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Isolation, identification, cultivation and determination of antimicrobial β-glucan from a wild-termite mushroom *Termitomyces heimii* RFES 230662

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ABSTRACT

During the monsoon season, a wild-termite mushroom *Termitomyces heimii* RFES 230662 (THR2) was successfully isolated from Negeri Sembilan, Malaysia. The mushroom was morphologically labelled based on its stipe, pileus, and the budding mycelia from the termites nest. Genetic component of THR2 (300 bp) was sequenced and found to be 99% identical to *Termitomyces* sp. strain. The evolutionary distance (K_{nuc}) of the isolate justified THR2 as *T. heimii* species. Two THR2 extracts were prepared from fruiting body (FB) and mycelial biomass (MB) for antimicrobial responses. THR2 was cultivated in a submerged-fermentation (SF) producing 8.55 g/L of MB, 0.80 g/L of endopolysaccharide (ENS) and 1.44 g/L of exopolysaccharide (EPS), respectively. For THR2-FB, two ENS extracts were obtained using hot (3.20 g/L) and cold-water (1.36 g/L) treatments. FTIR spectra analysis verified all polysaccharide as β-glucan when compared to laminarin standard. Those β-glucan possess antibacterial properties with highest response shown by ß-glucan-FB compared to ß-glucan-MB extracts. These findings serve as the blueprint for the production of β-glucan from a rare termite mushroom.

1. Introduction

The increase in the consumption of edible mushrooms food-based have been reported on a global basis and have gained public concern regarding the dietary and health benefits (Mahamat el at., 2018). One of the edible mushrooms that have been consumed commonly in Asia and Africa is termite mushrooms, scientifically known as *Termitomyces*. *Termitomyces* sp. is classified within the kingdom of fungi in the Basidiomycota division under the family of Lyophyllaceae (Ye et al., 2019; Seelan et al., 2020). The unique name of 'termites mushroom' is derived from the obligated symbiotic or mutualistic growth association behaviour between the mushroom and termites (Aanen et al., 2002; Aanen and Eggleton, 2005). In the symbiotic termites-mushroom growth association behav-

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iour, termites will provide a nest as a substrate for the mushroom to grow. The mushroom, in return, plays its role in digesting the substrates consumed by termites using its secreted lignocellulosic enzyme (Wisselink et al., 2020).

Fruiting bodies of *Termitomyces* mushrooms substantially contain high proteins (27–36%), ascorbic acid (10–18 mg/g) as compared to other edible mushrooms (Nakalembe et al., 2015; Thatoi and Singdevsachan, 2014), ergosterol (Malek et al., 2012; Sargunam et al., 2012) and important minerals like phosphorus, potassium, calcium, copper and iron (Aryal and Budhathoki, 2014). Apart from the reported medicinal values of *Termitomyces* such as rheumatism, lowering blood pressure, controlled obesity, kwashiorkor and treating diarrhoea, *Termitomyces* fruiting bodies are also rich in phenolic compounds (Woldegiorgis et al., 2014) and polysaccharides (Manna et al., 2015) which significantly exhibited antioxidant, antitumor and antibacterial properties (Rashid et al., 2018).

It has been observed that *Termitomyces* spp. contains ethnomedicinal qualities that are advantageous to human health (Singha et al., 2021). In India, *T. heimii* and *T. microcarpus* are used to treat fever, cold, and fungal infections (Venkatachalapathi and Paulsamy, 2016). *Termitomyces heimii* was also reported to act as blood tonics during wound healing and blood coagulation (Chandrawati et al., 2014), while *T. eurrhizus* is for curing rheumatic disorder and diarrhoea and for lowering high blood pressure (Sachan et al., 2013). In Tanzania, *T. microcarpus* are used for boosting the immune system for people with long illnesses, and *T. titanicus*, *T. letestui*, *T. eurrhizus*, and *T. aurantiacus* are as tonics for various gastrointestinal problems, such as abdominal pain, constipation, stomach ache and ulcers (Tibuhwa, 2012).

In Malaysia, *Termitomyces* mushrooms or locally known as '*cendawan busut*' and ''*kulat pusu*'' are seasonal, where the mushrooms grow effectively in the late rains season (September–December). However, because of the delicacy taste and seasonal factors, most collected mushrooms were normally consumed by the collectors (Ghorai et al., 2009). They gathered this termite mushroom after heavy thunderstorms and emerged in colony on termite nest. Only the long branch fruiting body can be eaten and sustain for only 4–5 days (Hamzah and Mohammad, 2021).

The earliest isolated Malaysian *Termitomyces heimii* Natarajan reported twenty-eight compounds of fatty acids (linoleic acid) and ergosterol were contained in the fruiting bodies extract (Malek et al., 2012). Ergosterol may act as a biological precursor to Vitamin D_2 which deficient in mushrooms. The ergosterol can formed viosterol by ultraviolet light, irradiation temperature and moisture (Jasinghe et al., 2007) which will then converted to ergocalciferol, a form of vitamin D_2 and applied in the pharmaceutical applications and food supplements (Malek et al., 2012).

Mushroom polysaccharides are a carbohydrate polymer generated from the cell wall of a mushroom. One of the polysaccharides found in mushroom fruiting bodies is β-glucan. Polysaccharides from fungal fruiting bodies and mycelium are known as endopolysaccharides (ENS). Exopoysaccharides (EPS) are polysaccharides that have dispersed throughout the liquid culture broth during fermentation process (Wan-Mohtar et al., 2016; Wiriya et al., 2014). Mushroom β-glucan possess a strong antibacterial, antiviral, and antitumor properties action due to the activation of the host's immune system (Villares et al., 2012; Friedman, 2016). Because of the vast spectrum of health benefits provided by mushrooms β-glucans, it could be used as a functional food, dietary supplement, or nutraceuticals (Giavasis, 2014).

Traditionally, mushroom fruiting bodies have been grown on artificial solid media, where this solid-state media cultivation method requires extensive incubation time to produce the first fruiting body. The cultivation of *Termitomyces* fruiting bodies tissue was reported as difficult to cultivate (Malek et al., 2012). However, the cultivation technique for mycelium growing was successfully reported recently (Tibuhwa, 2012; Hsieh et al., 2017; Yang et al., 2020). The production of *T. microcarpus* were only successfully grown into cultures and spawn from the basidiospore prints (Olila et al., 2007). This proved that the cultivation of *Termitomyces* fruiting bodies under artificial cultivation has been minimal; thus, more research work is needed in exploring the growth of the *Termitomyces* spp. mycelium under artificial medium.

It was previously reported that the mycelia of *Termitomyces* has been successfully grown on agar media (Hsieh et al., 2017). Due to the long cultivation period and unidentical substituents and lack of reproducibility from batch to batch of solid-state cultivation method, submerged-fermentation (SF) has been suggested as an alternative method to reduce the lack of reproducibility and maintain the identical compound substituents (Wan-Mohtar et al., 2016) in mushroom cultivation. SF could be beneficial to overcome some other difficulties related to the seasonal harvesting activities of *Termitomyces* mushrooms where the bioactive compounds from the fruiting bodies could be obtained continuously without facing the seasonal issues. SF offers a great deal in the cultivation method where the cultivation could be carried out within a shorter incubation time and less risk of contamination (Ahmad et al., 2015).

The aim of the present study was to isolate wild termite mushrooms and identify it using morphological, polymerase chain reaction (PCR) molecular sequencing and phylogenetic Molecular Evolutionary Genetic Analysis (MEGA) software. The mycelium of the isolated termite mushroom strain was cultured in SF to produce the mycelial biomass (MB) which was used for the extraction of β-glucan, subsequent spectrophotometrical characterization using Fourier-Transform Infrared Spectroscopy (FT-IR) and antibacterial analysis. To the best of our knowledge, this is the first report on the cultivation of *Termitomyces heimii* mycelia in SF for the production of mycelial biomass and β-glucan polysaccharide with antibacterial properties.

2. Materials and methods

2.1. Fungal source

Wild termite mushrooms *Termitomyces heimii* RFES 230662 (THR2) were isolated from Kg. Kuala Sungkak, Kuala Pilah, Negeri Sembilan, Malaysia at coordinate 2°45′06.6″N 102°16′49.0″E as shown in Fig. 1a. The strain was morphologically identified based on its stipe, pileus and the budding mycelia from the termite nest as according to Hsieh et al. (2017) and Jannual et al. (2020).

Fig. 1. (a): The locality of wild termite mushroom *Termitomyces heimii* strain RFES 230662 (THR2) found at Kg. Kuala Sungkak, Negeri Sembilan, Malaysia (red circle indicates the coordinates 2°45′06.6″N 102°16′49.0″E*), Source: Google Earth. (b): fruiting bodies of wild termite THR2 mushroom emerged on the surface of the soil; (c): Cross-section of soil-wild termite THR2 mushroom was observed growing from the termite nest; (d): termite nest; (e): inner comb of termite nest; (f): soils and cracked termite nest with white termites.
*Reported weather on 15th September 2019: isolation time (10 a.m. - 12 p.m.); humidity (84%); average temperature (26–33 °C); Cloudy; Southwest Monsoon (May–September), Source: weather.atlas.com.. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.2. Cultivation mushroom mycelium on selective medium

The fruiting bodies were washed with Clorox and rinsed with sterile distilled water and their internal tissues was cut using a sterile scalpel and placed onto Potato Dextrose Agar (PDA), 30 g/L added with 2% (w/v) yeast extract (YE). The mycelium grown after full plate obtained were sub-cultured onto another PDA + YE and stored at 4 °C for a long term used (Ahmad et al., 2018). Manipulation of selective agar medium composition for the enhancement of the mycelium growth were carried out and the formulation of the selective medium are shown in Table 1. Pure cultures of the mushroom mycelium were maintained and deposited at the Halal Action Laboratory, Kolej GENIUS Insan, Universiti Sains Islam Malaysia.

2.3. Molecular identification

2.3.1. gDNA extraction

Mycelium fine powder (50 mg) were suspended and lysed with 400 μL of Lysis solution (SLS), added with 20 μL of Proteinase K; mixed vigorously by pulsed vortexing for 5 s. The sample was incubated at 65 °C for 30 min, centrifuged at 11 000 rpm for 1 min. The lysed sample (supernatant) was added with 200 μL of binding solution (SBS); mixed by pipetting up and down several times. The sample was added with 650 μL of washing solution (MS), centrifuged at 11 000 rpm for 1 min and this step was repeated one time. DNA was precipitated using ethanol (95%) and washed three times in ice-cold ethanol (70%). The sample was centrifuged again at 11 000 rpm for 2 min to remove all traces of ethanol. The washed DNA was dried, dissolved in Tris-EDTA (100–200 μL) and stored at −20 °C (Supramani et al., 2019A).

Table 1
Different composition of fungal-growth medium for the enhancement of wild termite mushroom *Termitomyces heimii* strain RFES 230662 mycelium in a solid-state fermentation.

Formulated Medium	Composition	Amount (g/L)			Plate morphology
		PDA[a]	YE[b]	ME[c]	
Medium A	PDA	39	–	–	Thin mycelium, white colour mycelium
Medium B	PDA + YE	39	4		Thin mycelium, white colour mycelium
Medium C	PDA + ME	39	–	4	Somewhat compact mycelium, white colour mycelium
Medium D	PDA + YE + ME	39	4	4	Compact and dense mycelium, white colour mycelium

[a] Potato Dextrose Agar.
[b] Yeast extract.
[c] Malt Extract.

2.3.2. PCR amplification

The part of the nuclear ribosomal region including internal transcribed spacer (ITS) was amplified and sequenced using universal primers ITS1 (5′-CTTGGTCATTTAGAGGAAGTAA-3′) forward primer and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′). First, the solution (400 μL) was added to PCR tube (Eppendorf, Hamburg, Germany). Then, 0.5 *p*mol of ITS1 and ITS2 was added following by MyTaq red mix bioline (MyTaq red reaction buffer contains dNTPs, $MgCl_2$ and enhancers at optimal concentrations) supplied with water. The following process was done for performing PCR: for initial denaturation 1 cycle (98 °C for 2 min); for annealing and extension 25 cycles (98 °C for 15 s; 60 °C for 30 s; 72 °C for 30 s) and for final extension of the amplified DNA 1 cycle (72 °C for 10 min).

2.3.3. PCR-amplified product purification and sequencing

According to the manufacturer's protocol, an InnuPREP DOUBLEpure Kit (Applied Biosystem, ABI) was used the PCR products were purified and directly sequenced using 96-capillary 3730xl DNA Analyser.

2.3.4. Gel electrophoresis

The base pair of wild *Termitomyces* sp. was estimated using agarose gel electrophoresis under UV light. Before loaded into the wells of the prepared agarose gel, the PCR product was mixed with loading dye (bioline) and 100 bp ladder was used. Tris-Borate EDTA (TBE) buffer was used as the running buffer and the electrophoresis was run for 1 h.

2.3.5. Data analysis

The obtained gDNA was entered into BLAST. The NCBI Nucleotide Collection (nr/nt) database was selected and the query was submitted. Sequences producing significant alignment were identified, and the top 10 hit blast was selected for Multiple Sequencing Alignment (MSA) using Clustal Omega (Park et al., 2004).

2.3.6. Phylogenetic analysis

By using the neighbouring-joining (NJ), evolutionary distance (K_{nuc}) of identical fungal species was calculated in Molecular Evolutionary Genetic Analysis (MEGA-X), and a phylogenetic tree was generated. The species with closest K_{nuc} was considered as the same species (Park et al., 2004; Siddiquee et al., 2015).

2.4. Submerged fermentation (SF)

SF was carried out for the growth of mushroom mycelium and the production of polysaccharide from THR2 mycelium in liquid medium. A 5 mm plug of THR2 mycelia was removed with a cork borer from 7 day-old-culture of agar medium. The mycelia cut (10 plugs) were transferred into liquid medium consisted of glucose (30 g/L), yeast extract (1.0 g/L), KH_2PO_4 (0.5 g/L) K_2HPO_4 (0.5 g/L), $MgSO_4$ (0.5 g/L) and NH_4Cl (4.0 g/L), incubated in orbital shaker for 7 days at 28 °C, 150 rpm for mycelia growth (Ahmad et al., 2015). After 7 days, the mycelial were harvested, filtered using Whatman No 1 filter paper. The mycelium obtained were dried using oven (50 °C) until constant weight. Dried mycelia were then used for endopolysaccharide (ENS) hot-water and cold-water extraction method. Supernatants from SF were used for exopolysaccharide (EPS) extraction using ethanol (Fournier et al., 2001).

2.5. Extraction of exopolysaccharide and endopolysaccharide

Endopolysaccharide were extracted from both dried FB and MB. Briefly the dried FB and MB were extracted with distilled water at the ratio of 1:15 (w/v) with a specific temperature of 100 °C (hot water extraction) and 27 °C (cold water extraction) for 3 h in water bath (Ahmad et al., 2015). The extracts were cooled, filtered using filter paper (Whatmann No.1) and filtrate solution were mixed with absolute ethanol at the ratio of (1:4) (v/v) and kept constant at overnight at 4 °C in refrigerator (Fournier et al., 2001). The endopolysaccharide precipitate obtained were collected and dried at 50 °C in the oven yielding endopolysaccharide (ENS). For exopolysaccharide, the supernatant from submerged fermentation were mixed with 4 times of absolute ethanol (1:4 ratio), stirred vigorously and kept overnight at 4 °C (Fournier et al., 2001). The precipitated polysaccharide were collected and dried at 50 °C in the oven yielding exopolysaccharide (EPS).

2.6. β-glucan determination using Fourier-Transform Infrared Spectroscopy (FTIR)

The crude ENS, EPS, ENS-FB (hot water) and ENS-FB (cold water) were characterized using FTIR analysis, and the frequency range are measured as wave numbers in the range of 4000–650 cm^{-1}. Briefly, the samples were placed on the clean window of Agilent Cary 630 equipped with diamond ATR (Attenuated Total Reflectance). Then, the pressure clamp was closed until a click was heard and analysed using a real-time Micro-Lab software.

2.7. Determination of antibacterial properties

2.7.1. Test microorganisms

Five standard bacterial species, *Escherichia coli* (*E. coli*), *Ralstonia* sp., *Salmonella* sp. *Staphylococcus aureus*, and *Streptococcus* sp. were used as test organisms. The bacterial species were grown in 10 mL Nutrient broth (NB) (Sigma-Aldrich, Dorset, UK), at room temperature for 24 h and the test organisms were adjusted the concentration at 1.0 OD. Bacterial samples were provided by the Functional Omics and Bioprocess Development Laboratory, Biotechnology Program, Institute of Biological Sciences, Faculty of Science, Universiti Malaya, Kuala Lumpur, Malaysia and Department of Biotechnology, Manipal International University, Nilai, Malaysia.

2.7.2. Antibacterial analysis

Antibacterial activity of the aqueous extract were tested against two Gram positive bacteria and three Gram negative bacteria. Approximately, 100 μL of the bacteria liquid culture, grown in NB (13 g/L) (Sigma-Aldrich, Dorset, UK), for 24 h. The bacterial culture were then transferred onto the middle of nutrient agar (NA) (Sigma-Aldrich, Dorset, UK) plate. The bacteria were spread using 'L' shaped hockey stick (spread plate technique). Sterile paper discs were immersed in the crude ENS, EPS, ENS-FB (hot water) and ENS-FB (cold water) (1 mg/mL) and placed on the NA plate and labelled. Antibiotic (Penicillin-streptomycin solution ATCC® 30–2300™) was used as positive control while sterile distilled water was used as negative control. The bacteria plates were incubated at 37 °C for 24 h and the zone of inhibition was measured in millimetre (mm) (Ramesh and Manohar, 2010).

2.8. Statistical analysis

Values are expressed as mean ± standard deviation. Results obtained were statistically analysed by using one-way ANOVA with $p < 0.05$ was considered a significant value using Minitab software.

3. Results and discussion

3.1. Isolation and morphology observation of Termitomyces mushroom fruiting bodies

The isolation of wild termite mushrooms (THR2) was carried out during Southwest Monsoon in September 2019 (Fig. 1A). At the sampling site, the fruiting bodies of the wild termite mushrooms (THR2) were found scattered on the surface of the soil in high humidity and low range of temperature (Fig. 1B). From the weather analysis data, it was reported that a day before the isolation, Kuala Pilah had two times thunderstorms at 12 midnight and noon respectively, causing the high humidity, between 82 and 87%, range of average temperature of 25–28 °C (Source: weather.atlas.com). The high humidity weather a day before isolation with heavy rain probably provide a suitable condition for the stipe continuously growing until it emerged on top of the soil. This environment condition was similar to the previous work suggested that during rainy periods, the fungal mycelium of the termite combs produces mushrooms, eventually penetrate the termite nests and soil to spread their spores (Frøslev et al., 2003).

People frequent mistakenly believe that *Termitomyces* mushroom was developed from soil containing a termite colony based on the surface of the soil observation. In this research, we have successfully done the soil's cross section (Fig. 1C) to observe where the stipe budding originated. It was clearly shown that the fruiting body stipe was growing from the termite nest, pass through the soil and emerged on the surface of the soil as shown in Fig. 1C. The size of termite nest observed was between 10 and 15 cm diameter (Fig. 1D), white-brownish colour with non-matured stipe meanwhile black coloured nest with mushroom matured stipe (Fig. 1C). The inner of the fungus-growing termite nest looks like a comb covered with accumulated mycelium (Fig. 1E).

Previous research has reported that *Termitomyces* mushroom growing in the form of basidiomes developing from comb inside the nest not from the outside layer of the nest (Wisselink et al., 2020). The substrate compound in the termite nest would be the plant litter decomposed by *Termitomyces* secreted enzymes (Yamada et al., 2005), grasses, leaves, woody debris and forest litter (Pennisi, 2015). It also reported that fungus-growing termites consumed 90% of the dry woody litter in a tropical area of Kenya (Buxton, 1981). All the substrate compound collected by the old workers forage termites (Yang et al., 2020) were then depolimerized, degrade and mineralized the plant cell wall polysaccharides including hemicellulose, cellulose, lignin and pectin by lignocellulosic enzymes like cellulases, amylase, and xylanase produced by *Termitomyces* fungi (Liu et al., 2019). Fig. 1F shows the collected cracked termite nest, and the termites (white termites) or known as young workers to build the combs (Yang et al., 2020) have proven that both organisms grow together in the termite mound. The mutualistic symbiosis between *Termitomyces* mushrooms and termites has been reported widely (Turnbull and Watling, 1999; Wisselink et al., 2020). Soils and crack termite nest together with termites colonies observed in this study indicate that the termites' association in the growing phases of wild termite mushrooms occurs. Thus this observation proves the mutualistic symbiosis between *Termitomyces* mushroom and termites colony.

Fig. 2 shows the morphology stages of hyphal growing onto the termite comb. The accumulated mycelium onto the termite comb (Fig. 2A) and on-growing tips of hyphal with pin-holes (Fig. 2B) suggested that the absorption of the nutrients/substrates from the termite's nest by the mycelial and grows until it becomes matured stipes as shown in Fig. 2C. The morphology of wild THR2 fruiting bodies are shown in Fig. 2D. Morphologically, wild THR2 mushroom can be distinctively characterized by its long white-yellowish stipe with a small umbrella-shape of prominent perforatorium (pileus) on the tip of the long stipes (Fig. 2E) with white gills which centrally attached to the stipes. The isolated wild THR2 mushroom fruiting bodies exhibited different heights of fruiting bodies where the range was between 10 and 30 cm height as shown in Fig. 2E.

3.2. Cultivation of mycelium onto agar medium

The isolated fruiting bodies of THR2 were subsequently cultivated after collecting from the sampling site to maintain the freshness of the inner tissue of the fruiting bodies. The cultivation of the mycelium were conducted as followed the method of (Hsieh et al., 2017) with slight modification. Since scientific reports on the cultivation of *Termitomyces* mycelium onto agar medium were very limited (Wiriya et al., 2014; Hsieh et al., 2017; Yang et al., 2020), thus this research attempted to manipulate the composition of the agar medium (Table 1) to enhance the growth of the THR2 mycelium. This data will be the preliminary data to develop a culture method for *Termitomyces* edible mushroom. Table 2 demonstrated the diameter of the mycelium with different incubation period and compositions of selective agar medium. For PDA medium only, the highest mycelium diameter achieved was 5 mm from day 9–11. The fungal-growth medium of PDA + YE showed the most increased diameter of 29 mm at day 16 of incubation. For Medium C (PDA + ME), the diameter was 20 mm at day 9 and increased to 29 mm at day 16. It was observed that THR2 mycelium had the most

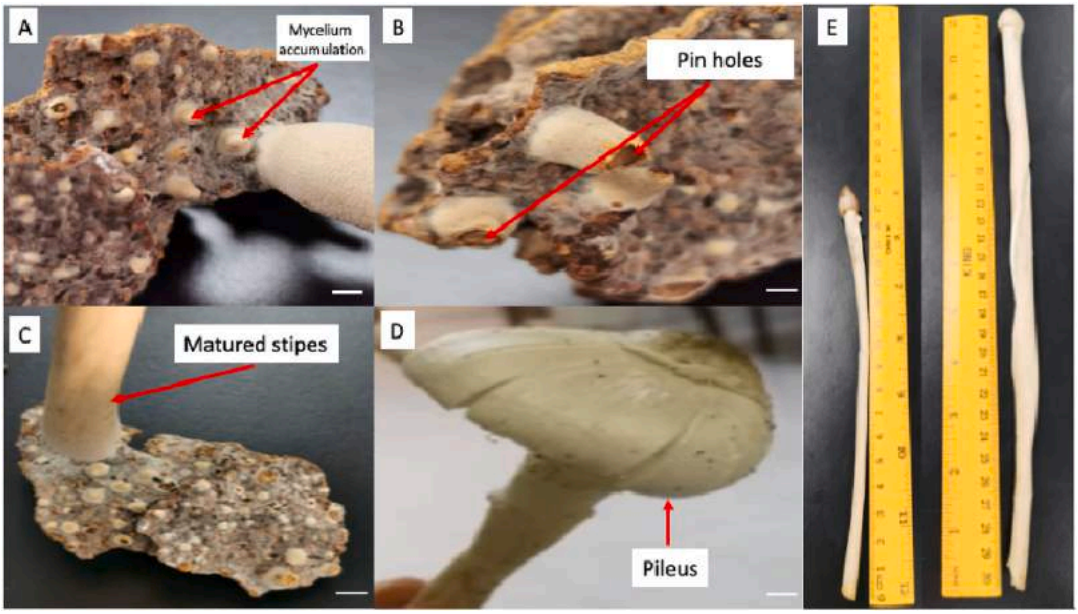

Fig. 2. (a): Accumulation of budding mycelium forming hyphal on top of the termite nest; (b): on-growing hyphal absorbed nutrients from termite nest and producing hyphal with pin-holes; (c): matured stipes of wild termite mushroom fully grown onto termite nest; (d): morphological structure of the prominent perforatorium (pileus), white gills under the pileus and centrally attached stipes (bar = 1 cm); (e): different height of matured stipes fruiting bodies range between 10 and 30 cm.

Table 2
Performance of different fungal-growth medium on wild termite mushroom *Termitomyces heimii* strain RFES 230662 mycelium diameter at different incubation time.

Incubation time	Medium Composition	Mycelium diameter
(Day)		(mm)
9	PDA	5 ± 0.8
	PDA + YE	17 ± 1.0
	PDA + ME	20 ± 1.0
	PDA + YE + ME	29 ± 1.3
11	PDA	5 ± 0.7
	PDA + YE	19 ± 1.0
	PDA + ME	25 ± 1.2
	PDA + YE + ME	33 ± 1.0
14	PDA	5 ± 0.6
	PDA + YE	25 ± 1.2
	PDA + ME	30 ± 1.0
	PDA + YE + ME	FP[a] (>33)
16	PDA	5 ± 0.2
	PDA + YE	29 ± 1.0
	PDA + ME	FP[a] (>33)
	PDA + YE + ME	FP[a] (>33)

[a] FP = full plate. Values are the average of triplicates with standard deviation (SD).

significant growth on the combination of PDA + YE + ME with the diameter observed 29 mm at day 9 of incubation, and keep on increased to 33 mm on the day 11 and reached full plate (45 mm) in a day of 14.

In this research, the selective medium of PDA + YE + ME was found to be the best medium where it could promote high mycelium density with a reasonable growth period within 14 days of cultivation. From the results, it was clearly showed that different combination of selective culture media exhibited different performance of the mycelial growth. This results was supported by previous work which described that different culture media has greatly influence the mycelial growth onto agar medium (Wiriya et al., 2014; Landingin et al., 2020). This is mainly because the different composition of fungal-growth medium provide different concentration of carbon and nitrogen source which essential for the growth of microorganisms (Wiriya et al., 2014). The PDA used act as carbon sources meanwhile malt extract and yeast extract play a role as nitrogen sources which significantly helps in enhancing the radial growth of the mycelial (Landingin et al., 2020).

3.3. Molecular identification

Molecular identification of a wild fungal sample is vital to determine the species of the isolated wild mushroom. Thus, molecular identification was performed on the isolated wild *Termitomyces heimii* RFES 230662 (THR2). The base pairs of THR2 was estimated

using agarose gel electrophoresis under ultraviolet (UV) light (Fig. 3). The marker (Lane 1) represented the standard curve and the estimated base pairs of RFES 230662.

Upon sequencing the product, it aligned with the top-10 related species as retrieved from NCBI BLAST. Based on the BLAST reference databases, RFES 230662 was found to be 99% similar to *Termitomyces* sp. Detailed phylogenetic analyses (Fig. 4) showed the evolutionary distances (K_{nuc}) values. Clade A showed that *Termitomyces* sp. RFES 230662 was closely related to *Termitomyces heimii*.

3.4. Production of endo and exopolysaccharides

SF was further carried out to determine the ability of the isolated mycelium (THR2) for growing and producing polysaccharides in liquid medium, as shown in Table 3. The medium was supplemented with a carbon source, nitrogen source and other micronutrients to support the growth of the THR2 mycelium. Table 3 demonstrated the production of mycelium biomass, ENS, and EPS after 7 days of incubation in batch SF. These results proved that THR2 mycelium was able to grow in SF and produced endopolysaccharide (ENS) and exopolysaccharide (EPS). In our research, the liquid medium which contained glucose act as carbon source was found to support the growth of mycelial biomass up to 8.55 g/L after 7 days of incubation. This is similar with previous work which reported that the

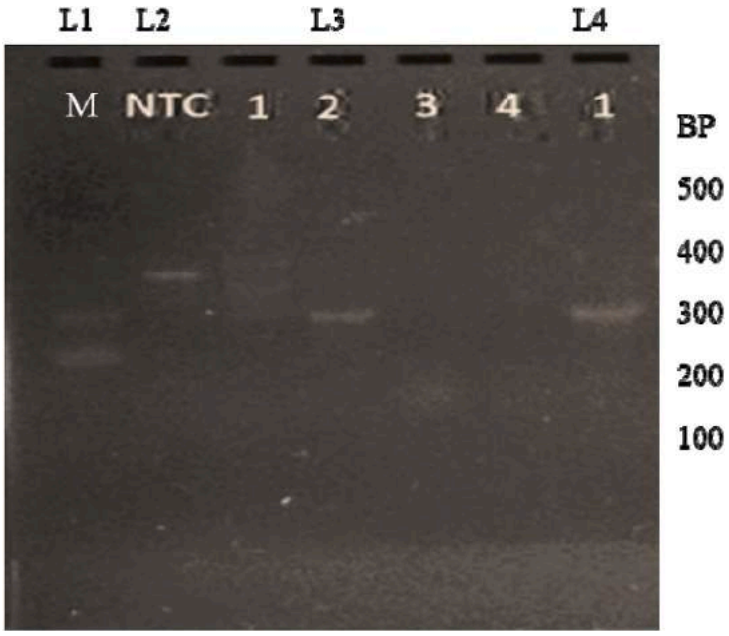

Fig. 3. DNA isolated from *Termitomyces heimii* strain RFES 230662 (THR2) mycelium in agarose gel electrophoresis. Lane 1 is DNA marker, Lane 2 is the no template control (NTC) as the negative control (-ve), Lane 3 is the positive control (+ve) and Lane 4 is the sample of THR2 (RFES 230662).

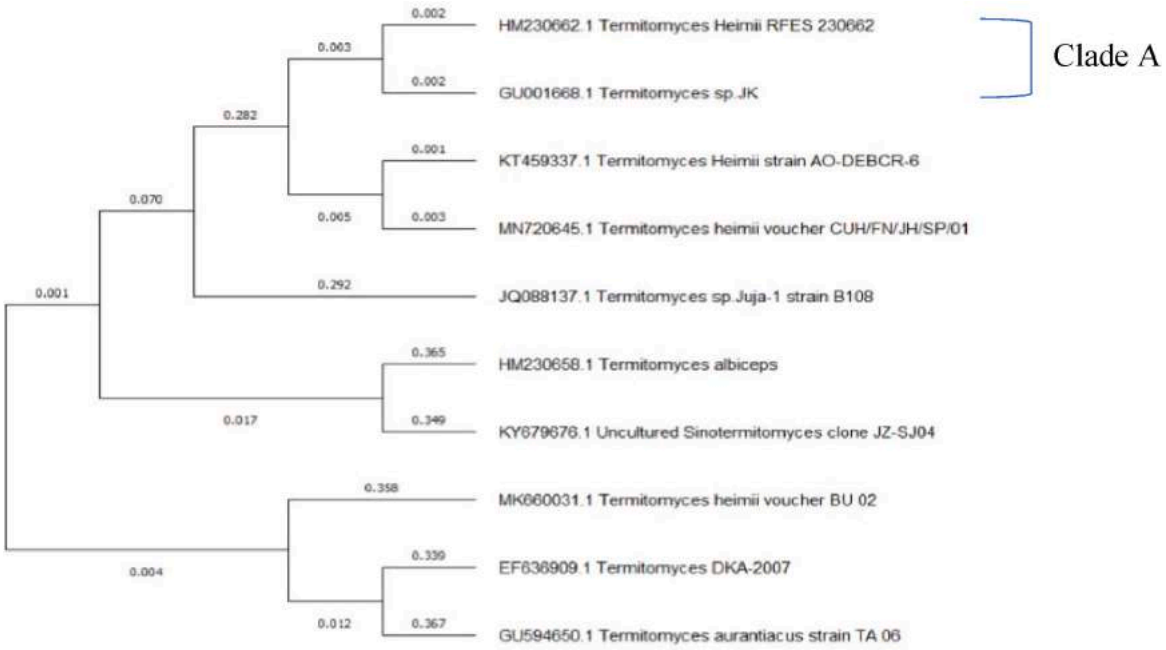

Fig. 4. Phylogenetic tree of *Termitomyces heimii* strain RFES 230662 with evolutionary distance.

Table 3
Endopolysaccharide and exopolysaccharide concentration (g/L) of wild termite mushroom *Termitomyces heimii* strain RFES 230662 extracts.

Source	MB	ENS	EPS	ENS-FB (hot water)	ENS-FB (cold water)
Mycelium	8.55 ± 0.20[a]	0.80 ± 0.20[d]	1.44 ± 0.15[c]	NA	NA
Fruiting body	NA	NA	NA	3.20 ± 0.30[b]	1.36 ± 0.20[c]

Each value is the mean ± SD of three replicates. Values with different small letters (a,b,c,d) are significantly different at the level of 0.05 ($p < 0.05$).
MB = Mycelium biomass, ENS = endopolysaccharide, EPS = exopolysaccharide.
ENS-FB = endopolysaccharide fruiting bodies. NA = Not Applicable.

Termitomyces fungal mycelial grew well in the medium containing glucose and subsequently produced high concentration of lignocellulosic enzymes (Yang et al., 2020). Another previous study also reported that the usage of D-glucose as carbon source showed significant results in supporting fungal mycelial growth (da.Costa et al., 2019). Basically, the carbon sources and nitrogen sources supplied in the medium is taken up by the mycelial as a source of nutrients to build up the structure of membranes and cell wall consequently generated a bio-polysaccharides (Steluti et al., 2004. Thus, the ENS which has been confirmed as β-glucan in this study, actually is bio-polysaccharides consisted in the structure of the fungal cell membrane or cell wall. Exocellular polysaccharides (EPS) are regularly excreted into the environment (medium), making them appear unattached, renewable, free, and easily extractable from orgasmic biomasses using specialised fermentation processes (Wu et al., 2014; Orlandelli et al., 2016). The mycelial obtained were dried and was further extracted in hot water extraction yielding ENS (0.80 ± 0.20 g/L) and EPS was extracted from the supernatant of the culture broth with 1.44 ± 0.15 g/L of crude extract.

Polysaccharides as one of the beneficial bioactive compounds existing in fruiting bodies of *Termitomyces* has been reported widely (Manna et al., 2015; Zhao et al., 2017; Hsieh and Ju, 2018). A water-soluble-glucan isolated from *T. heimii* with (1–3)-, (1–6)-, and (1–3,6)-linked and terminal-D-glucopyranosyl moieties in a nearly 2:1:1:1 ratio demonstrated powerful antioxidant activities and a protective effect against nicotine toxicity (Manna et al., 2015). Recently, a water soluble crude polysaccharide fraction from *Termitomyces heimii* dried fruit bodies were confirmed using ^{1}H Nuclear Magnetic Resonance analysis (Singha et al., 2021). However, in this study we examined the polysaccharide from dried fruiting bodies, mycelium as well as the polysaccharide which secreted from mycelium during fermentation or known as exopolysaccharides (EPS). The polysaccharide obtained from the SF were compared with polysaccharides extracted from fruiting bodies through hot and cold water (ENS-FB) extraction process. Comparatively, crude polysaccharides in fruiting bodies from ENS-FB (hot-water) and ENS-FB (cold-water) were higher than SF mycelium. ENS-FB (hot water) exhibited 3.20 ± 0.30 g/L while ENS-FB (cold water) showed a 1.36 ± 0.20 g/L of crude polysaccharide.

SF was reported as a promising method in producing high concentration of polysaccharides from mushroom mycelial (Wan-Mohtar et al., 2016; Ahmad et al., 2015). After 7 days of fermentation, THR2 mycelium was found to be small, circle pellet and dense. The small, circle pellet of mycelial in this study might contribute to the secretion of high EPS in the SF liquid broth. It was suggested that the small pellets of mycelium formed in this study lead the ease EPS secretion (1.44 ± 0.15 g/L) into the liquid broth thus associated with high EPS production. The results obtained were compared with the concept that have been proven by our research team which extensively investigated the influence of the pellet diameter and morphology on the production of EPS, where small pellets of mycelium were proved to produced high concentration of EPS in liquid broth. (Wan-Mohtar et al., 2016; Supramani et al., 2019B; Abdullah et al., 2020). Meanwhile, the dense of the mycelium pellet could be related to the high dry cell weight (8.55 ± 0.20 g/L) obtained in this study. The small pellet of fungal mycelial observed allows a high concentration of EPS due to the higher surface area of the mycelium pellet which resulted in the high secretion EPS to the culture medium (Wan-Mohtar et al., 2016; Supramani et al., 2019B; Abdullah et al., 2020). The successful submerged fermentation of THR2 remarks the value of the local isolated strain which might possibly overcome the seasonal drawbacks of this rare mushroom and provide a promising solution in producing β-glucan from time to time and contribute in pharmaceutical field.

3.5. *β-glucan determination using fourier- transform infrared spectroscopy (FTIR) analysis*

The existence of the position and anomeric configuration of the glycosidic linkage in the glucan targeted ENS, ENS-FB (hot-water), ENS-FB (cold-water) and EPS extracts were characterized and confirmed using FTIR analysis. The peaks exhibited from the ENS, ENS-FB (hot-water), ENS-FB (cold-water) and EPS were compared with standard laminarin where the absorption in the region between 1250 cm^{-1} and 1650 cm^{-1} indicated the sample was a polysaccharide (Wang et al., 2009; Chen et al., 2013). Laminarin is commonly known as storage β-1,3-glucan which is a group of polysaccharide comprising a β-1,3-linked glucose main chain and β-1,4-linked and/or β-1,6-linked glucose branches (Ji et al., 2013). Laminarin acts as an active component that shows beneficial activities such as antibacterial, antiviral, antitumor, immunomodulatory, blood lipid regulating, anti-oxidation, anticlotting, antiradiation and hypoglycaemic activities (Ji et al., 2012). Fig. 5 showed the FTIR peak of ENS, ENS-FB (hot-water), ENS-FB (cold-water) and EPS, respectively, and it was confirmed that β-glucan polysaccharides are all existed in the four crude extracts from THR2 mushroom mycelium.

Table 4 shows the absorbance peak from FTIR analysis and compares the peak exhibited from the extracts and standard laminarin. The presence of the poly-hydroxilic compounds was demonstrated by the strong, broad-peak in the absorption region between 3370 cm^{-1} indicated the stretching vibration of O–H groups in the sugar residue (Wan-Mohtar et al., 2016; Paulo et al., 2012). The absorption peaks at 2924 cm^{-1} (Figs. 5C) and 2920 cm^{-1} (Fig. 5D) were associated with the stretching vibration of C–H in the sugar ring, indicating the presence of a methylene group, $–CH_2$ (Usuldin et al., 2020). The absorption peak of 1641 in Fig. 5A, B, C and D

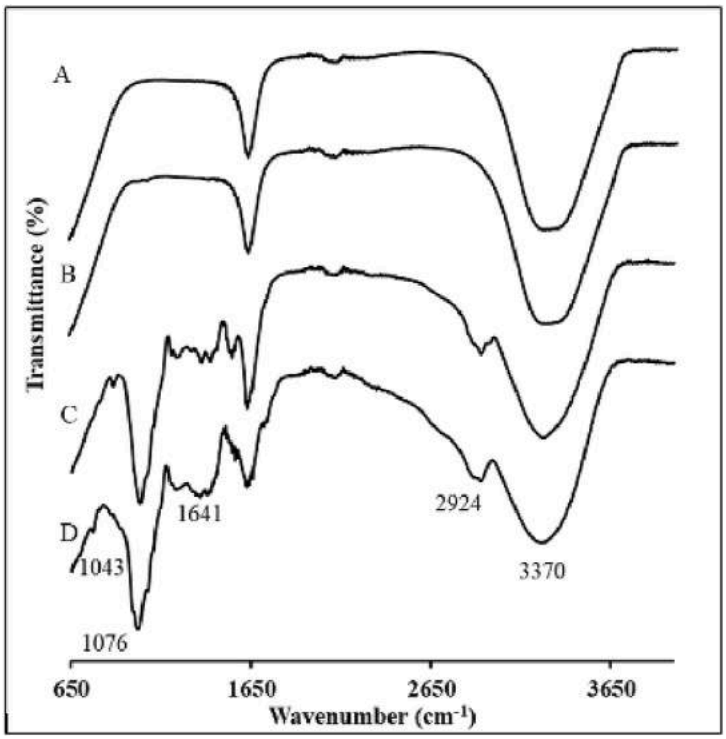

Fig. 5. A) FTIR peak of ENS-FB (hot water) of *Termitomyces heimii* strain RFES 230662 (THR2) fruiting bodies crude extracts, B) ENS-FB (cold water) of THR2 fruiting bodies crude extracts, C) ENS (mycelium) of THR2 mycelium crude extract, D) EPS crude extract of THR2 culture broth (supernatant).

Table 4
Spectral characteristics of endopolysaccharide and exopolysaccharide of wild termite mushroom *Termitomyces heimii* strain RFES 230662 extracts.

Group	Vibration Mode	Type of crude extracts				Standard
		ENS-FB (hot water)	ENS	ENS-FB (cold water)	EPS	Laminarin (Chen et al., 2013)
O–H	O–H stretching vibration	3370	3370	3370	3370	3370
-CH_2	C–H stretching vibration	–	2924	–	2924	2924
C = O	Symmetric and asymmetric stretching vibration	1641	1641	1641	1641	1641
C–O	C–O stretching vibration	1076	1076	–	1076	1043, 1076
–	Anomeric configuration of the glycosidic linkage (β-configuration)	860	890	–	889	889

indicated the water bending vibration in the polysaccharide (Usuldin et al., 2020; Miao et al., 2014). The absorption peaks at 1076 showed in Fig. 5A, C and D shows the presence of C–O–C and C–O bonds stretching vibrations and it was suggested that the presence of C–O, C–O–C and O–H stretching vibrations absorptions resembles the characteristics of a polysaccharide structure (Usuldin et al., 2020). It was previously reported that the absorbance region between 700 cm^{-1} to 950 cm^{-1} is reported for the anomeric region. Based on Fig. 5C and D, the absorption peak were arise in the range of the anomeric region at 890 cm^{-1} in and 889 cm^{-1} respectively where the anomeric configuration within this peak range was reported as the configuration of the polysaccharide and the glucosidic linkage was in β-configuration (Usuldin et al., 2020). Based on the absorption peaks of the three samples, it could be suggested that the structural characterization of the ENS, ENS-FB (hot-water) and EPS polysaccharides from the THR2 mycelium was a β-glucan.

3.6. Antimicrobial responses

The antibacterial activities of different crude extract from THR2 fruiting bodies, mycelium and fermentation broth were further investigated against two Gram-positive bacteria (*Staplylococcus aureus* and *Streptococcus* sp.) and three Gram-negative bacteria (*Ralstonia* sp., *E.coli* and *Salmonella* sp.) as represented in Table 5. Overall, compared to the positive control, all crude extracts exhibited antibacterial activities with the formation of a clear zone to all tested bacteria except EPS and ENS from mycelium towards *E.coli*. The average zone of inhibition against *Ralstonia* sp. obtained from ENS-FB (hot water) was 6.5 ± 0.1 mm and ENS from mycelium was 6.0 ± 0.1 mm as it was significantly different followed by ENS-FB (cold water) (4.5 ± 0.1 mm) and EPS (3.0 ± 0.2 mm) crude extract. ENS-FB (hot-water) maintain to exhibit the larger zone of inhibition against *Streptococcus* sp. and *E.coli*, and has similar value (not significant different) with ENS against *Salmonella* sp. Meanwhile, ENS showed a significant largest zone of inhibition to *Staplylococcus aureus* (5.7 ± 0.1 mm) compared to other crude extracts.

Table 5
Average zone of inhibition of ENS, ENS-FB (hot-water), ENS-FB (cold-water) and β-glucan polysaccharide against three Gram-negative and two Gram-positive bacteria.

Tested Samples	Tested Bacteria				
	Gram -ve Bacteria			Gram + ve Bacteria	
	Ralstonia sp.	*E.coli*	*Salmonella* sp.	*Streptococcus* sp.	*Staphylococcus aureus*
	(mm)	(mm)	(mm)	(mm)	(mm)
ENS-FB (hot water)	6.5 ± 0.1[a]	4.0 ± 0.1[a]	5.3 ± 0.1[a]	5.0 ± 0.1[a]	4.5 ± 0.1[a]
ENS-CW-Fb (cold water)	4.5 ± 0.1[c]	2.5 ± 0.1[b]	3.5 ± 0.2[b]	4.0 ± 0.1[b]	3.0 ± 0.1[b]
EPS	3.0 ± 0.2[b]	ND	3.8 ± 0.1[b]	2.7 ± 0.1[c]	4.0 ± 0.1[a]
ENS (mycelium)	6.0 ± 0.1[a]	ND	5.0 ± 0.1[a]	4.5 ± 0.1[b]	5.7 ± 0.1[c]
Negative control[a]	0.0 ± 0	0.0 ± 0	0.0 ± 0	0.0 ± 0	0.0 ± 0
Penicillin- Streptomycin[b]	10 ± 0.1	10 ± 0.1	10 ± 0.1	10 ± 0.1	10 ± 0.1

[a] Sterile distilled water as negative control.
[b] Penicillin-Streptomycin as positive control (100 μg/mL); The symbol (–) indicate that no inhibition was detected. ND = not detected. Each value is the mean ± SD of three replicates. Values with different small letters in the same column are significantly different at the level of 0.05 ($p < 0.05$). Average zone of inhibition is the value of total diameter – disk (5 mm).

Among the 4 tested crude extracts, ENS-FB (hot-water) is the most effective crude extract as this extract showed a larger zone of inhibition in all tested bacteria. It was followed by ENS from mycelium. Similar previous study also reported that *Termitomyces* spp. hot water extract has shown promising antimicrobial activities against *S.aureus* and *E.coli* with a minimum inhibitory concentration of 0.67 ± 0.29 mg/mL (Gebreyohannes et al., 2019). The highest inhibition effect from these two extracts was suggested due to the extraction process conducted, which allow more bioactive compounds were extracted at a high temperature, whereby the ENS-FB (cold-water) was only extracted at room temperature. The EPS was extracted from the fermentation broth of the SF. The low average zone of inhibition of EPS was particularly due to the secretion of EPS from mycelium pellet to the broth during the batch fermentation process itself. The increase of the EPS concentration could be obtained by optimizing the culture medium and other chemical and physical factors that affect the production of EPS in SF process.

The effect of the antibacterial activities from other *Termitomyces* sp. have been reported previously, for example, the aqueous extracts (hot water extraction process) from *Termitomyces clypeatus* fruiting bodies was reported to highly inhibited the growth of several bacteria such as *E.coli, S. aureus*, *Salmonella typhi* and *E.aerogenes* in the range of 2.6–10.55 mm zone of inhibition (Mahamat et al., 2018). Apart from water extracts, *T. hemii* acetone extract had demonstrated antibacterial effectiveness against *Staphylococcus aureus* and *Klebsiella pneumoniae* (Singha et al., 2017). Different concentration of crude methanolic extracts were also reported antibacterial activity against five pathogenic bacteria including *S. aureus, E. coli, K. pneumoniae* and *Pseudomonas* sp. (Kumari et al., 2017). Singha et al. (2021) further reported that antibacterial activities were found in two homogeneous fractions of polysaccharides isolated from *Termitomyces heimii*, with greater minimum inhibitory concentrations (MIC) against the examined species. From our study, we had successfully adding the value where polysaccharides extracted from mycelium and cultured broth of THR2 also demonstrated a significant antibacterial activities. Since the ENS and EPS characterized in this study were confirmed as β-glucan, thus under specific conditions, mushroom β-glucan with β-linkage has been shown to stimulate the human immune system and modulate the immunological response, making β-glucans popularly known as biological response modifiers (BRM) (Friedman et al., 2016; Minato et al., 2019). As a results of the activation of the host's immune system, the β-glucan showed significant antibacterial properties (Villares et al., 2012).

3.7. Biological activity comparison of Termitomyces spp. mushroom with available literature

Biological activities comparison of several *Termitomyces* spp. mushroom are shown in Table 6. Based on Table 6, all reported studies used fruiting bodies as the source of polysaccharides; meanwhile, current work was the first to exhibit β-glucan polysaccharide produce from mycelium and culture broth of *Termitomyces* sp. through SF. The biological activities reported were activating splenocytes Mondal et al., 2006), the immunomodulatory effect (Bhanja et al., 2012), antioxidant activities against nicotine toxicity (Manna et al., 2015), antioxidant properties (Pattanayak et al., 2015), natural drugs to treat atherosclerosis (Zhao et al., 2017). In contrast, the current study highlighted the antibacterial activity of the β-glucan polysaccharide.

3.8. Future challenge

T. heimii was reported as one of the world's most hardest mushrooms to cultivate (Malek et al., 2012; Rahmad et al., 2014; Jannual et al., 2020). Little is known about how these mushrooms grow, evolve and the information that is accessible is still insufficient. With the natural state of this mushroom that grows only during the monsoon season, this makes termite mushrooms increasingly difficult to obtain and cultivate the wild fruit bodies. Since there are limited report on the isolation, identification and cultivation of wild termite mushroom, this paper also highlight the current practice, limitation and suggested future work which can be carried out to increase the knowledge and scientific information regarding the wild termite mushrooms industry as shown in Table 7. The isolation of wild termite mushroom which randomly carried out during the monsoon season could be conducted with

Table 6
Comparison of the current work on *Termitomyces* sp. mushroom based on the biological activities of bioactive compound extracted from the fruiting bodies, mycelium and fermentation broth.

Termitomyces sp.	Source	Extracts	Bioactivities	References
Termitomyces heimii RFES 230662	FB MB	β-glucan	Antibacterial	*Current work*
Termitomyces sriatus	FB	Water soluble heteropolysaccharide	Activating splenocytes	Mondal et al. (2006)
Termitomyces robustus	FB	Linear water soluble β-glucan, Branched and water-insoluble β-glucans	Immunostimulating effect	Bhanja et al. (2012)
Termitomyces heimii	FB	Water soluble β-glucan containing (1,3)-(1,6) and (1,3,6) linked polysaccharide	Antioxidant activities against nicotine toxicity	Manna et al. (2015)
Termitomyces clypeatus	FB	Water soluble heteroglucan	Antioxidant properties	Pattanayak et al. (2015)
Termitomyces albuminosus	FB	Exopolysaccharide	Natural drugs to treat artherosclerosis	Zhao et al. (2017)

Table 7
Current practice, limitations and suggested future work for termite mushroom industry.

Current practice	Limitation	Suggested future work
Random isolation from wild area	• Seasonal (late monsoon season: June–November) • Specific weather such as after thunderstorm, heavy rains	• Weather forecast monitoring (Aryal and Budathoki, 2016)
Macro-morphological identification	• Not accurate groupings within an evolutionary framework (Olson and Stenlid, 2002). • Misleading due to hybridization (Hughes et al., 2013), crytic speciation (Lücking et al., 2014) and convergent evolution (Brun et al., 2010).	• Combined identification of micromorphological, cultural and molecular technique Raja et al. (2017)
Nuclear ribosomal genes identification	• Inconsistent phylogenetic reconstruction levels (Raja et al., 2017)	• NCBI-BLAST search-DNA barcoding Hennell et al. (2012) • Protein-coding genes Raja et al. (2017)
Cultivation on agar media and liquid medium	• Unknown suitable growth media composition • Unknown suitable cultivation parameters (temperature, shaking, pH)	• Optimization of using Response Surface Methodology (RSM) Ahmad et al. (2013) • Investigation of cultivation parameters on the biomass and polysaccharide yield (Supramani et al., 2019A; Balamurugan et al., 2021)

weather forecast particularly to get information regarding on the thunderstorms and heavy rains as similar reported by Aryal and Budathoki et al., 2016. For a precise species determination, the identification of mushroom fruit bodies must not only depended on the macro-morphological characterization, but a combination of micromorphological, cultural and molecular characterization need to be done for the newly isolated strain (Raja et al., 2017). In order to overcome the inconsistent phylogenetic reconstruction levels using nuclear ribosomal genes identification, NCBI-BLAST search for DNA barcoding with a cautionary note regarding its limitations is suggested to improve the results in species identification (Hennell et al., 2012). Besides, protein-coding genes which have intron sections, can be used for species identification via barcoding and could be applied in the phylogenetic analysis since they have better resolution at higher taxanomic levels than rRNA genes (Raja et al., 2017). Since the cultivation of wild termite mushroom mycelium in submerged fermentation has not been thoroughly investigated, only limited scientific data has been published. As a result, much study is needed to demonstrate the optimal growth media composition and cultivation parameters for biomass and polysaccharide production (Ahmad et al., 2013; Balamurugan et al., 2021).

4. Conclusion

The newly discovered wild THR2 was identified as *Termitomyces heimii* RFES 230662. The *T. heimii* mycelium was successfully grown in submerged fermentation with high concentration of mycelium biomass (8.55 g/L) and EPS concentration (1.44 g/L) in rapid incubation time (7 days). ENS of fruiting bodies and EPS of mycelium extracts were verified as β-glucan, with both possessing antibacterial properties. The optimization of culture condition is needed for large scale production of β-glucan from THR2 mycelial culture.

Declaration of competing interest

The authors whose names are listed in the manuscript certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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References

Aanen, D.K., Eggleton, P., 2005. Fungus-growing termites originated in African rain forest. Curr. Biol. 15 (9), 851–855. https://doi.org/10.1016/j.cub.2005.03.043.

Aanen, D.K., Eggleton, P., RoulandLefevre, C., 2002. The evolution of fungus- growing termites and their mutualistic fungal symbionts. Proc. Natl. Acad. Sci. U.S.A. 99 (23), 14887–14892. https://doi.org/10.1073/pnas.222313099.

Abdullah, N.R., Sharif, F., Azizan, N.H., Hafidz, I.F.M., Supramani, S., Usuldin, S.R.A., Ahmad, R., Wan-Mohtar, W.A.A.Q.I., 2020. Pellet diameter of *Ganoderma lucidum* in a repeated-batch fermentation for the trio total production of biomass-exopolysaccharide-endopolysaccharide and its anti-oral cancer beta-glucan response. AIMS Microbiology 6 (4), 379–400. https://doi.org/10.3934/microbiol.2020023.

Ahmad, R., Al-Shorgani, N.K.N., Hamid, A.A., Wan Yusoff, W.M., Daud, F., 2013. Optimization of medium components using response surface methodology (RSM) for mycelium biomass and exopolysaccharide production by *Lentinus squarrosulus*. Adv. Biosci. Biotechnol. 4, 1079–1085.

Ahmad, R., Clementine, I.N., Er, J.S., Syed-Mustaffa, S.N.A.B., Wan-Mohtar, W.A.A.Q.I., 2018. Incorporation of palm oil for the enhancement of biomass and polysaccharides production through submerged fermentation from locally isolated *Pleurotus* sp., mycelium. Int. J. Adv. Res. 6 (4), 223–232. https://doi.org/10.21474/IJAR01/6849.

Ahmad, R., Usyida, A.S.M., Hamid, A.A., Wan Yusoff, W.M., Ismail, E., Daud, F., 2015. Production and antiproliferative activity of various crude extract from *Lentinus squarrosulus* mycelium. Scholars Acad. J. Biosci. 3 (4), 377–385.

Aryal, H.P., Budhathoki, U., 2014. Ethnomycology of *Termitomyces* spp. R. Heim for its medicinal importance in Nepal. Int. J. Med. Plants 6 (2), 128–137.

Aryal, H.P., Budhathoki, U., 2016. Ethnomycology of *Termitomyces* R. Heim in Nepal. J. Yeast Fungal Res. 7 (4), 28–38.

Balamurugan, J.P., Supramani, S., Usuldin, S.R.A., Ilham, Z., Klaus, A., Ikram, N.K.K., Ahmad, R., Wan-Mohtar, W.A.A.Q.I., 2021. Efficient biomass-endopolysaccharide production from an identified wild-Serbian *Ganoderma applanatum* strain BG S6Ap mycelium in a controlled submerged fermentation. Biocatalysis and Agricultural Biotechnology 37, 102166. https://doi.org/10.1016/j.bcab.2021.102166.

Bhanja, S.K., Nandan, C.K., Mandal, S., Bhunia, B., Maiti, T.K., Mondal, S., Islam, S.S., 2012. Isolation and characterization of the immunostimulating β-glucans of an edible mushroom *Termitomyces robustus* var. Carbohydr. Res. 357, 83–89.

Brun, S., Silar, P., 2010. In: Pontarotti, P. (Ed.), Evolutionary Biology – Concepts, Molecular and Morphological Evolution. Springer, pp. 317–328 Berlin Heidelberg.

Buxton, R.D., 1981. Termites and the turnover of dead wood in an arid tropical environment. Oecologia 51, 379–384. https://doi.org/10.1007/BF00540909.

Chandrawati, P.S., Narendra, K., Tripathi, N.N., 2014. Macrofungal wealth of kusumhi forest of gorakhpur, UP, India. American Int Journal of Research in Formal, Applied & Natural Sciences 5, 71–75.

Chen-Fing, J., Yu-bin, J., De-you, M., 2013. Sulfated modification and anti-tumor activity of laminarin. Experimental and therapeutic medicine 6, 1259–1264. https://doi.org/10.3892/etm.2013.1277.

da Costa, R.R., Vreeburg, S.M.e., Shik, J.Z., Aanen, D.L., Poulsen, M., 2019. Can interaction specificity in the fungus-farming termite symbiosis be explained by nutritional requirements of the fungal crop?. Fungal Ecology 38, 54–61. https://doi.org/10.1016/j.funeco.2018.08.009.

Fournier, E., 2001. Colorimetric quantification of carbohydrates. In: Ronald, E.W., Terry, E.A., Eric, A.D., Michael, H.P., David, S.R., Steven, J.S., Charles, F.S., Denise, M.S., Peter, S. (Eds.), Current Protocol in Food Analytical Chemistry. John Wiley & Sons, Inc. Hoboken E1.1.1-E1.1.8.

Friedman, M., 2016. Mushroom polysaccharides: chemistry and antiobesity, antidiabetes, anticancer, and antibiotic properties in cells, rodents, and humans. Foods 5 (4), 80. https://doi.org/10.3390/foods5040080.

Frøslev, T.G., Aanen, D.K., Laessøe, T., Rosendahl, S., 2003. Phylogenetic relationships of *Termitomyces* and related taxa. Mycol. Res. 107, 1277–1286. https://doi.org/10.1017/S0953756203008670.

Gebreyohannes, G., Nyrere, A., Bii, C., Sbhatu, D.B., 2019. Investigation of antioxidant and antimicrobial activities of different extracts of *Auricularia* and *Termitomyces* species of mushrooms. Sci. World J. 7357048 2019 https://doi.org/10.1155/2019/7357048.

Ghorai, S., Banik, S.P., Verma, D., Chowdhury, S., Mukherjee, S., Khowala, S., 2009. Fungal biotechnology in food and feed processing. Food Res. Int. 42, 577–587. https://doi.org/10.1016/j.foodres.2009.02.019.

Giavasis, I., 2014. Bioactive fungal polysaccharide as potential functional ingredients in food and nutraceuticals. Curr. Opin. Biotechnol. 26, 162–172. https://doi.org/10.1016/j.copbio.2014.01.010.

Hamzah, H.N., Mohammad, A., 2021. A Study of Wild Mushrooms Utilised for Food and Medicine by the Orang Asli Communities of Sungai Berua, Terengganu. Resource Use and Sustainability of Orang Asli: Indigenous Communities in Peninsular Malaysia. p. 75.

Hennell, J.R., D'Agostino, P.M., Lee, S., Khoo, C.S., Sucher, N.J., 2012. Using GenBank® for genomic authentication: a tutorial Humana Press. In: Sucher, N.J. (Ed.), Plant DNA Finger Printing and Barcoding: Methods and Protocols. Springer, New York, pp. 181–200 2012.

Hsieh, H.M., Chung, M.C., Chen, P.Y., Hsu, F.M., Liao, W.W., Sung, A.N., Lin, C.R., Wang, C.R., Kao, Y.H., Fang, M.J., Lai, C.Y., Huang, C.C., Chou, J.C., Chou, W.N., Chang, B.C., Ju, Y.M., 2017. A termite symbiotic mushroom maximizing sexual activity at growing tips of vegetative hyphae. Botanical Studies 58 (39), 1–14. https://doi.org/10.1186/s40529-017-0191-9.

Hsieh, H.M., Ju, Y.M., 2018. Medicinal components in *Termitomyces* mushrooms. Appl. Microbiol. Biotechnol. 102, 4987–4994. https://doi.org/10.1007/s00253-018-8991-8.

Hughes, K.W., Petersen, R.H., Lodge, D.J., Bergemann, S.E., Baumgartner, K., Tulloss, R.E., Lickey, E., Cifuentes, J., 2013. Evolutionary consequences of putative intra- and inter specific hybridization in agaric fungi. Mycologia 105 (6), 1577–1594. https://doi.org/10.3852/13-041.

Jannual, N., Nipitwattanaphon, M., Hasin, S., Kaewgrajang, T., 2020. Morphological and molecular characterization of *Termitomyces* (Lyophyllaceae, agaricales) in Thailand. Biodiversitas 21 (6), 2481–2491. https://doi.org/10.13057/biodiv/d210620.

Jasinghe, V.J., Perera, C.O., Sablami, S.S., 2007. Kinetics of the conversion of ergosterol in edible mushrooms. J. Food Eng. 79, 864–869. https://doi.org/10.1016/j.jfoodeng.2006.01.085.

Ji, C.F., Ji, Y.B., Meng, D.Y., 2013. Sulfated modification and anti-tumor activity of laminarin. Experimental and Therapeutic Medicine 6, 1259–1264. https://doi.org/10.3892/etm.2013.1277.

Ji, Y.B., Ji, C.F., Zhang, H., 2012. Laminarin induces apoptosis of human colon cancer LOVO cells through a mitochondrial pathway. Molecules 17, 9947–9960. https://doi.org/10.3390/molecules17089947.

Kumari, S.G., Kumari, R.P.K., Geetha, S., 2017. In vitro antioxidant and antimicrobial activity of edible mushroom (*Termitomyces heimii*). Int J Development Research 7, 17531–17537.

Landingin, H.R.R., Francisco, B.E., Dulay, R.M.R., Kalaw, S.P., Reyes, R.G., 2020. Optimization of culture conditions for mycelial growth and Basidiocarp production of *Cyclocybe cylindracea* (Maire). The CLSU Int. J. Sci. Technol. 4 (1), 1–17. https://doi.org/10.22137/ijst.2020.v4n1.01.

Liu, N., Li, H., Chevrette, M.G., Zhang, L., Cao, L., Zhou, H., Zhou, X., Zhou, Z., Pope, P.B., Currie, C.R., 2019. Functional metagenomic reveals abundant polysaccharide-degrading-gene clusters and cellobiose utilization pathways within gut microbiota of a wood-feeding higher termite. ISME J. 13 (1), 104–117. https://doi.org/10.1038/s41396-018-0255-1.

Lücking, R., Dal-Forno, M., Sikaroodi, M., Gillevet, P.M., Bungartz, F., Moncada, B., Yanes-Ayabaca, A., Chaves, J.L., Coca, L.F., Lawrey, J.D., 2014. A single microlichen constitutes hundreds of unrecoqnized species. Proc. Natl. Acad. Sci. Unit. States Am. 111 (30), 11091–11096. https://doi.org/10.1073/pnas.1403517111.

Mahamat, O., André-Ledoux, N., Chrisopher, T., Mbifu, A.A., Albert, K., 2018. Assessment of antimicrobial and immunomodulatory activities of termite associated fungi, *Termitomyces clypeatus* R. Heim (Lyophyllaceae, Basidiomycota). Clinical Phytoscience 4, 28. https://doi.org/10.1186/s40816-018-0089-4.

Malek, A.S.N., Kanagasabapathy, G., Vikneswary, S., Abdullah, N., Yaacob, H., 2012. Lipid components of a Malaysiam edible mushroom, Termitomyces heimii Natarajan. Int J.Food Properties 15, 809–814. https://doi.org/10.1080/10942912.2010.506017.
Manna, D.K., Nandi, A.K., Pattanayak, M., Maity, P., Tripathy, S., Mandal, A.K., Roy, S., Tripathy, S.S., Gupta, N., Islam, S.S., 2015. A water soluble β- glucan of an edible mushroom *Termitomyces heimii*: structural and biological investigation. Carbohydr. Polym. 134, 375–384. https://doi.org/10.1016/j.carbpol.2015.07.099.
Miao, M., Li, R., Jiang, B., et al., 2014. Structure and digestibility of endosperm water-soluble a-glucans from different sugary maize mutants. Food Chem. 143, 156–162. https://doi.org/10.1016/j.foodchem.2013.07.109.
Minato, K.I., Laan, L.C., van Die, I., Mizuno, M., 2019. *Pleurotus citrinopileatus* polysaccharide stimulates anti-inflammatory properties during monocyte-to-macrophage differentiation. Int. J. Biol. Macromol. 122, 705–712. https://doi.org/10.1016/j.ijbiomac.2018.10.157.
Mondal, S., Chakraborty, I., Rout, D., Islam, S.S., 2006. Isolation and structural elucidation of a water-soluble polysaccharide (PS-I) of a wild edible mushroom, *Termitomyces striatus*. Carbohydr. Res. 341, 878–886. https://doi.org/10.1016/j.carres.2006.02.004.
Nakalembe, I., Kabasa, J.D., Olila, D., 2015. Comparative nutrient composition of seleted wild edible mushroom from two agro-ecological zones, Uganda. Spring 4, 433–447. https://doi.org/10.1186/s40064-015-1188-z.
Olila, D., Kyeyune, G., Kabasa, J.D., Kisovi, L., Munishi, P.K.T., 2007. Assessment of potential for domestication of *Termitomyces microcarpus*: an indigenous edible and medicinal mushroom from Lake Victoria basin. Agric. J. 2, 627–631. http://medwelljournals.com/abstract/doi=aj.2007.627.631.
Olson, A., Stenlid, J., 2002. Pathogenic fungal species hybrids infecting plants. Microb. Infect. 4 (13), 1353–1359.
Orlandelli, R.C., Vasconcelos, A.F.D., Azevedo, J.L., da Silva, M.L.C., Pamphile, J.A., 2016. Screening of endophytic sources of exopolysaccharides: preliminary characterization of crude exopolysaccharide produced by submerged culture of *Diaporthe* sp. JF 776998 under different cultivation time. Biochimie Open 2, 33–40. https://doi.org/10.1016/j.biopen.2016.02.003.
Park, H.G., Ko, H.G., Kim, S.H., 2004. Molecular identification of Asian isolates of medicinal mushroom *Hericium erinaceum* by phylogenetic analysis of nuclear ITS rDNA. J.Microbiol.Biotechn. 14, 816–821.
Pattanayak, M., Samanta, S., Maity, P., Sen, I.K., Nandi, A.K., Manna, D.K., Mitra, P., Acharya, K., Islam, S.S., 2015. Heteroglycan of an edible mushroom *Termitomyces clypeatus*: structure elucidation and antioxidant properties. Carbohydr. Res. 413, 30–36. https://doi.org/10.1016/j.carres. 2015.05.005.
Paulo, E.M., Boffo, E.F., Branco, A., et al., 2012. Production, extraction and characterization of exopolysaccharides produced by the native *Leuconostoc pseudomesenteroids* R2 strain. An. Acad. Bras. Cienc. 84, 495–507.
Pennisi, E., 2015. Africa's soil engineers: termites. American Association for the Advancement of Science 347 (6222), 596–597.
Rahmad, N., Al-Obaidi, J.R., Rashid, N.M.M., Zean, N.B., Yusoff, M.H.Y.M., Shaharuddin, N.S., Jamil, N.A.M., Saleh, N.M., 2014. Comparative proteomic analysis of different developmental stages of the edible mushroom *Termitomyces heimii*. Biol. Res. 47, 30. http://www.biolres.com/content/47/1/30 2014.
Raja, H.A., Miller, A.N., Pearce, C.J., Oberlies, N.H., 2017. Fungal identification using molecular tools: a primer for the natural products research community. J. Nat. Prod. 80, 756–770. https://doi.org/10.1021/acs.jnatprod.6b01085.
Ramesh, C.H., Manohar, G.P., 2010. Antimicrobial properties, antioxidant activity and bioactive compounds from six wild edible mushrooms of western ghats of Karnataka, India. Phcog Res 2, 12–107. https://doi.org/10.4103/0974-8490.62953.
Rashid, M.H., Rahman, M.M., Correll, R., Naidu, R., 2018. Arsenic and other elemental concentrations in mushrooms from Bangladesh. Health risks. Int. J. Environ. Res. Publ. Health 15, 919. https://doi.org/10.3390/ijerph15050919.
Sachan, S.K.S., Patra, J.K., Thatoi, H.N., 2013. Indigenous knowledge of ethnic tribe for utilization of wild mushrooms as food and medicine in similipal biosphere reserve, Odisha, India. J Agriculture Technology 9, 335–348.
Sargunam, S.D., Johnsy, G., Samuel, A.S., Kaviyarasan, V., 2012. Mushrooms in the food culture of the Kaani tribe of Kanyakumari district. Indian Journal of Traditional Knowledge 11, 150–153.
Seelan, J.S., Yee, C.S., Fui, F.S., Dawood, M., Tan, Y.S., Kim, M.J., Park, M.S., Lim, Y.W., 2020. New species of *Termitomyces* (Lyophyllaceae, Basidiomycota) from sabah (northern borneo), Malaysia. Mycobiology 48, 95–103. https://doi.org/10.1080/12298093.2020.1738743.
Siddiquee, S., Rovina, K., Naher, L., et al., 2015. Phylogenetic relationships of *Termitomyces aurantiacus* inferred from internal transcribed spacers DNA sequences. Adv. Biosci. Biotechnol. 6 (5), 358–367.
Singha, K., Hor, P.K., Soren, J.P., Mondal, J., Mondal, K.C., Pati, B.R., Mohapatra, P.K.D., 2021. Exploration of bioactive prospects of a polysaccharide fraction from *Termitomyces heimii* against colorectal cancer and broad spectrum bacteria. Bioactive Carbohydrates and Dietary Fibre 25, 100255 2021 https://doi.org/10.1016/j.bcdf.2020.100255.
Singha, K., Pati, B.R., Mondal, K.C., Mohapatra, P.K.D., 2017. Study of nutritional and antibacterial potential of some wild edible mushrooms from Gurguripal Ecoforest, West Bengal, India. Indian J. Biotechnol. 16, 222–227.
Steluti, R.M., Giese, E.C., Piggato, M.M., Sumiya, A.F.G., Covizzi, L.G., Job, A.E., Cardoso, M.S., Corradi da Silva, M.L., Dekker, R.F.H., Barbosa, A.M., 2004. Comparison of botryosphaeran production by the ascomyceteous fungus *Botryosphaeria* sp., grown on different carbohydrate carbon sources, and their partial structural features. J. Basic Microbiol. 44, 480–486. https://doi.org/10.1002/jobm.200410415.
Supramani, S., Ahmad, R., Ilham, I., Annuar, M.S.M., Wan-Mohtar, W.A.A.Q.I., 2019B. Optimisation of biomass, exopolysaccharide and intracellular polysaccharide production from the mycelium of an identified *Ganoderma lucidum* strain QRS 5120 using response surface methodology. AIMS Microbiology 5 (1), 19–38.
Supramani, S., Jailani, N., Ramarao, K., Mohd Zain, N.A., Klaus, A., Ahmad, R., Wan-Mohtar, W.A.A.Q.I., 2019A. Pellet diameter and morphology of European *Ganoderma pfeifferi* in a repeated-batch fermentation for exopolysaccharide production. Biocatal. Agric. Biotechnol. 19. https://doi.org/10.1016/j.bcab.2019.101118.
Thatoi, H., Singdevsachan, S.K., 2014. Diversity, nutritional composition and medicinal potential of India mushrooms: a review. Afr. J. Biotechnol. 13, 523–545. https://doi.org/10.5897/AJB2013.13446.
Tibuhwa, D.D., 2012. *Termitomyces* species from Tanzania, their cultural properties and unequalled basidiopores. J. Biol. Life Sci. 3, 1. https://doi.org/10.5296/jbls.v3i1.1723.
Turnbull, E., Watling, R., 1999. *Termitomyces* from old world rainforests. Kew Bull. 54 (3), 731–738.
Usuldin, S.R.A., Mahmud, N., Ilham, Z., et al., 2020. In-depth spectral characterization of antioxidative (1,3)-ß-D-Glucan from the mycelium of an identified tiger milk-mushroom *Lignosus rhinoceros* strain ABI in a stirred-tank bioreactor. Biocatal Agric Biotechnol 23. https://doi.org/10.1016/j.bcab.2019.101455.
Venkatachalapathi, A., Paulsamy, S., 2016. Exploration of wild medicinal mushroom species in walayar valley, the southern western ghats of coimbatore district Tamil nadu. Mycosphere 7, 118–130. https://doi.org/10.5943/mycosphere/7/2/3.
Villares, A., Mateo-Vivaracho, L., Guillamon, E., 2012. Structural features and healthy properties of polysaccharides occurring in mushrooms. Agriculture 2, 452–471. https://doi.org/10.3390/agriculture2040452.
Wan-Mohtar, W.A.A.Q.I., Latif, N.A., Harvey, L.M., 2016. Production of exopolysaccharides by *Ganoderma lucidum* in a repeated-batch fermentation. Biocatalysis Agriculture Biotechnology 6, 91–101. https://doi.org/10.1016/j.bcab.2016.02.011.
Wang, J., Zhang, L., Yu, Y., et al., 2009. Enhancement of antitumor activities in sulphated and carboxymethylated polysaccharides of *Ganoderma lucidum*. J. Agric. Food Chem. 57, 10565–10572.
Wiriya, J., Kavinlertvatana, P., Lumyong, S., 2014. Effects of different culture media, carbon and nitrogen sources and solid substrates on growth of *Termitomyces* mushrooms. Chiang Mai J. Sci. 41 (3), 542–556.
Wisselink, M., Aanen, K.D., Padje van', A., 2020. The longevity of colonies of fungus-growing termites and the stability of the symbiosis. Insects 11, 527. https://doi.org/10.3390/insects11080527.
Woldegiorgis, A.Z., Abate, D., Haki, G.D., Ziegler, G.R., 2014. Antioxidant property of edible mushrooms collected from Ethiopia. Food Chem. 157, 30–36. https://doi.org/10.1016/j.foodchem.2014.02.014.
Wu, Z., Yang, Z., Gan, D., Fan, J., Dai, Z., Wang, X., Hu, B., Ye, H., Abid, M., Zeng, X., 2014. Influences of carbon sources on the biomass, production and compositions of exopolysaccharides from *Paecilomyces hepiali* HN1. Biomass Bioenergy 67, 260–269. https://doi.org/10.1016/j.biombioe.2014.05.008.
Yamada, A., Inoue, T., Wiwatwitaya, D., Ohkuma, M., Kudo, T., Abe, T., Sugimoto, A., 2005. Carbon mineralization by termites in tropical forests, with emphasis on fungus-combs. Ecol. Res. 20, 452–460. https://doi.org/10.1007/s11284-005-0062-9.
Yang, G., Ahmad, F., Liang, S., Fouad, H., Gui, M., Gaal, H.A., Mo, J., 2020. Termitomyces heimii associated with fungus-growing termite produces volatile organic

compounds (VOCs) and lignocellulose-degrading enxymes. Appl. Biochem. Biotechnol.. https://doi.org/10.1007/s12010-020-03376-w.
Ye, L., Karunarathna, S.C., Li, H., Xu, J., Hyde, K.D., Mortimer, P.E., 2019. A survey of *Termitomyces* (Lyophyllaceae, Agaricales), including a new species, from a subtropical forest in Xishuangbanna, China. Mycobiology 47, 391–400. https://doi.org/10.1080/12298093.2019.1682449.
Zhao, H.J., Li, J., Zhang, J.J., Wang, X.X., Liu, M., Zhang, C., Jia, L., 2017. Hepatoprotective and in vitro antioxidant effects of native depolymerised-exopolysaccharides derived from *Termitomyces albuminosus*. Sci. Rep. 7 (3910), 1–13. https://doi.org/10.1038/s41598-017-04171-0.