a germ-tube (gt) from a germinating conidium before liberation of conidia. Fig.31(g,h). Fully developed conidial chain illustrating inflated, ellipsoid-ovoid conidia (ic) and a fully developed, inflated, liberated conidium (lc) (scale bar = 10 $\mu$m).

**Fig. 32** (a - h). Light micrographs illustrating conidium development in the cultures of *T. clypeatus*.
Fig.32(a - d,f). Developing, deeply-staining conidiogenous hyphae (ch) congregated into synnematous structures, followed by the formation of transverse septa (ts) and conidia. Fig.32(e). Monilioid sphaerocysts (aged ,inflated conidia) in the aerial mycelium. Fig.32(g,h). Fully developed conidiophores bearing cylindrical, broadly-ellipsoid, reniform conidia with polar vacuoles and a median transverse band (scale bar = 20 $\mu$m).

**Fig. 33** (a - h). Scanning electron micrographs illustrating conidiophore development in the cultures of *T. clypeatus*.
Fig.33(a). Developing, conidiogenous hyphae in the advancing zone with sympodial branches (sb). Fig.33(b - h). Illustrating different stages of conidiophore development with the formation of transverse septa (ts), sympodial branches (sb) and possible secondary holoblastic conidial chains (cc) (scale bar = 10 $\mu$m).

**Fig. 34** (a - f). Tessovar photomacrographs of cultures illustrating macromorphological characters.
Fig.34(a,b). Raised, cerebriform, soft, smooth, stromatic mycelium (st) of *T. reticulatus* covered by numerous, small, farinose sporodochia (sp) which develop directly on the raised cerebriform stroma. Fig.34(c). Raised, cerebriform, tough stroma of *T. umkowaani*. Fig.34(d). Farinaceous sporodochial mycelium growing on the stroma. Fig.34(e). Raised, cerebriform, tough stroma of *T. clypeatus* overlaid with a farinaceous layer of conidiophore producing mycelium. Fig.34(f). Short, erect synnematous structures (sy) containing congregated conidiophores, growing on the stroma (scale bar = 2 mm).

synnematous structures.

### 5.5 *Termitomyces microcarpus*

**Growth characters.** Growth is rapid, colony reaching a diameter of 50 mm in five days. Advancing zone even, appressed for 5-8 mm then raised with advancing hyphal strands conspicuous and fimbriate. Aerial mycelium cottony-woolly, raised 8-10 mm touching Petri dish lid, creamy white to 'buffy brown'. Reverse unchanged. Rapid growth on gallic and tannic acid with the formation of prominent, yellow hyphal strands on acid media. Strong diffusion zones are produced on gallic acid. Guaiacol reaction intense (Fig.19 e ; Fig.40 g).

**Hyphal characters.**

**Advancing zone.** Hyphae thin-walled with simple septa; deeply-staining hyphal tips. Hyphae seldomly branch but frequently develop into hyphal strands, 4-7 $\mu$m wide.

**Aerial mycelium.** Hyphae as in advancing zone. **Submerged mycelium.** Hyphae as in advancing zone.

Striking morphological features of the cultures are the cottony-woolly, creamy white to 'buffy brown' rapidly growing colonies with abundant aerial mycelium and well defined hyphal strands. The absence of conidiophores, conidia and sphaerocysts, distinguishes *T. microcarpus* from the other species of *Termitomyces*.

## 6. General discussion and conclusions

It is difficult to distinguish between cultures of different *Termitomyces* species by relying strictly on microscopic characters. The microscopic characters of the cultures investigated are almost identical. The only differences are reflected in the size,shape and distribution pattern of some hyphal elements for example : (a) slightly larger conidia of *T. umkowaani* (b) more pronounced

cylindrical conidia of cultures prepared from comb sporodochia of *M. natalensis* (c) large, numerous monilioid sphaerocysts in the aerial mycelium of *T. reticulatus* (d) and the conidiophores of *T. clypeatus* which are aggregated into synnematous structures. With the exception of *T. microcarpus* all the species produce conidiophores and holoarthric conidia which are loosely situated in either spherical sporodochia or synnematous structures. The different hyphal elements develop from germinating and enlarging conidia and are densely compacted to form a raised, cerebriform stroma. This stroma is covered with a sporodochial layer. Newly-produced conidia have a deeply-staining granular protoplasm and the conidial shape may vary from cylindrical (young conidia) to subglobose (aged conidia). The variable conidial length may be ascribed to holoarthric ontogeny and undulated conidiogenous hyphae with bent apices. Conidial chains usually comprise two to five conidia. Sympodially branched conidiogenous hyphae frequently may terminate further development at an early stage. Each branch develops into a single, subglobose conidium, a type which is frequently encountered. This method of development may appear to be blastic but is only the result of a single arthrospore delimited at an early stage of the growth of the conidiogenous hypha. In mature conidia the granular protoplasm is reduced to a thin peripheral layer at the cell poles and a transverse median band with two polar vacuoles. Cole & Samson (1979) recorded the occurrence of both holoarthric and holoblastic conidia in the cultures of two yeasts, *Trichosporon beigelii* (Küch. & Rabenh.) Vuill. and *Trichosporonoides oedocephalis* Hask. & Spencer where holoarthric conidia enlarged to produce secondary holoblastic conidia. A similar enlargement of holoarthric conidia occurred in cultures of *Termitomyces* and may indicate transformation of holoarthric to holoblastic conidia (Fig.31 g,h ; Fig.33 c,g,h), or normal enlargement of holoarthric conidia to form globose, allantoid, sphaerocysts as originally suggested. Transmission electron micrographs do not indicate transformation from holoarthric to holoblastic conidia (Fig.29 a-f).

It is evident that the mode of conidiogenesis and germination and the basic morphology of the conidiophores and conidia, as well as the branching pattern of the conidiogenous hyphae, conidial chains and conidiophores, are identical for all the species studied in this investigation. Fully developed conidiophores are frequently dendritic as a result of the sympodial branching pattern. This observation with regard to cultural similarity is in agreement with the observations recorded by Heim (1977) and Piearce (1987) for species in Africa and of Petch (1913) and Batra & Batra (1979) for the species in India. The illustration of Heim (1977) of the hyphal elements in the cultures of *T. mammiformis*, is similar to the conidiophores and conidial chains which I have observed in the cultures. This illustration is the only record available at present of the hyphal characters of cultures prepared from the context of fruit bodies. With the exception of *T. microcarpus*, all the species produce conidiophores and conidia in pure culture which are indistinguishable from conidiophores in the cultures of comb sporodochia. These cultural studies indicate that the production of conidiophores and conidia are not restricted to sporodochia either on the comb or in culture. Cultures prepared from the context tissue of fruit bodies are also capable of producing conidiophores and conidia similar to those present in sporodochia. It may therefore be suggested that mycelium of *Termitomyces*, whether taken from sporodochia on the comb or from the context of fruit bodies, contain the necessary genetic information to produce a well developed anamorph. These studies indicate that an intimate relation exists between the perfect and imperfect phases in the genus *Termitomyces* and that it may be possible to stimulate basidiome context cultures to revert to the perfect phase. Cultures prepared from the comb sporodochia of *Odontotermes badius* and basidiome context of *T. reticulatus* fruit bodies, are macro and microscopically identical. Both sporodochia and fruit bodies were collected simultaneously from the same comb and must therefore be genetically identical. This important observation will confirm my statement

that the anamorph and teleomorph of *Termitomyces* are genetically closely related if not identical. Transformation of one phase to the other occurs readily and without difficulty. In the natural environment of the termitarium this transformation is from imperfect to perfect phase (comb sporodochia to fruit bodies) and on nutrient media with context tissue from fruit bodies, the transformation is from perfect to imperfect phase. The anamorph will always develop in culture whether sporodochial or fruit body context mycelium is used. It would of course prove much more difficult to effect transformation from imperfect to perfect phase under laboratory conditions. The hyphal characters of *Termitomyces* cultures seem to be similar to other basidiomycetes where the anamorph is known e.g. *Arthroderma* Currey, *Gymnoascus* Baran. and *Peniophora gigantea* (Fr.) Mass. which display holoarthric ontogeny (Cole & Samson, 1979).

Despite the microscopical cultural similarity of *Termitomyces* species, growth characters of basidiome context cultures differ markedly and it is possible to distinguish between the cultures of different species. This may be accomplished by comparing growth characters such as mat texture, colour and odour. These characters remain unchanged when compared on the same medium and under identical incubation conditions. For example cultures of *T. umkowaani* emit a sharp pepper-like, mushroomy odour and patches of the stroma may become crustose and dark brown. Cultures of *T. reticulatus* have a creamy white, raised, cerebriform, tuberculous stroma which is covered with a thick farinose sporodochial layer. The soft, cinnamon brown cerebriform stroma of *T. sagittaeformis* is characteristic of this species while the striking features of *T. clypeatus* cultures are the tough, creamy white, cerebriform stroma with patches of erect synnematous structures. Cultures of *T. microcarpus* form a cottony-woolly mat with well defined hyphal strands. Cultures of *M. natalensis* type sporodochia have a creamy white, farinaceous stroma which is not cerebriform and is covered

**Fig. 35** (a - g). Macromarphology of *Termitomyces* basidiocarps. Fig.35(a). *T. umkowaani* ; Fig.35(b). *T. reticulatus* ; Fig.35(c). *T. sagittaeformis* ; Fig. 35(d). *T. clypeatus* ; Fig.35(e). *T. microcarpus* ; Fig.35(f). *T. schimperi* ; Fig.35(g). *T. striatus* (scale bar = 1cm).

by a thick layer of sporodochial mycelium.

7. **Description of fruit body morphology :**

7.1 **Termitomyces umkowaani** (Cooke & Mass.) Reid, *Contributions from the Bolus Herbarium* no. **7**: 163-164 (1975).
*Agaricus umkowaani* Cooke & Mass., *Grevillea* **17**: 70 (1889).
*Schulzeria umkowaani* (Cooke & Mass.) Sacc., *Syll. Fung.***9**: 11 (1901).

**Pileus** 8-22 cm diam, hemispherical at first then expanding to convex or conico-campanulate, at maturity almost plane with a slightly pointed to broadly umbonate perforatorium. **Surface** grayish to umber-brown changing to ochraceous or brownish buff, smooth at first then becoming radially striate to striate at the margin, viscid when moist, cuticle peeling readily to perforatorium, cracking radially and concentrically at maturity. **Margin** irregular to slightly undulate, remaining incurved, splitting radially and finely striate. **Lamellae** free, crowded, with lamellulae, pale cream to brownish pink when mature, thin, up to 12 mm broad, margins smooth to slightly uneven or unevenly sinuate. **Stipe** central,70-150 mm above ground level, creamy white, smooth, lacking annulus, attenuated towards pileus, 10-20 mm diam, expanding below ground level to 20-30 mm then attenuate to form an elongate pseudorhiza, 6-10 mm diam, 30-50 cm long below ground level which terminates in a termite nest; hypogeal section of stipe with dark grayish longitudinally fibrous striae which become closely appressed in the narrow pseudorhiza with a blackish-grey appearance, texture fibrous, tough, solid and is hollowed out by termites in older specimens. **Context** uniform, white, thin, 1-6 mm over the lamellae to 20 mm under the perforatorium, of firmly interwoven uninflated, thin-walled, nonamyloid, hyaline, ramose, septate hyphae, lacking clamp connections, 3-22 $\mu$m diam, aseptate lactiferous hyphae with deeply staining, vitreous contents, 7-9 $\mu$m diam . **Spore print** brownish pink. **Spores** 6-11 x 4-6 $\mu$m, smooth, broadly ovoid-

ellipsoid, hyaline, thin-walled to slightly thickened, nonamyloid, obliquely apiculate. **Basidia** 22-34 x 8-11 µm, clavate, bearing 4 sterigmata. **Lamellar-edge** heteromorphous. **Cheilocystidia** 22-64 x 12-43 µm, scattered pedicellate, thin-walled, hyaline, broadly clavate to pyriform. **Pleurocystidia** similar to cheilocystidia, few, scattered. **Hymenophoral trama** regular, consisting of 3.5-5 µm wide, uninflated, thin-walled, hyaline, parallel hyphae devoid of clamp-connections at the septa, with lactiferous ducts, 4-6 µm. **Subhymenial layer** thin. **Pileal surface** an epicutis consisting of uninflated, unbranched, narrow, hyaline, septate hyphae, 3-5 µm diam. Epicutis separated from the context by a hypodermial layer of radially parallel hyphae containing greatly inflated, hyaline to pale brownish doliform cells, 25 x 27-37 µm diam, lacking clamps at the constricted septa. **Hypogeal stipe surface** a cuticle consisting of hyphae with doliform cells resembling those on the pileal surface, interwoven with parallel strands of narrow, uninflated, thin-walled hyphae, 3.7-5.6 µm diam, with pale brownish walls and brown contents. **Caulocystidia** on black cortical layer of stipe, 18-30 x 10-16 µm, pyriform to cylindrical, pedicellate, with brown, slightly thickened walls and brown contents (Fig.36 a-i).

Basidiocarps emerge either as solitary specimens or dispersed on the ground over or in the vicinity of the nests of *Odontotermes badius* (Hav). and usually appear during the rainy season from approximately mid-November to mid-February. Specimens have been collected at the end of the rainy season in April and even as late as August from the Piet Retief district in south-east Transvaal. Collection records indicate the prevalence of *T. umkowaani* in the Pretoria and Johannesburg district but can also be found in eastern and south-eastern Transvaal, Kwa Zulu and northern Natal (Fig. 34 a).

**Specimens examined :** on termite nest, Arcadia Park, Pretoria, Nov.1953, PREM 40715; on termite nest, in grassland, Wakkerstroom, Transvaal, Dec. 1964, PREM 42699; on termite nest, Pretoria, Jan. 1965, PREM 42987; on termite nest, Bronkhorstspruit Road, Pretoria,

March 1966, PREM 43147; on ground, Lynnwood Manor, Nov. 1983, PREM 47309; on ground Brummeria, Pretoria, Dec. 1983, PREM 47353; on ground among grass, Donkerhoek, Pretoria district, Nov. 1983, PREM 47359; on ground, Sunnyside, Pretoria, 1958, PREM 49059; on termite nest of *Odontotermes badius* University of Pretoria, Pretoria, Nov. 1986, PRUM 2254; on termite nest of *O. badius* in sugar-cane field, Eshowe, Natal, Dec. 1986, PRUM 2407; on termite nest, Kiepersol, Transvaal, Feb. 1988, PRUM 2408; on termite nest, Derdepoort, Transvaal, Jan. 1989, PRUM 2643; on termite nest, Rustenburg, Transvaal, Feb. 1989, PRUM 2685; in soil over termitarium, Hoedspruit, Transvaal, March 1989, PRUM 2704; on lawn in contact with termite nest of *O. badius*, University of Pretoria, Pretoria, March 1989, PRUM 2705.

Reid (1975) conducted a detailed examination of the type material of *Agaricus umkowaani* Cooke & Mass. and subsequently made the transfer to the genus *Termitomyces*. According to Reid the pileus was 'shallowly convex, seemingly smooth, grey-brown and closely sulcate at the margin' and the cuticle consisted of 'repent hyphae, consisting of short, inflated, thin-walled, barrel-shaped segments, up to 20.8 µm wide, lacking clamp connections' (Reid, 1975). No mention was made of the extended, rooting base of the stipe. Characters of pileal surface hyphae of herbarium specimens were in agreement with those described by Reid (1975) from the type material. He did not observe any cystidia in the type material but cystidia were present in some of the specimens examined. Cheilocystidia were few and scattered while the lamellar-edges consisted mostly of immature basidia. Pleurocystidia were present in some specimens and were morphologically almost identical to cheilocystidia. Basidiospore dimensions corresponded to a considerable degree with the measurements recorded by Reid (1975) and Van der Bijl (1920) and were somewhat larger than the basidiospores of other species of *Termitomyces* as described by Heim (1977) and Pegler (1977). However basidia were significantly larger in relation to the measurements recorded by Reid (1975). *T. umkowaani*

displays certain similarities to *T. eurhizus* and *T. globulus* with regard to the blackish subterranean pseudorhiza and the form and colour of the pileus. *T. umkowaani* differs from both *T. eurhizus* and *T. globulus* by the presence of larger basidiospores and broadly clavate to pyriform cystidia and differs further from *T. globulus* by the presence of hyphae with short inflated, barrel-shaped cells in the pileal surface. This latter characteristic is shared by both *T. umkowaani* and *T. eurhizus*. None of the specimens examined had an annulus. Therefore there are several similarities between *T. umkowaani* and *T. eurhizus* as judged by the descriptions for *T. eurhizus* supplied by Pegler (1977), Pegler & Piearce (1980), Pegler & Rayner (1969) and Piearce (1987). The morphology of both species varies, a phenomenon which Piearce (1987) ascribed to the biological activities and identity of the host termite species. This variation in *T. umkowaani* is reflected in the different shapes of the perforatorium which can be broadly conical and rounded or small conical and conspicuous and may even be totally lacking. Pileal surface may be evenly dark brown to slight ochraceous brown while the periphery may be lighter coloured than the umbo. A long, slender cylindrical stipe may be present or may be reduced to a short, robust stipe which broadens towards a bulbous base. The black cortical layer of the narrow hypogeal pseudorhiza is not a variable character. Descriptions and illustrations of this species by Bottomley & Talbot (1954), Louwrens (1964) and Van der Bijl (1920) confirm this variability of certain characters. Therefore the present system of separating *T. umkowaani* and *T. eurhizus* might prove to be incorrect and they possibly should be included in a single species. As far as can be ascertained *T. umkowaani* is at present only associated with the widely distributed wood-destroying termite *Odontotermes badius*, which has not been recorded before. Consequently *T. umkowaani* may also be widely distributed throughout South Africa. To obtain information which may elucidate the taxonomic status of *T. umkowaani* would require the acquisition of more freshly collected specimens, a prerequisite which is not easily met because of the perishability and edibility of this species in the natural environment (Van der Bijl, 1920; Lastovica, 1974).

7.2 **Termitomyces reticulatus** v.d. Westh. & Eicker sp. nov., *Mycol. Res.* (in press).

**Pileus** 4-18 cm diam, fleshy, immature specimens ovoid with a widely conical, rounded umbo, enlarging to convex-applanate with an unobtrusive, flattened, broadly rounded perforatorium. **Surface** smooth, dark brown at first, finally with small verrucose, brown fragments of soil adhering to the transparent, grayish, reticulate cuticle in a concentric pattern over a glabrous white to silvery white, silky pileal surface, perforatorium remains grayish to dark brown. **Margin**, smooth, entire, at first incurved then turning upwards, splitting, sometimes adnexed to veil fragments. **Lamellae** crowded, free with lamellulae, frail, thin, with entire edges which change to irregular sinuate, creamy white when young, becoming brownish-pink at maturity, up to 12 mm wide. **Stipe** central, cylindrical, white, 15-40 mm diam, epigeal part 30-90 mm with erect annulus, ridge fringed, white to brownish with soil fragments. The enlarging pileus ruptures the upper surface of the stipe and leaves a dangling, veil-like membrane affixed to the uppermost part of the stipe or remaining partly affixed to the pileal margin and stretching across the lamellae. Surface underneath the annulus white to grayish with desultory bands of brownish markings and clinging soil particles, narrowing somewhat before it elongates for 30-80 cm towards termite nest where it tapers abruptly to a white, thin, short pseudorhiza, 5-15 x 3-6 mm, before it either terminates directly in the fungus comb or terminates in a sclerotic, hemispherical disc with a short, thin, 2-5 mm wide rhizomorphic strand to the fungus comb, tough, fibrillate, solid, then hollowed out by feeding termites. **Context** solid, white, uniform, 7 mm thick over lamellae to 15 mm underneath the perforatorium, nonamyloid, composed of ramose, septate, thin-walled, hyaline hyphae lacking clamp connections, 4-21 $\mu$m wide and ramose, aseptate, hyaline lactiferous elements with refractive contents, 3-12 $\mu$m wide, smell reminiscent of sour milk. **Spore print** brownish-pink. **Basidiospores** 6-8 x 4-6 $\mu$m, smooth, apiculate, broadly-ellipsoid, hyaline with deeply-staining contents, thin-walled to slightly thick-walled. **Basidia** claviform, bearing 4 sterigmata, hyaline, 23-33 x 8-10 $\mu$m. **Lamellar-edge** heteromorphous with immature basidia and cheilocystidia.

**Cheilocystidia** 40-54 x 17-32 µm, hyaline, wide claviform to pyriform, with frequently a short 1-celled pedicel. **Pleurocystidia** rare, similar to cheilocystidia. **Hymenophoral trama** bilateral, consisting of hyaline, branched, septate, inflated hyphae, lacking clamps, 4-11 µm and several lactiferous ducts. **Subhymenial layer** thin. **Pileal surface** a gelatinized epicutis condensed into a 21-57 µm layer, which becomes sclerotic when dry, composed of creeping, ramified, anastomosing, septate, thin-walled hyphae lacking clamp connections, 1-2 µm wide (Fig.37 a-g).

Sporocarps prevalent in the rainfall period which stretches from mid-November to February but specimens may also be found in April. Fruit bodies emerge scattered or concrescent in soil over termitaria of *Odontotermes badius* or at the base of the mounds of *O. transvaalensis* (Fig.35 b).

**Specimens examined :** on ground over nest of *Odontotermes transvaalensis* occupied by *O. badius*, Pretoria, Nov. 1986, PREM 2403; on ground over termite nest, National Botanical Gardens, Pretoria, Nov. 1986, PRUM 2404; on nest of *Odontotermes transvaalensis*, Lynnwood Glen, Pretoria, Nov. 1986, PRUM 2405; on termite nest, Pretoria, Nov. 1934, PREM 28667; on termite nest, Pretoria district, Nov. 1948, PREM 36932; on termite nest, Bronkhorstspruit road, Silverton, Nov 1954, PREM 41011; in soil over termitarium, Lynnwood Glen, Pretoria, Nov. 1981, PREM 47085; on ground under trees, Buccleugh, Transvaal, Dec. 1969, PREM 44648; on termite nest, Springbok Park, Pretoria, Nov. 1982, PREM 47247; on lawn, L.C. de Villiers Stadium, Pretoria, Nov. 1984, PREM 47691; on termite nest on lawn, University of Pretoria, Pretoria, Dec. 1984, PREM 47977; in soil, Horticultural Research Station, Roodeplaat, Transvaal, Dec. 1984, PREM 47978; on lawn, L.C. de Villiers stadium, Pretoria, Dec. 1983, PREM 48467; on termite nest, Kroonstad district, Orange Free State, Apr. 1988, PRUM 2693.

Heim (1977) created the stirpe Schimperi to accommodate *T. schimperi* and *T. Le Testui*. *T. reticulatus* shares two common characters with these species, namely a well differentiated annulus on the stipe and the dangling veil-like membrane affixed to the upper part of the stipe. It differs from *T. Le Testui* with respect to the manner in which the partial veil is formed as well as in the absence of a conspicuous

cylindrical perforatorium displayed by *T. Le Testui* (Heim, 1977). Further differences between *T. reticulatus*, *T. Le Testui* and *T. schimperi* are reflected in the anatomy of the pileal surface as well as in the presence of velar scales which consist of spherical cells. When both anatomical and morphological characters as well as the preponderantly white sporocarps of *T. reticulatus* are considered, this species is closer related to *T. striatus*. However according to Heim (1977) and Pegler (1977), *T. striatus* exhibits a pointed perforatorium and the pileal surface is radially striated. The hypogeal section of the pseudorhizal surface remains white to cream coloured and pseudorhizae may reach a length of 80 cm. Diameter of the pseudorhiza varies little and no subterranean enlargement occurs. It may narrow close to its rooting base on the fungus combs. The mode of attachment to the comb, either a rooting base or a hemispherical sclerotic disc, may be influenced by environmental conditions, the structure of the comb or by the biological behaviour of the specific host termite species. In the account by Louwrens (1964) of 'Some mushrooms of the Transvaal' she figured and described an 'Annulatus form of *Termitomyces* sp.' which resembles *T. reticulatus*. This species is prevalent in and around Pretoria but should have a much wider distribution due to its association with *Odontotermes badius* and *O. transvaalensis* which are common and widely distributed. In fact fresh specimens of *T. reticulatus* have recently (April 1988) been collected on a farm situated in the Kroonstad district, Orange Free State, PRUM 2693.

7.3 **Termitomyces sagittaeformis** (Kalchbr. & Cooke) Reid, *Contributions from the Bolus Herbarium* no. **7**: 117-118 (1976).
*Agaricus sagittaeforme* Kalchbr. & Cooke, *Grevillea* **9**: 114, (1881).
*Entoloma sagittaeformis* (Kalchbr. & Cooke) Sacc., *Syll. Fung.* **5**: 687, (1887).

**Pileus**, 2.5-6.5 cm diam at maturity, fleshy, at first conical then expanding to campanulate with a conspicuous spiniform perforatorium. **Surface** even, slightly glossy, with a grayish brown perforatorium, lighter coloured towards the periphery and tends to split radially and concentrically forming recurving grayish brown scales over the pale

brown subjacent tissue. **Margin** irregular, smooth, incurved, radially splitting and finally upturning. **Lamellae** thin, crowded, free to somewhat adnexed with lamellulae, cream coloured at first becoming yellowish-pink to pale brown, margins even, changing to irregular sinuate. **Stipe** central, irregular fusiform, 6-12 mm diam at the pileus and broadens to 10-39 mm below the soil level, sometimes branched, attenuates towards a bulbous fibrillate mass of tissue from where other stipes and primordia originate, lacking annulus or velar remains, either longitudinally striate or spirally tortuous 25-60 mm above soil level, white, subterranean section cream coloured and the bulbous base grayish brown with a pseudorhiza 180 mm long, robust, fibrillate and solid. **Context** white, uniform, 1-4 mm under the perforatorium, firm, thin, nonamyloid, with a slight mushroomy odour, of inflated, ramose, thin-walled, hyaline, septate hyphae lacking clamp connections, 14-33 µm diam and ramose, aseptate, hyaline, lactiferous ducts, 8-17 µm diam, with refractive contents. **Spore print** yellowish pink to pale brownish pink. **Spores** 8-10 x 6-7 µm, apiculate, smooth, thin-walled, ellipsoid, hyaline. **Basidia** 22-37 x 4-8 µm, long clavate, bearing 4 sterigmata. **Lamellar-edge** either sterile or heteromorphous. **Cheilocystidia** 26-61 x 13-28 µm, ovoid to pyriform, numerous, hyaline. **Pleurocystidia** rare, similar to cheilocystidia. **Hymenophoral trama** regular, consisting of parallel, thin-walled, septate, hyaline hyphae, 14-22 µm diam, with a thin subhymenium. **Pileal surface** an epicutis consisting of radially parallel, thin-walled, repent, septate hyphae lacking clamp connections, with brownish contents, 6-17 µm diam (Fig.38 a-f).

Basidiocarps appear in a concrescent manner in the soil over nests of *Odontotermes latericius* (Hav.) at the outset of the rainy season (Fig.35 c).

**Specimens examined :** on the ground, Inanda, Natal, J.M. Wood no. 344 (Lectotype) PREM 10258; on ground, Inanda, Natal, J.M. Wood no. 357, PREM 10262; on the soil over nest of *Odontotermes latericius*, National Botanic Gardens, Pretoria, Nov. 1987, PREM 49058; on soil over nest of *Odontotermes latericius*, National Botanical Gardens, Pretoria, November 1986, PRUM 2235.

Reid (1975) examined specimens collected by Dr. Medley-Wood and

he designated J.M. Wood no. 344 as the lectotype since this material was used for the original description. The original description was augmented by Reid with additional information on the microscopic characters and therefore the transfer to the genus *Termitomyces* was effected by Reid. Recently collected specimens compared favourably with the older material in Herb. PRUM, although the latter consisted only of longitudinal slices of sporocarps and fruit bodies collected in the National Botanical Gardens were significantly larger. These specimens had cystidia, which did not occur in the type material, but the size and shape of the basidiospores corresponded. Although only a limited amount of herbarium material were available for examination (two records by J.M. Wood and the collections at the Botanical Gardens during two successive seasons) it would seem warranted at present to allocate the recently acquired material to the species, *sagittaeformis*. As was the case with *T. umkowaani*, much more material would be required to obtain adequate information with regard to the morphology and taxonomic status of this mushroom. The association of *T. sagittaeformis* with *Odontotermes latericius* is a new record though, since neither J.M. Wood nor Reid mentioned the specific termite host. This species displays certain affinities to *T. clypeatus* but does not have such a conspicuous spiniform perforatorium. The presence of the branched, fusiform stipe which terminate in the fibrillate tissue mass is peculiar to *T. sagittaeformis*. It differs from *T. umkowaani* with respect to the morphology of the pileal surface.

7.4 **Termitomyces clypeatus** Heim, *Bull. Jard. Bot. Brux.* **21:** 207 (1951).

**Pileus** fleshy, conical at first enlarging to campanulate and convex at maturity with a conspicuous spiniform perforatorium, 20-90 mm diam. **Surface** even with small cavities below the perforatorium, sericeous, dry, tarnished, pale grayish or yellowish brown to brown at first, becoming paler towards the periphery, cracking radially and concentrically when enlarging in dry weather, the cuticle curls into long straw coloured scales, exposing the subjacent white tissue. **Margin** incurved, undulate, irregular to irregular-lobate, splitting at maturity. **Lamellae** creamy white, changing to pinkish cream, thin,

free, crowded with lamellulae, margins even becoming slightly sinuate, 8 mm wide. **Stipe** 60-150 x 7-14(22) mm, either long cylindrical or broadening somewhat towards the base, from where it narrows towards a subterranean rooting base and finally tapers to a pseudorhiza 2-4 mm diam, creamy white to pale brown, glabrous or longitudinally striate, occasionally spirally curved, with some striae darker coloured and sometimes with recurved scales on the upper parts, no annulus, fribillate, solid and tough. **Context** uniform, nonamyloid, solid, creamy white, thin, 10 mm thick under the perforatorium, emitting a slight mushroomy odour and consisting of ramose, thin-walled, septate, hyaline, parallel, inflated hyphae, lacking clamp connections, 6-15 µm wide and with lactiferous ducts, 4-12 µm, sometimes branched, aseptate, containing refractive contents. **Spore print** deep pinkish cream. **Basidiospores** 6-9 x 4-6 µm, obliquely apiculate, ellipsoid, glabrous, hyaline, containing one large oil drop, applanate above hilum and thin-walled. **Basidia** 19-30 x 8-14 µm, clavate, hyaline and bearing four sterigmata. **Lamellar-edge** sterile or heteromorphous. **Cheilocystidia** 17-30 x 12-20 µm, broadly clavate to pyriform, hyaline, thin-walled, numerous, frequently with 1-4 short pedicellate cells. **Pleurocystidia** 24-36 x 16-27 µm, rare, similar to cheilocystidia but lacking pedicels. **Hymenophoral trama** regular, consisting of thin-walled, uninflated, hyaline hyphae, lacking clamp connections, 5-8 µm. **Subhymenial layer** narrow and undifferentiated. **Pileal surface** an epicutis with radially parallel hyphae in a layer 32-49 µm wide, consisting of thin-walled, narrow, septate hyphae with brownish contents and lacking clamp connections, 3-7 µm (Fig.39 a-h).

Pilei concrescent to caespitose and abundant in soil over termite nests. No record exists of the termite host species in South Africa (Fig. 35 d).

**Specimens examined :** on ground in *Pennisetum clandestinum* lawn, Blairgowrie, Johannesburg, Jan. 1987, PRUM 2402; on ground, Pretoria, Jan. 1929, PREM 233663; among grass, Ashlea Gardens, Pretoria, Jan. 1983, PREM 48452.

Characters peculiar to *T. clypeatus* include sporocarps with conspicious spiniform perforatoria and the tall, tough, cylindrical stipes bearing grayish brown pilei, growing in caespitose groups on soil over termite nests. The only records in South Africa are limited

to the three collections mentioned above and they fit the descriptions of this species by Pegler (1977) and Heim (1977) quite well. The host termite species is unknown in South Africa at present although the association of this mushroom with *Odontotermes badius* and *Macrotermes falciger* (Gerstecken) in Zambia has been recorded by Piearce (1987). Only the latter species occurs in South Africa, from districts in the north-eastern Transvaal and then in a southwardly direction along the foothills of the Drakensberg to Swaziland (Fuller, 1915). It is therefore possible that *T. clypeatus* also may occur in these areas.

7.5 **Termitomyces microcarpus** (Berk. & Br.) Heim, *Mem. Acad. Sci. Instit. Fr.* **64**: 72 (1941).
*Agaricus microcarpus* Berk. & Br., *Journ. Linn. Soc. Bot.* **11**:537 (1871).
*Entoloma microcarpum* (Berk. & Br.) Sacc., *Syll. Fung.***5**:687 (1887).
*Collybia microcarpa* (Berk. & Br.) Höhn., *Akad. Wiss. Wien. Math. Naturw. Klasse* **117**:993 (1908).
*Mycena microcarpum* (Berk. & Br.) Pat., *Bull. Soc. mycol. Fr.* **29**:210 (1913).
*M. termitum* Beeli, *Rev. Zool. Bot. Afr.* **21**:327 (1932).
*Podabrella microcarpa* (Berk. & Br.) Sing., *Lloydia* **8**:144 (1945).
*Termitomyces narobiensis* Otieno, *Proc. E. Afr. Acad.* **2**:110 (1966).

**Pileus**, 5-20 mm diam, campanulate, enlarging to convex and finally almost plane, umbonate with mucronate papilla, firm and fleshy. **Surface** smooth, dry, white to cream, changing to yellowish brown at the center. **Margin** at first incurved, finally upturned at maturity, entire to slightly partite. **Lamellae** crowded, thin, adnexed to free with lamellulae, margin entire, 1-2 mm broad, white becoming pale pink. **Stipe**, 25-60 x 1.5-3 mm, cylindric, thin, terminating in a bulbous base, lacking annulus, surface smooth, white, fibrillate, solid. **Context** thin, 1 mm wide, tough, white, consisting of interlaced, septate, hyaline hyphae lacking clamp connections, somewhat inflated with thickened walls, 6-12 µm diam. **Spore print** rose pink to flesh pink. **Basidiospores** 6-8 x 4-4.8 µm, apiculate, thin-walled, hyaline, nonamyloid, ovoid to ellipsoid, containing one or more oil droplets. **Basidia** 21-32 x 6-8 µm, 4-spored, clavate. **Lamellar-edge** fertile. **Cheilocystidia** 17-40 x 9-14 µm, hyaline,

pyriform to cylindric, rare. **Pleurocystidia** few, similar to cheilocystidia. **Hymenophoral trama** regular, composed of thin-walled, hyaline hyphae. **Subhymenial layer** pseudoparenchymatous. **Pileal surface** an epicutis, composed of radially parallel, uninflated, thin-walled, hyaline, septate hyphae, 3-5 µm diam, lacking clamp connections (Fig.40 a-f).

Sporocarps occur in dense clusters on soil brought up from the nests of *Odontotermes badius* (Haviland), *O. transvaalensis* (Sjöstedt) and *O. vulgaris* (Hav.) (Bottomley & Fuller, 1921 ; Sands, 1960) (Fig. 35 e).

**Specimens examined :** on termite soil, Pretoria, Jan. 1921, PREM 14683; on termite soil, Klerksdorp, May 1928, PREM 23358; on termite soil, Johannesburg, Apr. 1941, PREM 33133; on soil, Arcadia, Pretoria, Jan. 1950, PREM 38911; on termite soil, Pretoria, Feb. 1964, PREM 42492; on termite soil, Brummeria, Pretoria, March 1976, PREM, 43697; on termite over termite nest, Pretoria, Jan. 1980, PREM 45564; on termite nest, Pretoria, Feb. 1982, PREM 47098; on soil Queenswood, Pretoria, Jan. 1983, PREM 47242; on soil, Ashlea Gardens, Pretoria, Feb. 1986, PREM 48273.

Singer (1949,1962) placed this species in the genus *Podabrella* Sing. and from the description of the type species *Collybia microcarpa* (Berk. & Br.) Höhn., renamed it *Podabrella microcarpa* (Berk. & Br.) Sing. Singer (1962) stated that development of the carpophores of *P. microcarpa* might be hemiangiocarpous (Heim, 1977) and he considered differences such as (i) absence of a pseudorhiza (ii) a true perforatorium (iii) pileal surface structure (iv) the near absence of pigment in the carpophores and (v) the small carpophore size, as sufficient differences to justify the placement of *T. microcarpus* in the genus *Podabrella*. In addition to these differences the presence of context hyphae with slightly thickened walls is a character peculiar to *T. microcarpus* and is not present in any of the other species investigated. Furthermore no conidiophores and conidia have been observed in the cultures of *T. microcarpus* and growth characters of the cultures (par.5.5) differ completely from those of the other species. The only characters this species has in common with species in the subgenus Eutermitomyces are the close association of this mushroom with the specific termite host on which it depends for fructification and dis-

persal and the presence of the umbonate pilei. The intimate relationship of *T. microcarpus* with the fungus-growing Macrotermitinae may be the result of convergent evolution. The present taxonomic status of *T. microcarpus* is therefore still unsatisfactory and although this species is retained in the genus *Termitomyces* in this investigation, Singer's (1962) interpretation of this species may present a better approach and would reflect a more natural taxonomic position than the present one.

Descriptions of *T. microcarpus* by Heim (1977) and Pegler (1977) correspond well with both the macro and micromorphology of sporocarps collected in South Africa. The occurrence of cystidia is a variable character in this species (Heim, 1977; Pegler, 1977) and cystidia were rare in the specimens examined. *T. microcarpus* has the widest distribution of all *Termitomyces* species both in Asia and in Africa of which southern Africa is no exception. It has been collected in the western Transvaal near Klerksdorp (Bottomley & Fuller, 1921) and in Natal near Pietermaritzburg (Lastovica, 1974). *Odontotermes badius*, *O. transvaalensis* and *O. vulgaris* which are widely distributed in the southern African region are associated with this mushroom and indicates the prevalence and wide distribution of this mushroom in all four provinces of South Africa.

7.6 **Termitomyces schimperi** (Pat.) Heim, *Arch. Mus. Hist. Nat. Paris* **(6)18:**114 (1942).

**Pileus**, 100-130 mm diam, solid fleshy , subglobose enlarging to spheroid-applanate, Lacking conical or spicular perforatorium. **Surface** favosely cracked with thick verrucose scales, yellowish brown to rust brown and roughly disposed in a concentrical manner over the disc where a tuberose unbroken plate persists. **Margin** entire, even, finally turning upwards. **Lamellae** crowded, free with lamellulae, margin entire to sinuate, creamy white and up to 12 mm broad. **Stipe** central, 80-140 x 10-30 mm, above ground level, cylindric, broadening to 30-40 mm below soil level then narrowing downwards to form a thin pseudorhiza 8 mm diam or less, surface overlaid with thick membranous scales of velar remains, white to creamy white, solid, fibrillate,

lacking annulus. **Context** firm, white, up to 16 mm thick, composed of interlaced, thin-walled, septate, hyaline, inflated hyphae, lacking clamp connections, up to 16 µm wide and with several lactiferous ducts, 5-14 µm diam. **Spore print** not observed, according to Pegler (1977) pinkish cream. **Spores** 6-9 x 4-5 µm, smooth, thin-walled, hyaline, nonamyloid, broad ellipsoid, apiculate. **Basidia** 25-33 x 5-9 µm, clavate, bearing 4 sterigmata. **Lamellar-edge** heteromorphous. **Cheilocystidia** 33-64 x 8-17 µm, abundant, cylindric-fusiform to lageniform, hyaline, occasionally with thickened walls or two transverse septa. **Pleurocystidia** similar to cheilocystidia. **Hymenophoral trama** bilateral, hyaline. **Subhymenial layer** pseudoparenchymatous. **Pileal surface** an epicutis, composed of radially parallel gelatinized hyphae, 2-3 µm diam, velar scales consist of sphaerocysts with deeply-staining contents and thin or thickened brownish walls, 14,0-47 x 11-28 µm (Fig.41 a-g).

Basidiocarps emerge as solitary specimens in sandy soil above termite nests in arid regions. At present the host termite species in southern Africa is not known (Fig.35 f).

**Specimens examined :** on ant heap in sandveld, South West Africa, Jan. 1953, PREM 27811; on termite nest, Simkue (Tsumkwe) South West Africa, Story no. 6278 & 6290, 1958, PREM 41964; on termite nests north-western Transvaal, 1959, PRUM 2406.

During the period of investigation no fresh specimens could be located and therefore *T. schimperi* was identified with the aid of the herbarium material mentioned above. According to Piearce (1987) *T. schimperi* is associated with *Odontotermes patruus* (Sjöstedt) in Zambia which does not occur in South Africa (Fuller, 1915) but the other host termite species *Macrotermes natalensis* (Hav.) occurs in all four provinces of South Africa, as well as Botswana, Swaziland and South West Africa. Therefore in spite of limited information with regard to its distribution in southern Africa it is possible that *T. schimperi* may have a larger distribution in South Africa. Only a few herbarium specimens exist and the acquisition of many more fresh specimens is a necessity before sufficient information regarding its distribution in South Africa can be obtained. Nonetheless there are certain striking features which are peculiar to *T. schimperi*. These are : (i)

favosely cracked, thick, brown velar scales on the large white pileus (ii) absence of a true perforatorium and (iii) lageniform, septate cystidia.

7.7 **Termitomyces striatus** (Beeli) Heim, *Mem. Acad. Sci. Instit. Fr.* **64**:74 (1941).
*Schulzeria striata* Beeli, *Bull. Jard. Bot. Brux.* **15**:29 (1938).

**Pileus**, 32-60 mm diam, at first campanulate then expanding to convex-applanate with a small pointed, broadly conical perforatorium. **Surface** pale brownish grey and dark brown over the perforatorium, hygrophanous, glabrous over umbo and central part, indistinctly radially striate from about halfway towards margin. **Margin** entire, regular, when fully expanded inflexed at maturity splitting radially. **Lamellae** creamy white, free, 5 mm broad, crowded or adnexed to creamy white collar at pileus-stipe junction, with numerous lamellulae, margins slightly crenulate becoming laciniate. **Stipe** central, 100 x 3-5 mm, pale cream, cylindrical, solid, tapering into a thin, subterranean pseudorhiza, surface glabrous, fibrillate striate often spirally twisted, without annulus. **Context** thin, white, consisting of thin walled, unbranched, septate, nonamyloid hyphae, lacking clamp connections, 6-8 µm inflated to 29 µm diam. **Spore print**, pale brownish pink. **Basidiospores** 6-8 x 5-6 µm diam, ovoid to ellipsoid, smooth, hyaline, nonamyloid, thin-walled, apiculate and containing one large oil drop. **Basidia** 21-24 x 4-8 µm diam, clavate, hyaline, bearing four sterigmata. **Lamellar-edge** heteromorphous with scattered cheilocystidia. **Cheilocystidia** 12-43 x 12-29 µm diam, hyaline, inflated pyriform to almost globose, occasionally with a short 1-celled pedicel. **Pleurocystidia** 22-32 x 7-8 µm, hyaline, clavate-cylindrical, thin-walled. **Hymenophoral trama** indistinctly bilateral to subregular, consisting of parallel, thin-walled, hyaline hyphae, 3-10 µm diam. **Subhymenial layer** well defined, interwoven. **Pileal surface** an epicutis consisting of uninflated, hyaline, septate, thin-walled, radially parallel hyphae, 3.4-8 µm diam, overlaying a layer of greatly inflated, pale brownish, thin-walled, irregular, monilioid cells, 21-55 x 15-36 µm diam (Fig.42 a-h).

**Specimens examined :** scattered on lawn in the vicinity of

termite nest, Baberton, Transvaal, Feb. 1989, PRUM 2683 (Fig. 17g).

Macro and microscopic characters of the examined material concurs with the descriptions supplied by Pegler (1977), Heim (1977) and Beeli (1938) of *T. striatus* in most aspects. Characteristics of the examined material somewhat resemble the description of *T. tyleranus* Otieno, Otieno (1964). However according to him the carpophores grow crowded together, has a pileus diameter of 25-30 mm and the perforatorium is prominent and distinct from the pileus as in *T. clypeatus*. The examined specimens are however significantly larger with respect to pileus diameter (32-60 mm), fruiting habit is scattered and the perforatorium is broadly conical and not so prominent. In the specimens examined the pileus margin turned upwards when fully expanded and split towards the center. These characters are also mentioned by Beeli (1938) in the original description of *T. striatus* and differ from the pileus of *T. tyleranus* which remains inflexed and entire at maturity. Pegler (1977) also mentions the radially striate, incised pileus of *T. striatus* which is slightly hygrophanous, but he does not mention the presence of greatly inflated, thin-walled, irregular, monilioid cells in the pileal surface. This conspicuous microscopic character of *T. striatus* is peculiar to this species and is described by Beeli (1938) as large, vesicular cap cells and by Heim (1977) as chains of ovoid cells. Heim's (1977) illustration of these cells concurs with my description. The membranous, whitish velar squamules described by Pegler (1977) were not present in the examined specimens. Although the examined specimens resemble the descriptions for both *T. tyleranus* and *T. striatus* it would seem more appropriate to assign the collected material to the latter species which it resembles closely. According to Pegler (1977) this species is wide spread throughout equatorial Africa and is frequently encountered whereas *T. tyleranus* seem to be restricted to the eastern region of Africa.

Only a few specimens were available for examination and more freshly collected material would be required to provide a more detailed and thorough investigation of the morphology of *T. striatus*. Apart from the descriptions of the authors already mentioned, no other descriptions of *T. striatus* are available at present and a detailed illustration is also lacking. To aggravate the situation, specimens

of *T. striatus* are extremely variable and do occasionally develop abnormally (Pegler, 1977). The geographical distribution pattern of *T. striatus* in South Africa as well as the associated termite host species are at present unknown. Heim (1977) reported its association with *Pseudoacanthotermes militaris* (Hagen) which does not occur in South Africa and probably *Macrotermes* spp. However the small slightly elevated termite heaps, scattered over a large area (± 20 $m^2$) are reminiscent of the termitaria of *Hodotermes mossambicus* (Hagen) which is wide-spread in South Africa (Fuller, 1915). Consequently the possibility exists that it may occur over a much greater area. At present no collection records exist for *T. striatus* in the southern African region and therefore this appears to be the first occurrence in South Africa.

## 8. Germination studies (basidiospores)

Although natural and cultural conidia germinate readily, basidiospores do not germinate under artificial laboratory conditions. Several attempts were made to induce germination of basidiospores which were prepared from spore prints of *T. reticulatus*, *T. microcarpus* and *T. umkowaani* utilizing the same semi-solid medium and incubation conditions applied to conidiospores (see material and methods, par.3). All attempts failed and no germ tubes were observed even after an incubation period of four weeks. Although Johnson *et al.* (1981) failed to induce germination of basidiospores, Sands (1960,1969) and Sieber (1983) considered basidiospores to be important propagules for inoculating newly constructed fungus combs. Research conducted by Akhtar (1978), Batra & Batra (1966), Grassé & Noirot (1955) and Johnson (1981) indicated the importance of conidiospores as a means of inoculating freshly constructed combs in newly founded colonies. At present the germination of basidiospores has not been observed in the natural environment and the optimal conditions for germination remain to be ascertained. Experimental results favour conidiospores and not basidiospores as a means of perpetuating the symbiotic relationship between the fungus-growing termites and *Termitomyces*. The possibility even exists that basidiospores have lost the ability to germinate altogether.

**Fig. 36** (a -i). Light micrographs of the hyphal characters of *T. umkowaani* fruit bodies.

Fig.36(a). Radial, longitudinal section of pileal surface hyphae illustrating unbranched, septate, narrow, uninflated hyphae (hy) over doliform cell layer (dc) and context hyphae (ch) below. Fig.36(b). Epicutis hyphae consisting of unbranched, narrow, septate, uninflated hyphae. Fig.36(c). Doliform cells in the pileal surface cuticle. Fig.36(d). Barrel-shaped, doliform cells in the stipe surface cuticle with constricted septa. Fig.36(e). Developing caulocystidia (ca) on the stipe cuticle surface overlaying the doliform cell layer (dc). Fig.36(f). Lactiferous duct (ld) in the hymenophoral trama. Fig.36(g). Hymenium (h) and subhymenial layer (sh). Fig.36(h). Basidiospores. Fig.36(i). Scattered, pedicellate, pyriform cheilocystidia (scale bar = 20 $\mu$m).

**Fig. 37** (a - g). Light micrographs of the hyphal characters of *T. reticulatus* fruit bodies.
Fig.37(a). Radial, longitudinal section of pileal surface illustrating the gelatinized epicutis (ep) and overlaying context hyphae (ch). Fig.37(b). Hyphae of the reticulate cuticle consisting of creeping, ramified, anastomosing, septate, narrow hyphae. Fig.37(c). Branched lactiferous duct (ld) in the hymenophoral trama. Fig.37(d). Lamellar-edge with scattered cheilocystidia. Fig.37(e). Lactiferous duct (ld) interwoven with context hyphae. Fig.37(f). Pedicellate, pyriform cheilocystidium (ch) with developing basidia. Fig.37(g). Basidiospores (scale bar = 20 $\mu$m).

**Fig. 38** (a - f). Light micrographs of the hyphal characters of *T. sagittaeformis* fruit bodies.
Fig.38(a). Pileal surface hyphae. Fig.38(b). Cheilocystidia intermingled with developing basidia. Fig.38(c). Hymenium (h) and subhymenial layer (sh). Fig.38(d). Hymenophoral trama. Fig.38(e). Basidiospores. Fig.38(f). Lactiferous duct(ld) interwoven with context hyphae (scale bar = 20 $\mu$m).

**Fig. 39**(a - h). Light micrographs of the hyphal characters of *T. clypeatus* fruit bodies.
Fig.39(a). Vertical radial section of pileal surface illustrating the layer of radial parallel hyphae comprising the epicutis (ep) and the context hyphae (ch) below. Fig.39(b). Lamellar-edge with scattered cheilocystidia. Fig.39(c). Basidium bearing one basidiospore with an oil drop. Fig.39(d). Basidiospores. Fig.39(e,f). Cheilocystidium with short pedicellate cell (pc). Fig.39(g). Hymenium (h) and subhymenial layer (sh). Fig.39(h). Thin-walled, septate, parallel, inflated hyphae of the context (scale bar = 20 $\mu$m).

**Fig. 40** (a - f). Light micrographs of the hyphal characters of *T. microcarpus* fruit bodies.
Fig.40(a). Pileal surface hyphae with scattered basidiospores. Fig.40(b). Hymenium, subhymenium and hymenophoral trama. Fig.40(c). Lamellar-edge with one pyriform cheilocystidium. Fig.40(d - f). Basidia, basidiospores and cystidia ; (scale bar = 20 $\mu$m). Fig.40(g). Advancing zone of mycelium in culture, showing hyphal strands (scale bar = 2mm).

**Fig. 41** (a - g). Light micrographs of the hyphal characters of *T. schimperi* fruit bodies.
Fig.41(a). Surface of pileal scales illustrating sphaerocysts with slightly thickened walls. Fig.41(b). Scattered sphaerocysts with thickened walls and deeply-staining contents from velar scales. Fig.41(c - f). Cylindric-fusiform, septate (sp), cheilocystidia (ch) intermingled with developing basidia (bs) of the hymenial layer. Fig.41(g). Lamellar-edge with scattered cheilocystidia (scale bar = 20 $\mu$m).

**Fig. 42** (a - h). Light micrographs of the hyphal characters of *T. striatus* fruit bodies.
Fig.42(a). Pileal surface hyphae illustrating the uninflated, septate, thin-walled, radial parallel hyphae (ph) overlaying a layer of greatly inflated, thin-walled, irregular, monilioid cells (mc). Fig.42(b). Lamellar-edge with scattered cheilocystidia. Fig.42(c). Hymenium, subhymenial layer and hymenophoral trama. Fig.42(d). Inflated, pyriform, pedicellate cheilocystidium (sc). Fig.42(e). Unbranched, thin-walled, septate, inflated context hyphae situated near the stipe surface. Fig.42(f). Immature basidium (ib) and developing pleurocystidia (pl) in the hymenial layer. Fig.42(g). Basidium bearing basidiospores with one conspicious oil droplet. Fig.42(h). Globose cheilocystidia intermingled with developing basidia (scale bar = 20 $\mu$m).

## 9. General discussion and conclusions

Seven species of *Termitomyces* occur in South Africa of which *T. clypeatus*, *T. schimperi*, *T. striatus* and *T. microcarpus* also occur in Kenya (Pegler, 1977; Pegler & Rayner, 1969), Nigeria (Alasoadura, 1966), Zambia (Pegler & Piearce, 1980; Piearce, 1987), Malawi (Morris, 1984) and in several Equatorial and Central African states (Heim, 1977). The species, *T. reticulatus*, *T. umkowaani* and *T. sagittaeformis* seem to be restricted to southern Africa. Like *T. clypeatus*, *T. sagittaeformis* has small pilei with spiniform perforatoria and share certain characteristics with *T. eurhizus*, *T. globulus* and *T. umkowaani*, namely a bulbous stipe and the dark-coloured cuticle on the pseudorhiza. *Termitomyces reticulatus* shares certain microscopic and macroscopic characters with *T. striatus*. This species has a wide distribution in Africa (Pegler, 1977) and its occurrence in South Africa is a new record for this region. The termite host species *Odontotermes latericius* which is associated with *T. sagittaeformis* is also a new record. As far as can be ascertained no other species of *Termitomyces* is associated with this termite.

Heim (1977) described the African species of *Termitomyces* by utilizing mainly macroscopic characters of fruit bodies, but these characters are variable and not reliable taxonomic criteria (Pegler, 1977; Piearce, 1987). Microscopic characters in this genus tend to be strikingly similar. The present investigation has shown that microscopic characters of the pileal surface and velar remains on the pileus in combination with basidiospore and cystidial characters seem to be reliable taxonomic criteria when identifying species of *Termitomyces*. These characters differed to a marked degree between the species investigated and could therefore prove to be of substantial aid for the future identification and description of species in this genus.

To summarize : (i) there seems to be more variation and difference in both macro and microscopic characters of *Termitomyces* fruit bodies than in the case of basidiome context cultures, consequently species differences are less pronounced when cultures are compared and

more conspicuous when fruit bodies are compared (ii) microscopic characters of cultures are very similar (iii) growth characters of the various species exhibit definite differences when compared on the same medium (iv) when mycelium is grown on different nutrient media, growth characters change to a certain degree (v) when compared on the same medium different isolates of the same species display no differences with respect to macro and microscopic characters. Although only a limited number of isolates of each species were investigated, the results obtained indicated that growth characters were reliable and invariable taxonomic criteria if the nutrient medium and incubation conditions were standarised. However it would be essential to obtain many more isolates of the various species from different geographical localities to increase the number of isolates and to broaden the scope of this investigation. Therefore my deductions are only valid for the species evaluated in this investigation and may not be directly applicable to other species in this genus. Limitations which greatly restricted the availability of fruit bodies and the number of isolations which could be prepared of each species, comprised the following : (i) strictly seasonal appearance of fruit bodies (ii) high perishability of the fruit bodies in nature (iii) popularity of the highly edible fruit bodies amongst the indigenous black population and (iv) great distances which had to be covered to the localities where the termitaria occur. Due to these limitations and the restricted period of time available for this investigation it is possible that additional described and even undescribed species of *Termitomyces* may occur in the southern African region which will obviously not be included in the present investigation.

One of the objectives of this investigation was to assess the usefulness of biochemical methods in resolving differences between species. Analysis of proteins and nucleic acids are usually effective when screening for interspecies differences. An attempt was made to discriminate between the different species of *Termitomyces* with the aid of SDS polyacrylamide gel electrophoresis of general proteins and computer assisted analysis of the numerical data obtained from spectrophotometric evaluation. It was also attempted to isolate intact DNA of high molecular weight from grown mycelium to determine the melting point (Tm) and GC contents of the purified DNA. No

satisfactory results were obtained even after several attempts due to factors unknown at the time the extractions were performed. This setback does not exclude the possibility of differentiating between species of *Termitomyces* on purely molecular level. If the practical problems of extraction and analysis can be resolved and many more isolates of the different species obtained the above-mentioned techniques would prove to be a valuable aid in identifying species of *Termitomyces*. It has been proved beyond any doubt by numerous mycologists and molecular biologists that methods involved in numerical taxonomy can be a valuable taxonomic tool when the fungi under investigation are morphologically very similar. The possibility also exists to employ even more refined biochemical techniques such as ribosomal protein separation with the aid of SDS PAGE and DNA : DNA reassociation studies between species to determine the percentage homology of the reassociated DNA.

## V. SUMMARY

# A MORPHOLOGICAL AND PHYSIOLOGICAL STUDY OF THE GENUS *TERMITOMYCES* HEIM IN SOUTH AFRICA

**BY**

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Eight different species of the genus *Termitomyces* Heim were collected in the Republic of South Africa viz. : (i) *T. umkowaani* (Cooke & Mass.) Reid (ii) *T. reticulatus* v. d. Westh. & Eicker sp. nov. (iii) *T. sagittaeformis* (Kalchbr. & Cooke) Reid (iv) *T. clypeatus* Heim (v) *T. microcarpus* (Berk. & Br.) Heim (vi) *T. schimperi* (Pat.) Heim (vii) *T. striatus* (Beeli) Heim and (viii) sporodochial mycelium from the combs of *Macrotermes natalensis* Hav. and *Odontotermes badius* Hav. The basidiocarp morphology of all the species was described and cultural studies were conducted on six species. Macroscopic characters of cultures differed markedly between species but microscopic characters appear to be very similar. Microscopic characters of the pileal surface, basidiospores and cystidia coupled with the velar remains on the pileus proved to be reliable taxonomic criteria for the identification of species in this genus. Microscopic characters of sporodochia from the combs of *M. natalensis* and *O. badius* displayed some small but conspicuous differences and may indicate that microscopic characters of the anamorph of *Termitomyces* where it grows on the comb, is not completely identical according to presently held views. With the exception of *T. microcarpus* all the species in pure culture produced conidiophores and holoarthric conidia similar to those of comb sporodochia and

indicate an intimate relation between the anamorph and teleomorph of this genus. Whether cultures are prepared from fruit body context or comb sporodochia, the anamorph will always develop in culture and microscopic characters will be very similar. Conidia from comb sporodochia or cultures germinated readily *in vitro* but basidiospores did not. A new species of *Termitomyces*, *T. reticulatus*, was described from South Africa. The association of this species with *O. badius* and *O. transvaalensis*, *T. umkowaani* with *O. badius* and *T. sagittaeformis* with *O. latericius* are new records. Micro and microscopical characters of *T. microcarpus* cultures and fruit bodies differed greatly from other species in the subgenus Eutermitomyces. It is therefore suggested that this species should be placed in the genus *Podabrella* Singer.

The influence of three environmental factors on mycelium growth was assessed. Optimal mycelium growth was observed in the pH range 5 to 7, in the temperature range 20 $^{0}$C to 30 $^{0}$C and under completely dark incubation conditions. Natural cellulose fibres supported maximal mycelium growth of all the species. Hemicellulose (xylan), starch, casein, natural and synthetic lipids and pectic substrates were degraded as well and was coupled with secretion of the relevant exo-enzymes into the culture media. Mycelium growth was possible on a wide range of carbon sources with the exception of certain amino sugars, sugar alcohols, dicarboxylic acids and uronic acids. Several nitrogen sources were utilized e.g. complex amino acid mixtures and inorganic ammonium sources supported good mycelium growth. The ability of *T. umkowaani* mycelium to grow on natural substrates was investigated. Excellent growth was supported by a mixture of wheat, sorghum, sawdust and digestive bran. Growth remained vegetative and no fruit body formation occurred. The biological value of mycelium protein grown on a defined medium was assessed for three species and was compared with that of *Agaricus bisporus*. The essential amino acid contents of *T. sagittaeformis* and *T. umkowaani* compared favourably with that of *A. bisporus* and a standard reference protein, whereas the biological value of *T. reticulatus* protein appeared to be of a mediocre to poor quality. Mycelium protein of both *T. sagittaeformis* and *T. umkowaani* exceeded the essential amino acid requirements of adults.

**SAMEVATTING**

Agt verskillende spesies van die genus *Termitomyces* Heim is versamel in die Republiek van Suid Afrika te wete : (i) *T. umkowaani* (Cooke & Mass.) Reid (ii) *T. reticulatus* v. d. Westh. & Eicker sp. nov. (iii) *T. sagittaeformis* (Kalchbr. & Cooke) Reid (iv) *T. clypeatus* Heim (v) *T. microcarpus* (Berk. & Br.) Heim (vi) *T. schimperi* (Pat.) Heim (vii) *T. striatus* (Beeli) Heim en (viii) sporodogium miselium vanaf die fungustuine van *Macrotermes natalensis* Hav. en *Odontotermes badius* Hav. Die basidiokarp morfologie van al die spesies is beskryf en kultuur studies van ses spesies is uitgevoer. Die makromorfologiese eienskappe van die kulture verskil merkbaar maar die mikromorfologiese eienskappe van die kulture is verbasend eenders. Die mikromorfologiese eienskappe van die pileus oppervlak, basidiospore en sistidiums asook oorblyfsels van vliese op die pileus is betroubare taksonomiese hulpmiddels vir die identifisering van spesies. Klein dog merkbare verskille is sigbaar in die mikromorfologie van sporodogiums afkomstig van die tuine van *M. natalensis* en *O. badius* en hiérdié waarneming mag moontlik daarop dui dat die anamorf van hierdie genus waar dit op die fungustuine groei, nie mikromorfologies eenders is soos wat tans aanvaar word nie. Die reinkulture van al die spesies met die uitsondering van *T. microcarpus* het konidiofore en holoartriese konidiums geproduseer wat amper identies is aan die konidiofore en holoartriese konidiums van die fungustuin sporodogiums. Daar bestaan 'n noue verwantskap tussen die anamorf en teleomorf in die genus *Termitomyces*, omdat die anamorf altyd in kultuur gevorm word ongeag of die miselium vanaf fungustuin sporodogiums of vanaf basidiokarp konteks verkry is. Hierdie kulture se mikromorfologiese eienskappe is amper identies. Konidiums vanaf sporodogiums en in kultuur het maklik *in vitro* gekiem maar basidiospore kon nie gestimuleer word om te kiem nie. 'n Nuwe spesie van *Termitomyces*, naamlik *T. reticulatus* is in Suid Afrika versamel en beskryf. Die assosiasie van hierdie spesie met *O. badius* en *O. tranvaalensis*, *T. umkowaani* met *O. badius* en *T. sagittaeformis* met *O. latericius* word vir die eerste keer aangeteken. Die makromorfologiese en mikromorfologiese eienskappe van *T. microcarpus* kulture en

vrugliggame verskil drasties van dié van die ander spesies en daar word aanbeveel dat hierdie spesie in die genus *Podabrella* Singer geplaas word.

Die invloed van drie omgewings faktore op die groei van miselium is bepaal. Optimale groei kom voor in die pH gebied 5 tot 7, by temperature wat gewissel het van 20 $^{0}$C tot 30 $^{0}$C en onder donker inkuberingstoestande. Al die ander ondersoekte spesies groei optimaal op natuurlike sellulose vesels. Hemisellulose (xilaan), stysel, kaseïen, natuurlike en sintetiese lipiede en pektiese substrate is afgebreek en die betrokke hidrolitiese ekso-ensieme is in die voedingsmediums afgeskei. 'n Wye reeks van koolstofbronne is benut met die uitsondering van sekere aminosuikers, suikeralkohole, dikarboksielsure en uronsure. Verskeie stikstofbronne is benut veral komplekse aminosuur mengsels en anorganiese ammonium bevattende bronne. Die vermoë van *T. umkowaani* om op natuurlike substrate te groei is ondersoek. Welige miselium groei is waargeneem op 'n mengsel van koring, sorghum, houtsaagsels en semels. Die ontwikkeling van basidiokarpe is nie waargeneem nie. Die biologiese waarde van *in vitro* gekweekte miselium proteïen is bepaal vir drie spesies en is vergelyk met dié van *Agaricus bisporus*. Die essensiële aminosuur inhoud van *T. sagittaeformis* en *T. umkowaani* vergelyk gunstig met dié van *A. bisporus* en 'n standaard verwysings proteïen, terwyl die biologiese waarde van *T. reticulatus* proteïen van 'n mindere tot swak gehalte is. Die biologiese waarde van *T. sagittaeformis* en *T. umkowaani* proteïen het die essensiële aminosuur vereistes van 'n volwassene oortref.

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# VII. APPENDIX 1

## 1. Recipes for nutrient media

### 1.1 Yeast extract, soytone, soluble starch medium (YESS)

| | | |
|---|---|---|
| Dipottasium hydrogen phosphate | $K_2HPO_4$ | 0,90 g |
| Potassium dihydrogen phosphate | $KH_2PO_4$ | 0,70 g |
| Magnesium sulfate heptahydrate | $MgSO_4.7H_2O$ | 0,75 g |
| Potassium chloride | KCI | 0,30 g |
| Soytone | | 2,00 g |
| Yeast extract | | 1,00 g |
| Soluble starch or (potato dextrose agar) | | 10,0 (39,0 g) |
| Agar | | 15,0 g |
| Chloramphenicol | | 250 mg |
| Double distilled water | | 1 $dm^3$ |

### 1.2 Basal medium

| | |
|---|---|
| $K_2HPO_4$ | 0,90 g |
| $KH_2PO_4$ | 0,70 g |
| $MgSO_4.7H_2O$ | 0,75 g |
| KCI | 0,30 g |
| Ammonium nitrate $NH_4NO_3$ | 2,00 g |
| Double-distilled water | 1 $dm^3$ |

### 1.2 Hagem nutrient agar modified by Modess

| | |
|---|---|
| $KH_2PO_4$ | 0,50 g |
| $MgSO_4.7H_2O$ | 0,50 g |
| Ammonium chloride $NH_4CI$ | 0,50 g |
| Ferric chloride $FeCI_3$ (1 % $w.v^{-1}$) | 10 drops |
| D-glucose | 5,00 g |
| Malt extract | 5,00 g |
| Double-distilled water | 1 $dm^3$ |

## 2. Recipes for reagents and buffers

### 2.1 Dinitrosalicylic acid reagent (Miller, 1959)

| | | |
|---|---|---|
| 3,5-Dinitrosalicylic acid | $C_7H_4N_2O_7.H_2O$ | 1,0 g |
| Phenol | $C_7H_5OH$ | 0,2 g |
| Sodium Hydroxide | NaOH | 1,0 g |
| Potassium sodium tartrate | $KNaC_4H_4O_6.4H_2O$ | 20,0 g |
| Sodium sulfite | $NaSO_3$ | 0,05 g |
| Double-distilled water | | 100 $cm^3$ |

### 2.2 Copper reagent (Duncombe, 1963)

Triethanolamine hydrochloride $(HOCH_2CH_2)_3N.HCL$ 18,6 g in 7 $cm^3$ distilled water ; Copper (II) nitrate trihydrate $Cu(NO_3)_2.3H_2O$ ; 6,45 g in 100 $cm^3$ distilled water ; mix both solutions and adjust to pH 7,5 with 5 N NaOH and dilute to 200 $cm^3$ with distilled water. Sodium diethyldithiocarbamate trihydrate $(C_2H_5)NCS_2Na.3H_2O$ ; 0,25 g in 100 $cm^3$ sec-butanol.

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