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Research Article

Evaluation and Characterization of Pellet Morphology of Genus *Termitomyces* Heim of a Wild Tropical Edible Mushroom

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ABSTRACT
In view of difficulty of domestication of the tropical, wild edible, *Termitomyces* species, the mycobiont of fungus grower termites were subjected to submerged conditions. The present work was aimed at morphological assessment of pellets obtained from 13 submerged cultures of seven *Termitomyces* species– *T. microcarpus, T. albuminosus, T. striatus, T. aurantiacus, T. heimii, T. globulus,* and *T. clypeatus*, in order to characterize pellet morphology under submerged conditions as a future source of mycoprotein. Pelletization was achieved in 100 ml Czapek Dox Solution, pH 5.5, with 1% v/v inoculum at 150 rpm in dark after incubation for 20 days at 28°C. All species produced good pelletization evidenced by micro and macro pellets. Spiky, loose and dispersed mycelial morphology was common. The most promising strains were *T. albuminosus* (0.72 g/L), and *T. globulus* (0.7 g/L), which produced brown to black, spherical spiky pellets (diameter 1–12 mm), and white to brown spherical oval compact pellets (diameter 3–12 mm) respectively. Pellet morphology of promising strains were analyzed using Digital Image Analysis and Scanning Electron Microscopy. FTIR studies were also carried out for detection of chemical groups of *T. albuminosus*, and *T. globulus* pellets

KEYWORDS: Submerged; palletization; mycoprotein; FTIR; SEM

INTRODUCTION

Despite knowledge of 2000 wild edible fungi in world very few wild edible species have been domesticated [1]. Mushrooms are highly prized for their utilization as nutritional and medicinal foods and to meet the need of future food security of world's growing population. The six big commercial species e.g. *Agaricus bisporus, Lentinula edodes, Pleurotus* spp., *Auricularia* spp., *Volvariella volvacea,* and *Flammulina velutipes* have a major share of global market [2]. Attempts have been made using submerged fermentations to commercially produce useful food and pharmaceuticals [3]. Submerged fermentation is a fast and attractive technology giving rise to potential advantages of higher mycelial biomass production in a compact space and within short time, which is otherwise produced by traditional time and labour consuming methods [4]. However, wild tropical edible mushrooms such as *Termitomyces* spp. popular in sub-Saharan Africa and South East Asia including India for centuries have proved difficult to domesticate. Olila et al. [5] reported failure in domestication of *Termitomyces* due to their complex mutualistic nature [6] leaving the only option of edible biomass production by submerged culture [7]. Nutritionally, these

species indicate a lot of promise for Mycoprotein production as they contain (w/w) 31% protein and 32% carbohydrate [8]. The lipid content of these mushrooms is reported to vary from 2.5–5.4 g/100 g dry weight with high proportion of polyunsaturated fatty acids (45.1–65.1% of total acid methyl esters) and the crude fiber content showed remarkable proportions (17.5–24.7 g/100 g dry weight). The protein content varied between 15.1–19.1 g/100 g dry weight [9]. *Termitomyces* species are also reported as a rich source of useful Cerebrosides –Termitomycesphins, Termitomycamides, saponins, and polysaccharides showing neuritogenic, analgesic, and anti-inflammatory activities [10,11]. This was another attractive impulse which might be important for food processing and biopharmaceuticals industries since submerged culture technique permits the utilization of both mycelial biomass as well as cell free culture filtrate as a source of important biomolecules. The present work was aimed in this direction with focus on morphological evaluation of pelletized biomass to identify strains suitable for future mycoprotein production.

MATERIALS AND METHODS

Cultures

Pure mycelial cultures were isolated from sterile context tissue of healthy specimens of identified [12], wild edible *Termitomyces* samples during monsoon from local habitats in Goa included *T. microcarpus* (TMIC), *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) and were deposited in Goa University Fungus Culture Collection (WFCC Reg. no. 946). Cultures showing good growth and morphologically stable colonies, were maintained on slants on sterile 2% (w/v) Malt Extract Agar medium comprising Malt extract powder and 2% (w/v) Agar bacteriological grade, pH 7.5 at 28±1°C in incubator (Modern Industrial Corporation, Mumbai, India). All chemicals used were obtained from HiMedia Chemicals Ltd., Mumbai, India.

Inoculum preparation and submerged conditions

For purpose of innoculum identical culture discs were aseptically excised using flame sterilized cork borer of 5 mm diameter from peripheral growth zones of the six days old *Termitomyces* colonies grown on sterile modified (5 g/L Sucrose) Czapek Dox Agar containing 0.5% (w/v) Sucrose, 0.2% (w/v) Sodium Nitrate, 0.1% (w/v) Dipotassium Phosphate, 0.05% (w/v) Magnesium Sulfate Heptahydrate, 0.05% (w/v) Potassium Chloride, 0.001% (w/v) Ferrous Sulphate Heptahydrate, and 2% (w/v) Agar bacteriological grade (pH 5.5, at 28±1°C in dark). Ten identical culture plugs were inoculated from each culture into 250 ml Erlenmeyer flask containing 100 ml of the modified Czapek Dox Solution (CDS). Flasks were kept on rotary shaker (Scigenics Biotech, Orbitek model LETT-A, Tamil Nadu, India) at 150 rpm and incubated at 28±1°C, in dark for six days. Mycelial suspensions were prepared by centrifuging liquid cultures on a bench centrifuge at 5000 rpm for 20 min (R-24, Remi Instruments Ltd, Vasai, India) in sterile centrifuge tubes. After removal of supernatant the pellets were rinsed with sterile distilled water and resuspended in approximately the same volume of distilled water, containing 100 sterile glass beads (4.5–5.5 mm diameter, Loba Chemie, Mumbai, India). The suspensions were shaken in an orbital shaker at 300 rpm for 20 min to provide fragmented mycelia suspensions. Inoculum was added (1% v/v) in 100 ml of CDS and kept on shaker at 150 rpm and incubated at 28±1°C, in dark for 20 days.

Characterization of pellet morphology

After 20 days of incubation final pH of each flask was determined (Elico, Water Quality Analyser, PE138, Hyderabad, India) and then the pellets were transferred aseptically to sterile Petri plates in laminar air flow using sterile stainless steel mesh with pore size 100 µm. Pellets were repeatedly washed three times with sterile distilled water to remove any debris or traces of culture filtrate.

Biomass estimation

Dry pellet weight was measured after harvesting fresh biomass by filtration of 100 ml cultivation medium through a pre sterilized stainless steel mesh with a pore size of 100 µm, washed thrice with sterile distilled water and placed on a pre-weighed Whatman filter paper 1 (Wipro GE Whatman products and filter papers), followed by drying at 75°C for 48 h (Bio-Technics, Mumbai, India) and measuring constant weight.

Biomass yield was expressed in gram of dry biomass per liter of culture medium [13].

Viscosity

Culture medium was centrifuged at 5000 rpm for 10 min and this cell free culture medium was used to determine viscosity using Ostwald's viscometer (Borosil, Mumbai, India).

Statistical analysis

The data were analyzed using One-way Analysis of Variance (ANOVA) to determine the significance of individual differences at $p<0.05$ level by the Duncan's multiple range test. All statistical analyses were carried out using XLSTAT statistical software package (Version 2015.6).

Stereomorphology of pellets

Pellet morphology was examined stereo-microscopically (Olympus SZ51, Tokyo, Japan) and characterized as per Lawton et al. [14] and recorded using Nikon Coolpix S9300 camera in order to distinguish different morphological forms based on parameters such as color, shape, size.

Digital Image Analysis (DIA)

Images of pellets from *Termitomyces* species which yielded high biomass were converted to 24 bitmapped images and then processed using SCION image analysis software (USA beta freeware version 4.0.2) to get distinct image panels with respective DIA output-original image, surface pixel plot density (SPPD), histogram profile (HP) and pixel profile plot (PPP) to gain further details of pellet morphology [15].

Scanning Electron Microscopy (SEM) of pellets

SEM studies using technique modified from Tyagi & Malik**,** [16] were attempted only for strains which yielded high dry pellet biomass. Harvested pellets were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer at 4°C for 4 h then dehydrated using ethanol in water series with increasing concentrations of 30%, 50%, 70%, 90%, and final step with absolute ethanol. Samples were stored at -20°C (Modern Industrial Corporation, Mumbai, India) overnight and freeze dried in lyophilizer (Scanvac Coolsafe 110-4, Denmark). Samples were mounted on double side carbon tape on Aluminum stub. Sputter coated with Palladium for 10 s (Quorum SC7620 Sputter Coater, UK) and examined by SEM at 5 kV (Vega 3 SB TeScan, USA).

FTIR characterization of pellets

This technique has been widely applied in biological field for rapid identification of bacteria [17], yeast [18], and fungi [19] using bands diagnostic of cell wall components. Dried biomass was powdered using a sterile porcelain mortar and pestle, transferred to a desiccator to avoid moisture absorption. About 1 mg of pellet dried biomass was used for FTIR analysis. FTIR spectra were recorded between 4000 and 400 cm^{-1} in transmission/absorbance mode on FTIR spectrometer (Shimadzu IR Prestige 21, Japan) averaging of 40 scans. Spectral resolution was 4 cm^{-1}, encoding interval 1 cm^{-1}, Happ-Genzel apodization and scanning speed 2.8 mm s^{-1}. Distinct bands were identified from the spectra.

RESULTS

Characteristic of submerged biomass morphologies and rheology

The present study reports for the first time the successful production of pellets and morphological characterization of 13 cultures of seven *Termitomyces* species in shaken submerged conditions. Figure 1(A-H) depicts morphology of pellets. *Termitomyces* species produced pellets and free mycelial fragments in submerged fermentation. The biomass yield varied across species (Table 1).

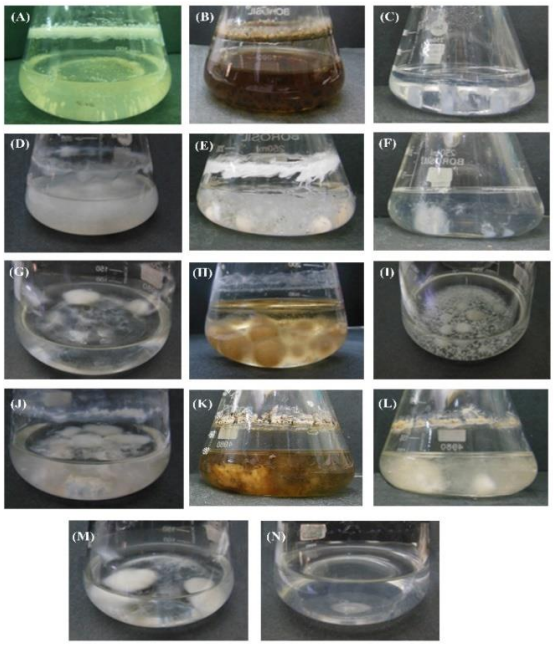

Fig. 1: Submerged *Termitomyces* biomass (**A**) *Termitomyces microcarpus* TMIC; (**B**) *T. albuminosus* TAL1; (**C**) *T. albuminosus* TAL2; (**D**) *T. striatus* TSTR; (**E**) *T. aurantiacus* TAUR; (**F**) *T. heimii* THE1; (**G**) *T. heimii* THE2; (**H**) *T. globulus* TGLO; (**I**) *T. clypeatus* TCL1; (**J**) *T. clypeatus* TCL2; (**K**) *T. clypeatus* TCL3; (**L**) *T. clypeatus* TCL4; (**M**) *T. clypeatus* TCL5; (**N**) Control medium

Table 1: Quantitative aspects of *Termitomyces* pelletization

Strain	Pellet morphology	Pellet size (mm)	Pellet number (Range/ 100ml)	Dry Biomass (g/L)	Viscosity (cP)	Final medium pH
TMIC	Spherical to oval compact smooth pellets, flakes	1–7	173–490	0.20±0.06[1d]	0.86±0.01	8.6±0.09[bcd]
TAL1	Spherical spiky pellets, Aggregates, fragmented mycelium	1–12	146–167	0.72±0.03[a]	0.85±0.04	8.4±0.07[cd]
TAL2	Fluffy pellets	2–10	32–35	0.07±0.04[e]	0.94±0.01	5.2±0.04[g]
TSTR	Aggregates	–	–	0.66±0.03[ab]	0.93±0.04	7.2±0.04[f]
TAUR	Fluffy and smooth pellets	1–7	124–158	0.22±0.07[d]	0.87±0.01	8.6±0.24[bc]
THE1	Fluffy and smooth pellets, flakes, aggregates	1–6	132–406	0.58±0.05[b]	0.87±0.02	8.5±0.05[cd]
THE2	Aggregates	–	–	–	0.89±0.02	3.7±0.02[h]
TGLO	Spherical to oval compact, spiky pellets, dispersed mycelium	3–12	56–69	0.71±0.08[a]	0.85±0.04	8.1±0.54[e]
TCL1	Spherical spiky, fluffy pellets	1–9	529–1286	0.30±0.06[d]	0.93±0.06	9.2±0.02[a]
TCL2	Aggregates, fragmented mycelium	5–60	250–860	0.44±0.04[c]	0.90±0.02	8.9±0.04[b]
TCL3	Aggregates	5–65	34–63	0.44±0.06[c]	0.87±0.01	8.7±0.05[bc]
TCL4	Fluffy and aggregates	3–45	42–58	0.67±0.05[a]	0.91±0.04	8.2±0.15[de]
TCL5	Aggregates, fragmented mycelium	5–80	12–38	0.64±0.06[ab]	0.85±0.01	9.2±0.03[a]

[1]*Values in the column are means of three replicates and same column with different letters are significantly different at 0.05 probability level according to Duncan Multiple Range Test*

Termitomyces strains TAL1 and TGLO was found to produce highest biomass yield 0.72±0.03 g and 0.71±0.08 g dry weight per liter respectively after 20 days of incubation. TAL2 found to produce high viscosity in medium which was recorded as 0.9398 cP and TGLO produced least as 0.8482 cP compared to control medium which showed 0.8229 cP. Pellets with high Exopolysaccharide (EPS) produced high viscosity into medium such as TAL2, TSTR, TCL1, and TCL4 whereas pellets with low EPS produced less viscosity into medium. Pellet number increased with increase in pH of medium from acidic to alkaline except TAL2 (Table 1). Pellet morphology in general appeared spiky, fluffy, compact, and smooth with some irregular aggregates / clumps, flakes, and free mycelium fragments (Table 1 and Figure 2). Among the 13 cultures belonging to seven *Termitomyces* species five strains produced aggregates i.e. THE2, TSTR, TCL2, TCL3, TCL5.

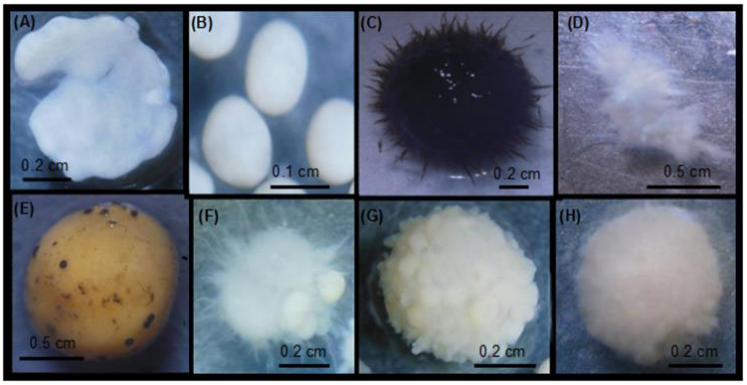

Fig. 2: Stereomorphology of pellets in submerged culture of *Termitomyces* (**A**) Flake; (**B**) Compact oval; (**C**) Melanized, spherical, spiky; (**D**) Aggregate; (**E**) Smooth, oval, compact; (**F**) Spiky with budding compact, smooth; (**G**) Hyaline, fluffy; (**H**) fluffy, spherical, hyaline

Image analysis of submerged biomass

Among all these, two strains TAL1 and TGLO emerged to be candidate strains which were studied in detail. Figure 3 shows their surface variations using DIA. SPPD gave a characteristic cross-sectional view of the smooth, undulating and rough pellet topography. TAL1 showed less number of surface spikes than TGLO on DIA. TAL1 pellets were found to be spherical, reflexed whereas TGLO formed the oval pellets with smooth surfaces based on the SPPD analysis. HP output of both the pellets produced different profiles indicating the distribution of gray values with in the selection. PPP output gives a 2D plot profile with fixed Y values and shows distinct cross-sectional differences of pellet surface.

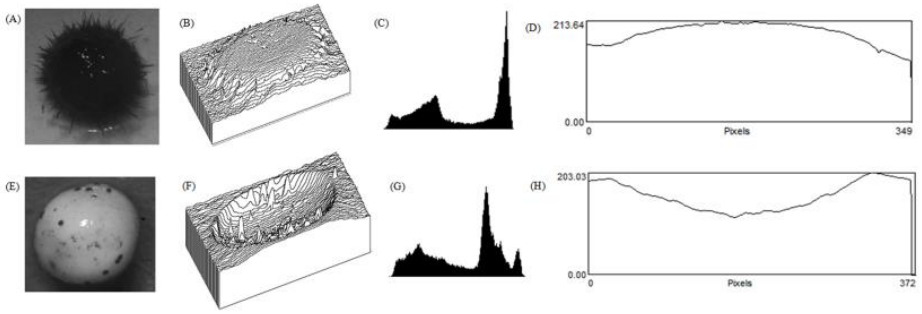

Fig. 3: Distinct digital characteristics of pellets TAL1 (A-D) and TGLO (E-H) (A, E) Processed 24 bitmapped image; (B, F) Surface Plot; (C, G) Histogram Profile; (D, H) Pixel Profile Plot

SEM based pellet tropographic studies

SEM based scans (Figure 4) revealed topographic microheterogeneity of TAL1 and TGLO pellets. TAL1 pellet appeared more porous with lose arrangement of hyphal

morphology whereas TGLO mycelial biomass uniformly cemented with EPS.

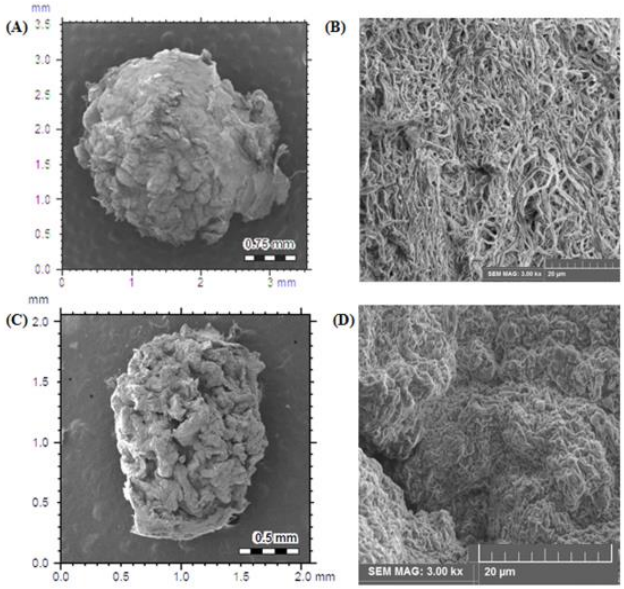

Fig. 4: SEM typology of pellets (**A**) Complex folds on TAL1 pellet; (**B**) Microporous nature of TAL1 pellet; (**C**) Cerebroidal surface of TGLO pellet; (**D**) Microporous nature of TGLO pellet

FTIR based pellet characterization: Figure 5A and 5B shows the characteristic spectra of *Termitomyces* TAL1 and TGLO pellets indicating the distinct diagnostic bands.

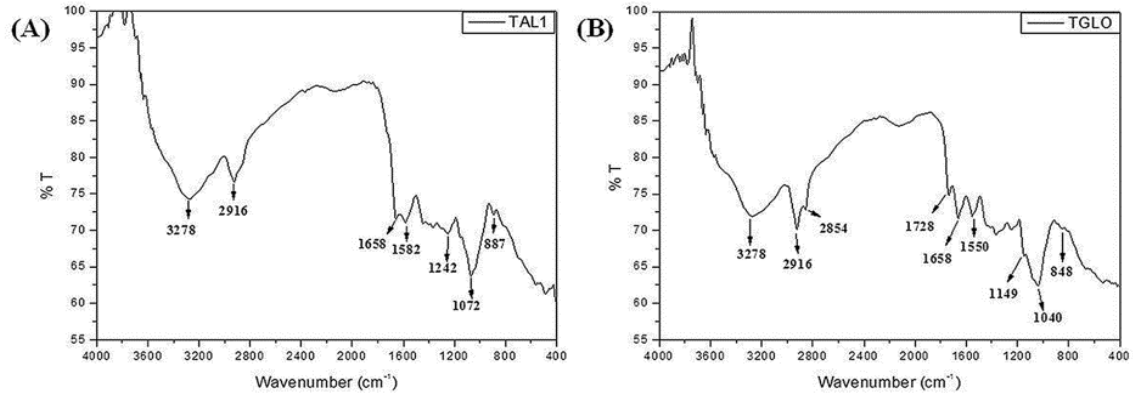

Fig. 5: FTIR spectral characterization of dry pelletized biomass (**A**) *T. albuminosus* TAL1 spectrum; (**B**) *T. globulus* TGLO spectrum

DISCUSSION

Globally only 15 *Termitomyces* species are available in world culture collections. Several popular basidiomycetes species such as *Auricularia polytricha*, *Agaricus brasiliensis* have been subjected to submerged cultivation for wide food, medicinal and industrial applications [20]. These two typical morphologies are known to be produced by most of the basidiomycetes cultures in submerged conditions [21]. According to Horincar et al. [22] *Pleurotus ostreatus* cultivation under submerged condition produced best biomass yield as 7.58 g dry biomass L^{-1} with 0.2% innoculum concentration whereas with 5% innoculum at pH 5.5 yield was found to be 2.25 gL^{-1}. This indicate that type of innoculum used have strong influence on yield of biomass production. High viscosity of the medium is known to be caused by mycelial fragments and the viscous nature is attributed to presumptive exopolysaccharides (EPS) production [23]. The morphological properties in fungal fermentations play an important role in their metabolism during the fermentation process and pellet morphology found to be one of the key factors directly affecting fermentation

productivity [21]. Various features of morphological interest have been incorporated in key given at the end. Using all DIA functions such as SPPD, HP, and PPP gives a whole insight to the cultural morphologies as previously studied [15]. DIA method found to be a potent tool to obtain a wide variety of detailed quantitative structural information of fungal morphologies [21]. FTIR spectroscopy is widely used to monitor quality in food industry. *Termitomyces* species TAL1 and TGLO showed a common band at 1658 cm^{-1} due to amide I / chitin stretching and 3278 cm^{-1} for OH stretching whereas bands at 1582 cm^{-1} and 1550 cm^{-1} is due to N-H bending and C-N stretching in amide II band for TAL1 and TGLO respectively [24]. The band at 1658 cm^{-1} and 1550 cm^{-1} are characteristic bands for chitin and chitosan [25,26]. The absorbance peaks at 1072 cm^{-1} and 1040 cm^{-1} is major structural polysaccharide in mushrooms [27]. TGLO showed band at 1728 cm^{-1} for presence of C=O stretching from esters functional groups such as lipids, fatty acids and carboxylic acids. The C-H stretching at 2854 cm^{-1}, 2916 cm^{-1}, and 887 cm^{-1} and 848 cm^{-1} band is due to N-H wag revealing the presence of carboxylic group [28]. These diagnostic spectral markers can be used to detect any possible biochemical changes of cell wall components [29,30].

A key for morphological characterization of *Termitomyces* pellets

1. Distinct pellets absent ……. 3
2. Distinct pellets present…….4, 5
3. Only mycelial Aggregates……..THE2, TSTR, TCL2, TCL3, TCL5
4. Isomorphic/Uniform pellet morphology……6, 7
5. Polymorphic/ Mixed pellet morphology……8, 9, 10, 11, 12, 13
6. Spiky pellets……..TAL1
7. Fluffy pellets ……TAL2
8. Compact pellets, flakes……TMIC
9. Fluffy and spiky pellets……..TCL1
10. Compact cerebroid and spiky pellets……TGLO
11. Fluffy and compact smooth pellets ……TAUR
12. Fluffy, compact smooth pellets, flakes and aggregates……...THE1
13. Fluffy and aggregates ……TCL4

CONCLUSION

This study revealed that there are morphological changes in pellets of *Termitomyces* species. The knowledge gained is useful for further studies on morphological, ultra structural, and biochemical characterization of pelletized biomass useful for bioprocess standardization and scaled up mass production using standard bioreactors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest in this research article.

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