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Three dimensional typological studies using scanning electron microscopy for characterization of *Termitomyces* pellets obtained from submerged growth conditions

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Abstract

There are gaps in existing understanding of fungal pellet growth dynamics. We used scanning electron microscopy (SEM) for morphological characterization of the biomass organization of *Termitomyces* pellets for seven species: *T. microcarpus* (TMI1), *T. albuminosus* (TAL1, TAL2), *T. striatus* (TSTR), *T. aurantiacus* (TAUR), *T. heimii* (THE1, THE2), *T. globulus* (TGLO) and *T. clypeatus* (TCL1, TCL2, TCL3, TCL4, TCL5). We assessed the utility of SEM for morphological and structural characterization of *Termitomyces* spp. in three dimensional (3D) pellet form to identify ideal pellet morphology for industrial use. Typological classification of *Termitomyces* species was based on furrows, isotropy, total motifs and fractal dimensions. The pellets formed were entangled and exhibited highly compacted mycelial mass with microheterogeneity and microporosity. The mean density of furrows of *Termitomyces* species was between 10,000 and 11,300 cm/cm^2, percentage isotropy was 30–80 and total motifs varied from 300 to 2500. TGLO exhibited the highest furrow mean density, 11243 cm/cm^2, which indicated a compact, cerebroid structure with complex ridges and furrows, whereas TAL2 exhibited the lowest furrow density. TMI1a exhibited a high percentage isotropic value, 74.6, TSTR exhibited the lowest, 30.9. Total motif number also was used as a typological classification parameter. Fractal values were 2.64–2.78 for various submerged conditions of *Termitomyces* species. TAL1 exhibited the highest fractal dimension and TAL2 the lowest, which indicates the complexity of branching patterns. Three-dimensional SEM image analysis can provide insight into pellet micromorphology and is a powerful tool for exploring topographical details of pellets.

Key words: isotropy, microheterogeneity, pellets, *Termitomyces*, scanning electron microscopy, three dimensional structure, typology

Fungal fermentation is used commercially to produce useful products including organic acids, enzymes, antibiotics and cholesterol lowering drugs (Liao et al. 2007, Barry and Williams 2011). Fungal fermentation studies have been applied to a wide range of industrially important microfungi including *Aspergillus terreus* (Lopez et al. 2005, Porcel et al. 2005), *Rhizopus oryzae* (Liao et al. 2007), *Penicillium citrinum* (Gomaa and Bialy 2009) and macrofungi including *Grifola frondosa* (Lee et al. 2004), *Phellinus* spp. (Hwang et al. 2004), *Pleurotus ostreatus* (Ha et al. 2001) to understand pellet morphology, because in most fungal fermentation processes, pellet formation due to rheological aspects affect productivity (Hwang et al. 2004, Villena et al. 2010).

Filamentous fungi under submerged conditions show two typical mycelial growth

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morphologies: pellets or free mycelium forms. These morphologies are affected by various preparation conditions including size, nature of inoculum, medium composition, pH, temperature, rpm, volume of medium, shear stress and genetic variability (Papagianni 2004, Teng et al. 2009, Zhou et al. 2011). Pellets are densely interwoven mycelial masses (Papagianni 2004).

Fungal morphology is typically described by hyphal length, total mycelium length, diameter of mycelium, number of tips, tip extension rate, hyphal growth unit and branching frequency (Lejeune and Baron 1997, Papagianni 2006). Although parameters such as area, compactness, perimeter, circularity and roughness have been used to describe aggregates and pellets of fungi, the need remains to identify new morphological parameters to distinguish growth more precisely (Ryoo 1999, Papagianni 2006). Fractal geometry and fractal dimensions have been used to quantify mycelial systems for describing growth patterns and morphology similar to naturally occurring irregular structures (Papagianni 2006, Park et al. 2007). Fractal values have been used to determine complex morphology especially in the industrially well-known fungus, *Aspergillus niger* (Ryoo 1999).

Three dimensional (3D) image analysis of scanning electron microscopy (SEM) images using 3D SEM commercial software packages has become a useful tool, because it provides great magnification scale, high spatial resolution and depth of focus, and hence is widely used for medical, biological and industrial applications (Marinello et al. 2008, Glon et al. 2014).

Termitomyces is a popular genus of wild edible mushrooms. It is characterized by obligate mutualistic symbiosis with Termitidae that belong to the subfamily, Macrotermitinae, distributed in the Asian and African continents (Wood and Thomas 1989, Bingham and Bingham 2002). The genus, *Termitomyces*, comprises 56 species and 86 taxa (http://www.indexfungorum.org) however only 12 species are available as pure cultures in the world culture collections (http://www.wfcc.info). *Termitomyces* fruitbodies are collected in the wild and consumed by locals. These species have high nutritive content (Ogundana and Fagade 1982, Kansci et al. 2003) including macro- and micronutrients (Adejumo and Awosanya 2005, Zoho et al. 2016).

Termitomyces are mutualistic fungi that are maintained in subterranean fungus gardens as sporodochial anamorph by fungus cultivator mound building termites, which control the complex life cycle of the holomorph. Artificial production of *Termitomyces* teleomorph, the much desired edible, protein rich fruitbodies have failed (Heim 1977, Olila et al. 2007). Because artificial cultivation by solid state fermentation is not possible, the only option is to obtain pelletized biomass by submerged culture, which could serve as a source of nutritious mycoprotein (Zhu et al. 2015). *Termitomyces* species produce industrially important enzymes (Kumari et al. 2012, Jonathan and Adeoyo 2011, Faulet et al. 2006) and novel compounds, such as termitomycesphins and termitomycamides, which exhibit neuritogenic activity (Choi et al. 2010, 2012, Qi et al. 2000, 2001, Qu et al. 2012). Pure cultures of *Termitomyces* have been obtained from anamorphic comb sporodochia (Botha and Eicker 1991a, Licht et al. 2005), germination of basidiospores (Licht et al. 2005) or basidiome context tissue cultures (Botha and Eicker 1991b).

Although some culture studies using SEM on comb sporodochial cultures and basidiome context cultures have been conducted (Botha and Eicker 1991a,b), we have found no reports concerning *Termitomyces* pellet morphology and characterization. We applied biometrologic techniques (http://www.digitalsurf.com/brochures/3D_Advanced_Surface_Texture_Mountains_7_Optional_Module.pdf) based on parameters such as furrow mean density, isotropy, number of motifs (open and closed), fractal dimensions using 3D SEM images to obtain fine details of *Termitomyces* pellet typology.

A similar biometrologic approach can be used to investigate hyphal typology of natural comb sporodochia maintained in termite mounds by fungus growing termites, which differentiate to produce fruitbodies only during the monsoon season in India. A biometrological approach may assist identification of common or uncommon cellular elements involved in development and differentiation of the *Termitomyces* from asexual, hypogeal sporodochial stage to sexual epigeal fruitbodies. Detailed analysis of hyphal typology using an approach similar to ours should improve our understanding of the *Termitomyces* holomorph. Availability of SEM image analysis and biometrologic tools, such as MountainsMap® SEM software (Digital Surf), 3D surface modelling (3DSM) (Carl Zeiss Microscopy), MeX (Alicona), Scandium Height (Olympus Soft Imaging Solutions), 3D SEM Three Dimensional Image Software (JEOL), should make it possible to undertake such work in future.

Material and methods

Collection and identification of fruitbodies

Fresh *Termitomyces* fruitbodies were obtained from local habitats of Goa including Banastarim, Corlim, Taleigao, Mardol, Molem, Old Goa, Priol, Santa Cruz and Loliem. Collected fresh fruitbodies were identified by distinct macromorphological characteristics according to standard published *Termitomyces* keys and micromorphological characters such as hymenophoral trama, basidia, cystidia and basidiospores (Botha and Eicker 1991a, Heim 1977, Karun and Sridhar 2013, Morris 1986, Pegler and Vanhaecke 1994, Tibuhwa et al. 2010, 2012).

Source of cultures

Only healthy young *Termitomyces* fruitbodies including *T. microcarpus* (TMI1), *T. albuminosus* (TAL1, TAL2), *T. striatus* (TSTR), *T. aurantiacus* (TAUR), *T. heimii* (THE1, THE2), *T. globulus* (TGLO) and *T. clypeatus* (TCL1, TCL2, TCL3, TCL4, TCL5) were used to obtain pure mycelial cultures. Young healthy fruitbodies were surface sterilized and the internal context tissue was exposed using a flame sterilized scalpel. Small tissue explants (0.5 cm) were removed aseptically and placed on 2% (w/v) malt extract agar (MEA) medium (2% refined bacteriological grade malt extract and 2% bacteriological grade agar) with 0.01 mg/ml conc. nalidixic acid and neomycin (HiMedia Chemicals Ltd., Mumbai, India) to generate fresh vegetative mycelial growth (Stamets and Chilton 1983). Cultures were checked microscopically for purity. The growth plates were incubated in an incubator (Modern Industrial Corp., Mumbai, India) and maintained at 28° C in the dark with weekly inspection for three successive weeks. Working cultures were maintained on fresh 2% MEA plates without antibiotics. Cultures were deposited in Goa University Fungus Culture Collection (WFCC Reg. no. 946).

Submerged growth conditions

Pellet to pellet inoculations were prepared according to Kalisz et al. (1986). Inocula were prepared in 1% (v/v) Czapek Dox liquid medium (0.5% sucrose, 0.2% sodium nitrate, 0.1% dipotassium phosphate, 0.05% magnesium sulfate heptahydrate, 0.05% potassium chloride, 0.001% ferrous sulfate heptahydrate). The medium was adjusted to pH 5.5 using 1 N sodium hydroxide or 1 N hydrochloric acid as needed. Flasks were incubated in dark at 28° C on a rotary shaker (Orbitek model LETT-A; Scigenics Biotech, Tamil Nadu, India) at 150 rpm for 20 days. Pellets were harvested aseptically under laminar air flow using a 100 µm pore size sterile stainless steel mesh and washed three times with sterile distilled water.

SEM of pellets

Pellet samples were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer. After refrigeration for 4 h, the samples were dehydrated through increasing concentrations of ethanol (Tyagi and Malik 2010). Samples were freeze dried in a lyophilizer (Scanvac Coolsafe 110-4; LaboGene, Lynge, Denmark) and mounted using double sided carbon tape on an aluminum stub. Samples were coated with palladium for 10 sec (SC7620 "Mini", Polaron Sputter Coater; Quorum Technologies, Newhaven, England)) and viewed in an SEM operating at 5 KV (Vega 3 SB, TeScan, Brno, Czech Republic).

SEM biometrologic image analysis

SEM images were processed using MountainsMap® 7.3 version SEM software (Digital Surf, Besancon, France) surface imaging and metrology software for 3D SEM biometrologic image analysis. SEM images at 3,000 x were used for measuring furrow density, isotropy, number of motifs and fractal dimensions. For each species three SEM images of pellets were used to calculate each parameter and statistical significance was determined.

Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA) to determine the significance of differences among individual pellets from different species using Tukey's honest significant difference (HSD) test (XLSTAT statistical software package, version 2016.7). Bar graphs and 3D scatter plots were created using SigmaPlot software version 13 (Systat Software Inc., Chicago, IL). Values for $p \leq 0.01$ were considered significant.

Results

Seven species of *Termitomyces* formed pellets after shaking under submerged conditions. Morphological differences among pellets from the different species were not clear from the two-dimensional (2D) SEM images. SEM pellet morphology varied among species from compact, smooth (Fig. 1A, H) to cerebroid (Fig. 1J), spiky (Fig. 1C, K), fluffy (Fig. 1D, F), flaky (Fig. 1B) and aggregates (Fig. 1E, G, L–O). Pellet shapes appeared to be spherical, oval and sometimes irregular. Surface topography of pellets viewed in 3D exhibited microheterogeneity and microporosity differences among *Termitomyces* species (Fig. 1). TMI1 and THE1 exhibited mixed pellet morphology. TMI1 appeared as compact pellets (TMI1a) and flakes (TMI1b), while THE1 formed aggregates (THE1a) and compact (THE1b) pellets. Typological classifications based on furrow mean density and fractal dimension were of two types, types I and II, whereas five types were identified based on isotropy percent and total motifs (Table 1). Typology based on fractal dimensions were classified as type I (2.64–2.71)

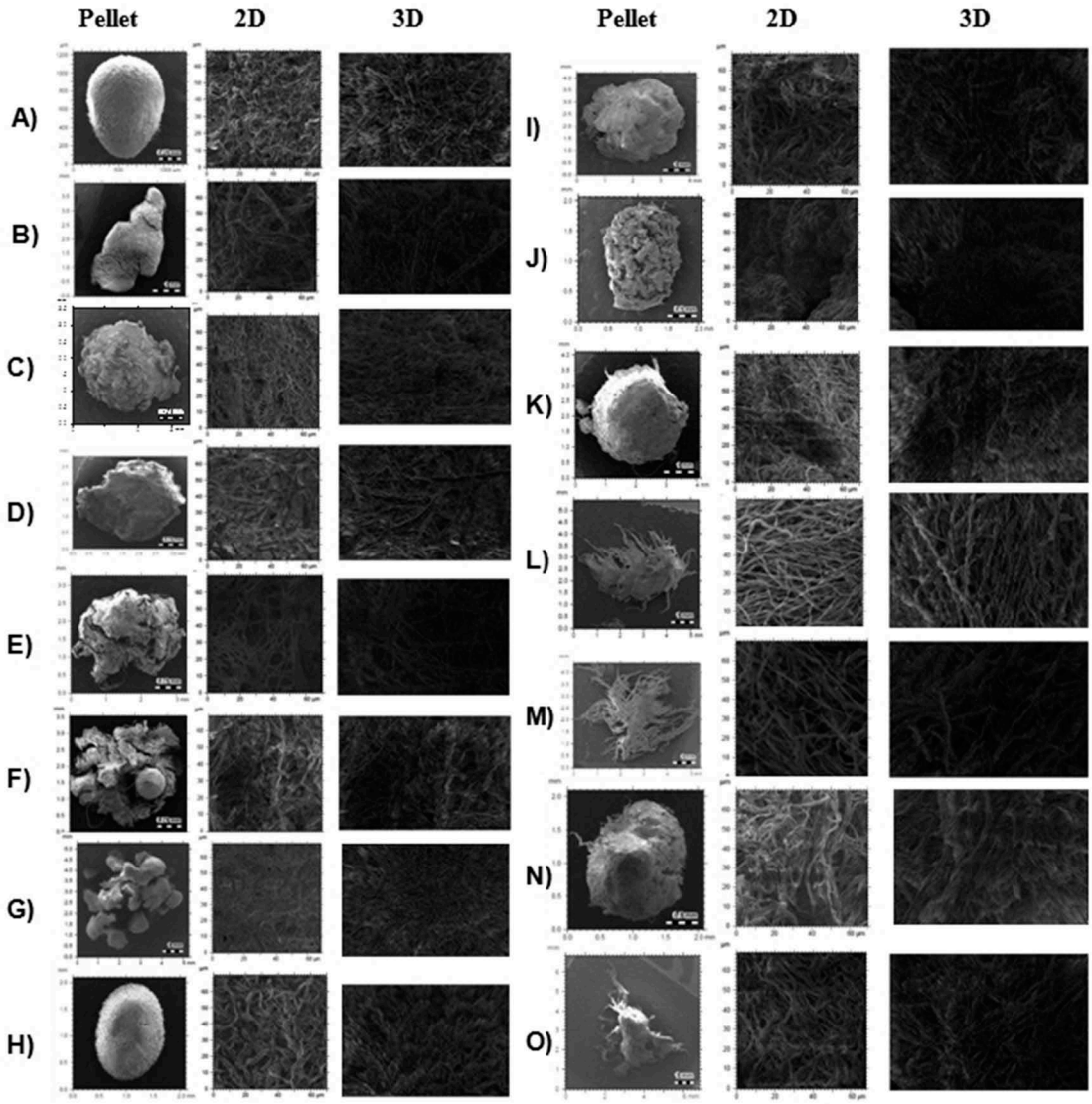

Fig. 1. Scanning electron pellet morphology in 2D and 3D view of *Termitomyces* spp. A) *T. microcarpus* TMI1a. B) *T. microcarpus* TMI1b. C) *T. albuminosus* TAL1. D) *T. albuminosus* TAL2. E) *T. striatus* TSTR. F) *T. aurantiacus* TAUR. G) *T. heimii* THE1a. H) *T. heimii* THE1b. I) *T. heimii* THE2. J) *T. globulus* TGLO. K) *T. clypeatus* TCL1. L) *T. clypeatus* TCL2. M) *T. clypeatus* TCL3. N) *T. clypeatus* TCL4. O) *T. clypeatus* TCL5.

Table 1. Typological classification of pellets based on biometrology

Category	Value	Corresponding strains
Typology based on fractal dimensions		
Type I	2.64–2.71	TMI1a, TMI1b, TAL2, TCL4,TCL5
Type II	2.72–2.78	TAL1, TSTR, TAUR, THE1a, THE1b, THE2, TGLO, TCL1, TCL2, TCL3
Typology based on furrows mean density cm/cm^2		
Type I	> 10000	TAL2, TAUR, THE1b, THE2, TCL2, TCL3, TCL4, TCL5
Type II	> 11000	TMI1a, TMI1b, TAL1, TSTR, THE1a, TGLO, TCL1
Typology based on isotropy %		
Type I	30–40	TSTR, TAUR, THE2
Type II	41–50	TAL1, TCL2, TCL3
Type III	51–60	THE1a, TCL1, TCL4, TCL5
Type IV	61–70	TMI1b, TAL2, THE1b, TGLO
Type V	71–80	TMI1a
Typology based on total motifs		
Type I	1–500	TCL3
Type II	501–1000	TAL2, TSTR, THE2
Type III	1001–1500	TMI1b, TAUR, TCL2, TCL4, TCL5
Type IV	1501–2000	THE1a, THE1b, TGLO
Type V	2001–2500	TMI1a, TAL1, TCL1

and type II (2.72–2.78) whereas furrow mean density was classified into type I (> 10,000) and type II (> 11,000). Five typologies were observed based on isotropy percentage, which included type I (30–40), type II (41–50), type III (51–60), type IV (61–70) and type V (71–80) whereas total motif typologies were type I (1–500), type II (501–1000), type III (1001-1500), type IV (1501–2000) and type V (2001–2500). The furrow mean density in TMI1a, TMI1b, TAL1, TSTR, THE1a, TGLO and TCL1 was high owing to complex network and branching patterns of hyphae seen in 2D images and microporosity seen in 3D images (Figs. 1, 2, 5A). Compact pellets, such as TMI1a and TGLO, and fluffy pellets for TAL2 and aggregates for THE1b exhibited high isotropy, i.e., > 60% belonging to types IV and V (Figs. 3, 5B; Table 1). TMI1a, TAL1, THE1a, THE1b, TGLO, and TCL1 exhibited high motif value (Fig. 4A, C, G, H, J, K), whereas TAL2, TSTR, THE2, and TCL3 formed the least motif values (Fig. 4D, E, I, M). The relation among furrow mean density, isotropy and number of motifs is shown in a 3D scatter plot (Fig. 5E). The fractal dimension value for *Termitomyces* pellets varied from 2.64–2.78 (Fig 5D). Species with isotropy values > 60 and fractal dimensions > 2.72 indicated complex topography, whereas the number of motifs indicates non uniformity/surface heterogeneity. Pellets exhibiting greater surface heterogeneity also exhibited a greater number of motifs (> 1,500) (Fig. 5C).

Discussion

The use of SEM 2D images to reconstruct 3D sample surfaces makes it possible to characterize surface morphology quantitatively (Mikhutkin and Vasiliev 2014). We found that topographic micro-features of pellets are useful markers for selecting fungus strain types in submerged cultures to obtain pellet forms. Biometrologic image analysis could aid quantification of topographic microheterogeneity of pellets. Kim et al. (2002) reported that all mushrooms exhibit pellet growth in submerged culture, but there is little information regarding the environmental factors that affect mycelial growth under submerged conditions. Papagianni (2004) reported various factors that affect pellet morphology in filamentous fungi including size, age, type of inoculum, medium composition, temperature and viscosity of the medium.

Three dimensional SEM based parameters, such as mean furrow density, isotropy value and number of motifs have been applied to different surfaces to understand different topographical features related to specific functions, properties or applications (Mezghani and Zahouani 2004, Schulz et al. 2013, Alipour et al. 2015). Three dimensional SEM images also can be used for surface characterization of *Termitomyces* pellets as shown in present study. Mean density of furrows can be used to quantify the microporous nature of pellets and total number of motifs, which include open and

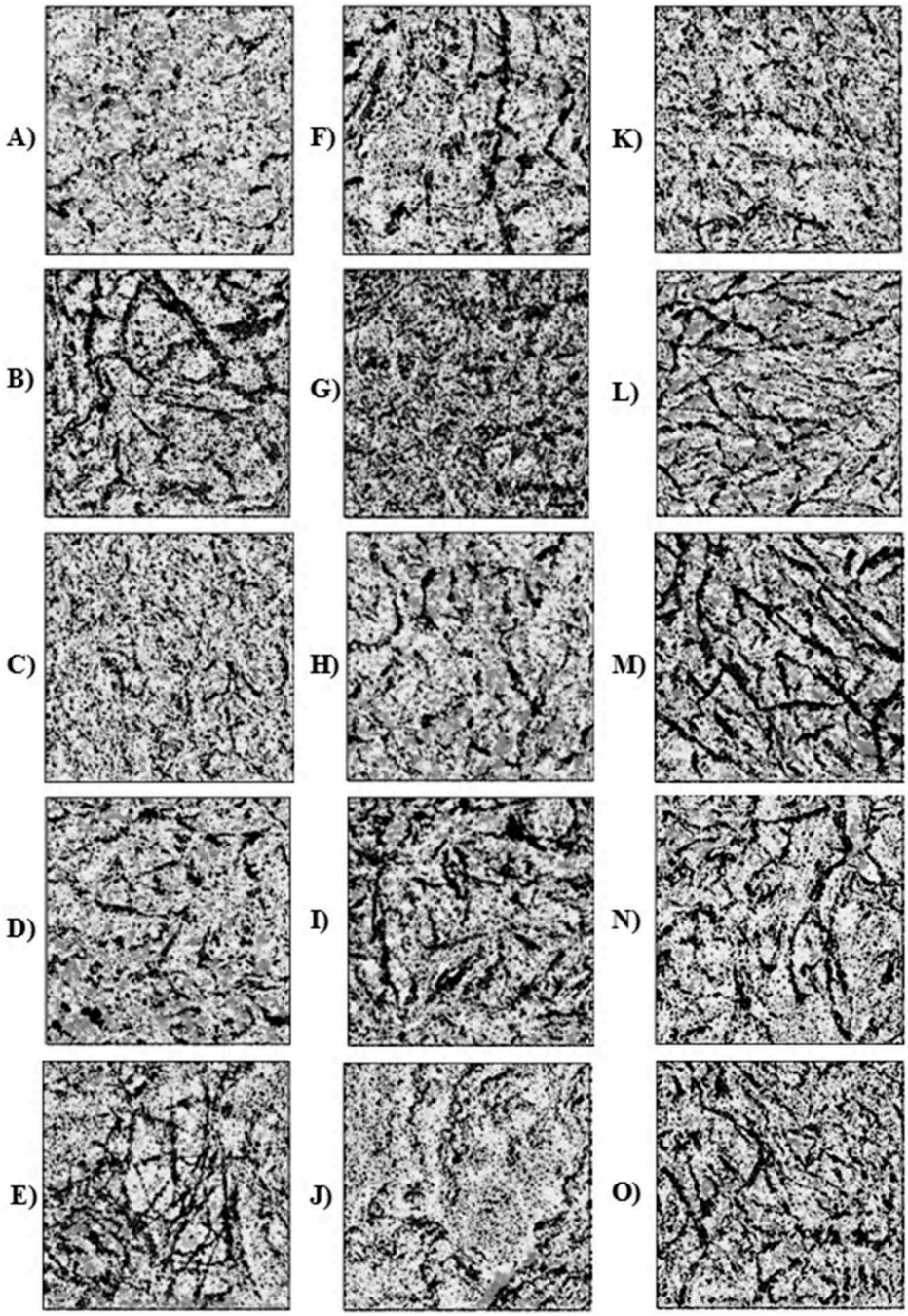

Fig. 2. Furrows view of *Termitomyces* spp. A*) T. microcarpus* TMI1a. B) *T. microcarpus* TMI11b. C) *T. albuminosus* TAL1. D) *T. albuminosus* TAL2. E) *T. striatus* TSTR. F) *T. aurantiacus* TAUR. G) *T. heimii* THE1a. H) *T. heimii* THE1b. I) *T. heimii* THE2. J) *T. globulus* TGLO. K) *T. clypeatus* TCL1. L) *T. clypeatus* TCL2. M) *T. clypeatus* TCL3. N) *T. clypeatus* TCL4. O) *T. clypeatus* TCL5.

closed types and can be used to estimate the total surface area. We found that mean density of pellet furrows and isotropic values were independent of pellet morphology. Only spiky and compact pellets exhibited high total motif numbers, possibly due to the microporous nature of compactly arranged hyphae. Fractal value is a reliable parameter for mycelial morphology, because it is inversely related to the average mycelial growth unit, which is the mean length of hypha (Ryoo 1999).

The fractal dimension depends only on the pellet mass and is not affected by parameters

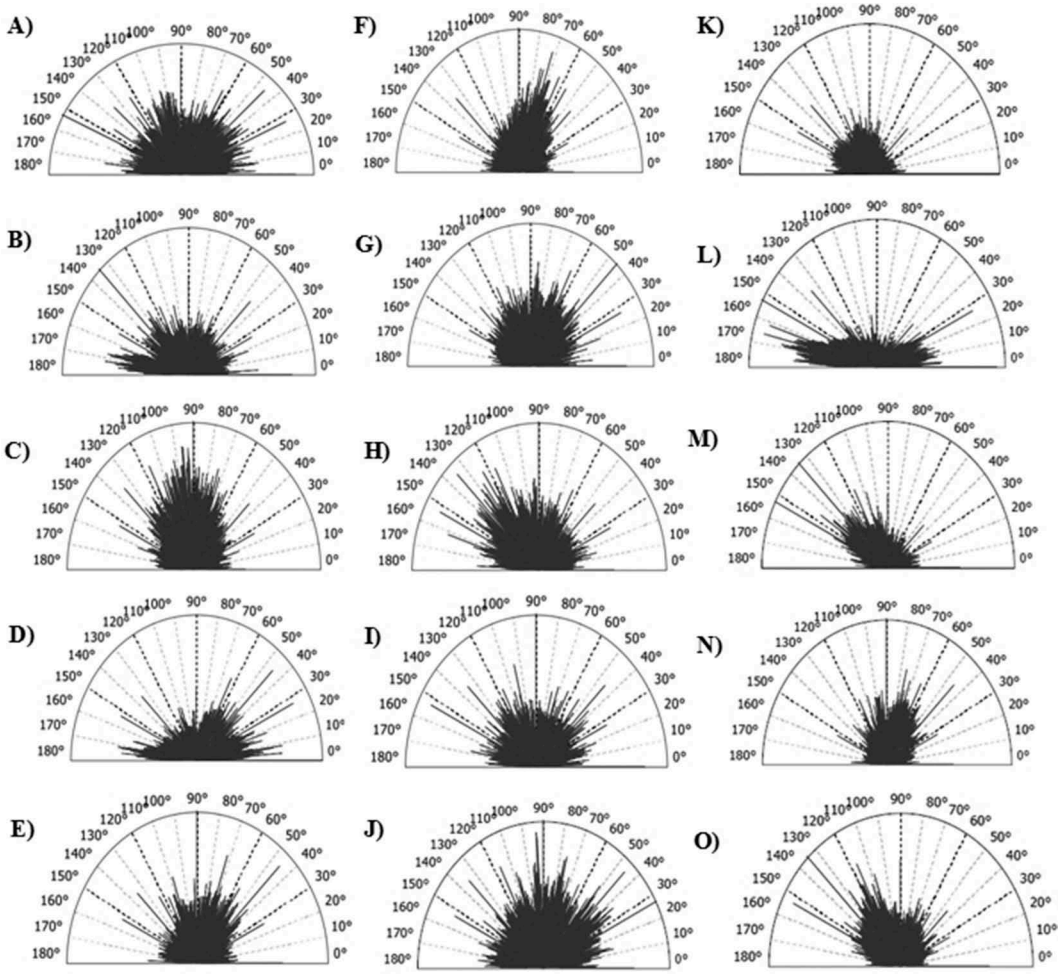

Fig. 3. Isotropy percent views of *Termitomyces* spp. A) *T. microcarpus* TMI1a. B) *T. microcarpus* TMI1b. C) *T. albuminosus* TAL1. D) *T. albuminosus* TAL2. E) *T. striatus* TSTR. F) *T. aurantiacus* TAUR. G) *T. heimii* THE1a. H) *T. heimii* THE1b. I) *T. heimii* THE2. J) *T. globulus* TGLO. K) *T. clypeatus* TCL1. L) *T. clypeatus* TCL2. M) *T. clypeatus* TCL3. N) *T. clypeatus* TCL4. O) *T. clypeatus* TCL5.

such as tip extension rate and branching frequency (Lejeune and Baron 1997). Filamentous suspensions exhibit fractal dimensions of approximately D= 2.5 (Patankar et al. 1992). Fractal dimensions in filamentous morphology that are closer to 3 indicate uniform and homogenous distribution of mycelia in the interior (Patankar et al. 1993).

Our criteria for a model pellet or choice of a strain that produces model pellets are as follows: it should be identifiable from a bank of pure culture strains based on its growth in a defined growth medium under defined growth conditions; it should exhibit consistently stable pellet morphology in terms of shape, size and biomass yield. Also, stable mycelial packing density is important, because it may provide structural heterogeneity and determine oxygen and nutrient uptake, which overall determines yield/unit volume of the medium (Saraswathy and Hallberg 2005). An ideal pellet could be defined using ideal physical parameters, such as spherical, a homogenous pellet population with 10 –20 mm pellet size, lacking appendages, a smooth or finely fibrillose surface and lacking buoyancy. Another characteristic of an ideal pellet is negligible production of potentially viscous extracellular polysaccharides or sediment. Dense mycelial packaging with high microporosity as indicated by high furrow mean density and motif number, surface hyphae that exhibit a fractal dimension > 2.5 and isotropy > 60% as revealed by SEM would be additional characteristics.

We found that for industrial purposes it is advisable to obtain a population of morphologically

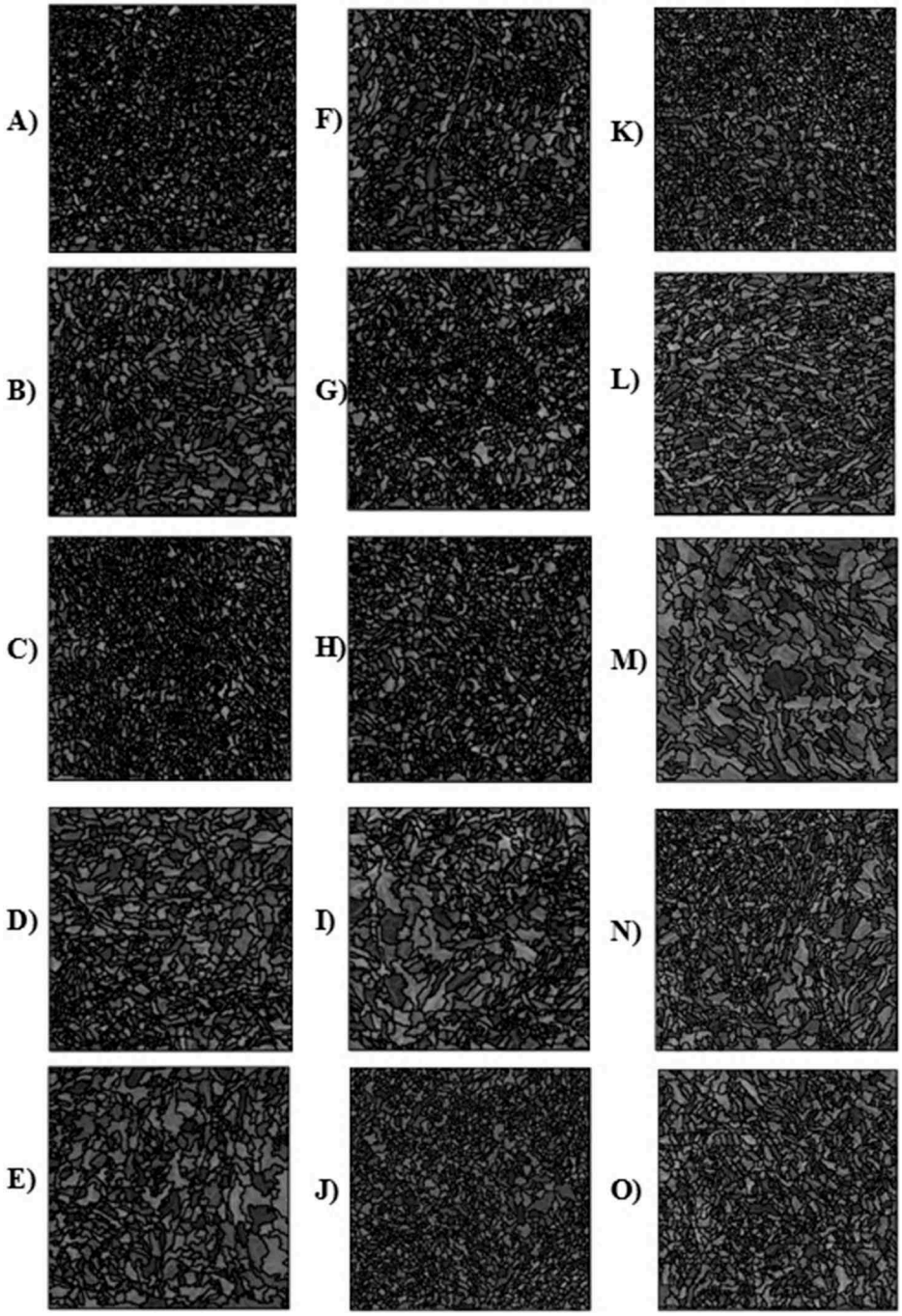

Fig. 4. Total motifs view *Termitomyces* spp. A*) T. microcarpus* TMI1a. B) *T. microcarpus* TMI1b. C) *T. albuminosus* TAL1. D) *T. albuminosus* TAL2. E) *T. striatus* TSTR. F) *T. aurantiacus* TAUR. G) *T. heimii* THE1a. H) *T. heimii* THE1b. I) *T. heimii* THE2. J) *T. globulus* TGLO. K) *T. clypeatus* TCL1. L) *T. clypeatus* TCL2. M) *T. clypeatus* TCL3. N) *T. clypeatus* TCL4. O) *T. clypeatus* TCL5.

homogeneous pellets that meet our definition of an ideal pellet. We also found that typologies used for sporulating fungi based on coagulative (Metz and Kossen 1977, Jimenez-Tobon et al. 1997) and noncoagulative (Znidarsic et al. 1998, Abd-Elsalam 2009) pellet types growing in submerged culture are not useful for mutualistic species such as *Termitomyces,* which is a nonsporulating fungus with a complex life cycle. We found that more typologies are discernible using biometrologic analysis. We agree with Zhang and Zhang (2015), however, that more research on fungal species will

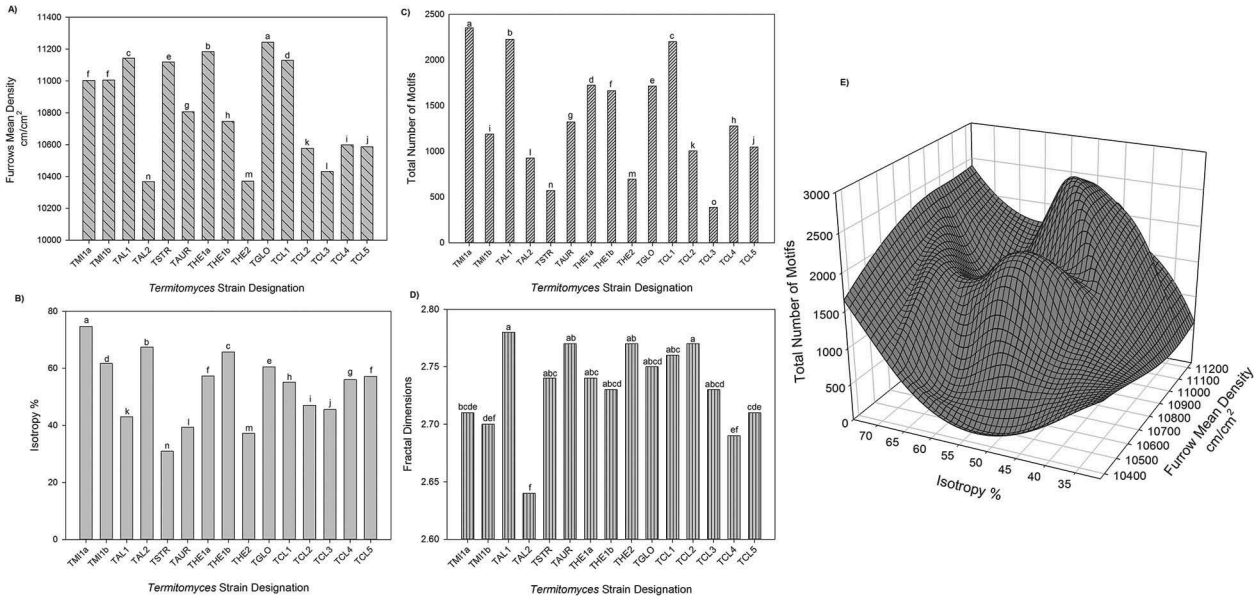

Fig. 5. A) Mean density of furrows (cm/cm^2). B) Isotropy percent. C) Total number of motifs: open and closed types. D) Fractal dimension. Different letters on bar graphs indicate significant difference between groups ($p < 0.01$). E) 3D scatter plot of furrow mean density, isotropy and total number of motifs.

clarify our understanding of the mechanisms of fungal pellet formation.

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