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Isolation and Identification of Carbonoclastic Fungi Causing Damage to Prehistoric Paintings in the Maros-Pangkep Karst Area

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Abstract

The carbonoclastic fungi known as a fungi that can produce calcium carbonate crystals ($CaCO_3$) through the urease enzyme produced to hydrolysis of urea contained in the substrate or growth medium. The presence of carbonoclastic fungi is frequently observed in the karst areas cave wall painting, which are a type of ancient artifact. The destruction to the ancient cave artwork is brought on by the fungus. The purpose of this study was to obtain and determine the types of carbonoclastic fungi that cause damage to cave wall paintings in the Maros-Pangkep karst area. Isolation and selection of carbonoclastic fungi were carried out using Christensen Urea Agar medium. The $CaCO_3$ precipitate potential test was carried out by calculating the mass $CaCO_3$ precipitates formed and analysis of ammonia levels and cell biomass produced during the growth period. Fungi identification was performed using the 18S rRNA gene molecular markers. Twenty four fungi isolates obtained from swab samples in Parewe and Bulu Sipong caves, 7 positive isolated belonged to carbonoclastic fungi. The results of $CaCO_3$ precipitates, namely isolate Ps3 producing precipitates of 80,30 mg with ammonia content value of 701,7064 ppm and cell biomass of 333,80 mg. The identification results showed that Ps3 isolate belongs to *Aspergillus* sp. strain BW1.

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Introduction

Karst areas are known for their expanses of high-lying limestone rocks found throughout the world. In Indonesia, karst regions cover an area of approximately 154,000 km^2, which accounts for only 0.08% of the country's landmass. One of the most renowned karst areas in Indonesia is the Maros-Pangkep Karst Region located in South Sulawesi. The Karst area of Maros-Pangkep has numerous unique features that have earned it a place in the UNESCO heritage site list with the status of Geopark, known as the Maros-Pangkep National Geopark Area (MPNGA). One of its unique features is the cave wall rock art known as "rock painting". According to Brahmantara (2016), rock paintings are a type of prehistoric legacy that holds extraordinary cultural and artistic value as a reflection of self and past artistic expression. This is why they are designated as UNESCO cultural heritage sites.

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Unfortunately, the condition of the cave paintings has greatly deteriorated due to both human and natural factors. According to Suhartono (2009) and Suhartono (2012), human factors that contribute to the damage of the cave paintings include ecosystem degradation and vandalism. On the other hand, natural factors are influenced by climate, rainfall, temperature, and the growth of microorganisms such as bacteria and fungi. The moist and cool cave conditions are the primary catalysts for the accelerated growth of fungus.

Fungal growth on karst cave paintings results in damage such as peeling, changes in paint pigmentation, and the covering of paintings due to precipitation or calcium carbonate ($CaCO_3$) precipitate. The process of $CaCO_3$ precipitation by fungi occurs through the enzymatic activity of urease hydrolyzing urea present in the substrate. White carbonate crystals precipitate and coat cave paintings (Phang *et al.*, 2018). According to Wazny and Rudniewsky (1972), this process is found in carbonoclastic fungi. Among these fungi, *Aspergillus* spp. (36.4%), *Penicillium spp*. (18.2%), *Talaromyces spp*. (3.6%), and *Paecilomyces variotii* (3.6%) are some of the groups that belong to the Ascomycota division, which dominates fungi in karst regions (Vanderwolf *et al.*, 2013; Wasti *et al.*, 2021). Precipitation is the cause of damage to cave karst paintings. Currently, research on the relationship between prehistoric cave painting damage and the activity of carbonoclastic fungi is lacking. This lack of information led to the undertaking of this study to determine the type of carbonoclastic fungi that cause damage to paintings on the walls of the Maros-Pangkep karst area cave.

Materials and Methods

Sample Collection

Samples were taken from the Karst Maros-Pangkep rock area, specifically the Parewe and Bulu Sipong caves. The collection process involved swabbing the Karst rocks with cotton swabs, which were then placed in a physiological NaCl solution and stored in a cool box. The isolation and identification processes were conducted at the Microbiology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Hasanuddin University, Makassar.

Isolation of Carbonoclastic Fungi

One milliliter of the sample is added to 9 milliliters of physiological NaCl solution and vortexed. Then, one swab sample is evenly inoculated into a Petri dish containing Malt Extract Agar (MEA). It is then incubated at 34°C for 3x24 hours, followed by the detection and counting of fungal growth colonies (Wasti *et al.*, 2021; Zhao *et al.*, 2022). From each distinct colony, pure isolates are obtained by inoculating them onto the same medium.

Carbonoclastic Fungi Selection

The purified fungal culture was grown on Christensen Urea Agar medium. One isolate swab of the fungus was streaked onto a slanted Christensen Urea Agar medium and incubated at 34 ^{o}C for a total of 72 hours. Phenol red on the medium acted as pH and color indicator (Zhao *et al.*, 2022).

Characteristics of Carbonoclastic Fungi

Observation of Colony Morphology

Observation of colony morphology is performed by directly observing the morphology of fungal colonies while considering colony characteristics such as color and surface (granular, powdery, rolled, smooth; presence or absence of exudate droplets), radial lines from the

center of the colony towards the edge, and the presence or absence of concentric circles (Gandjar *et al.*, 1999).

Cell Morphology Observation

Observation of cell morphology is done by making slide culture which is then observed under a microscope. Slide culture is made by aseptically taking the fungal isolate using a swab, then placing it on a sterile glass object and previously dripped with liquid MEA medium until it solidifies. Fungal slides were then inserted into a Petri dish covered with sterile filter paper that had been slightly moistened with sterile distilled water, then incubated in an incubator for 2x24 hours at room temperature 28°C. Then the fungal slides were dripped with lactophenol cotton blue and observed under a microscope with a magnification of 400x. The morphological characteristics of the cells observed include hyphal structure and reproductive structure (spores). The identification of fungi refers to the identification book Illustrated Genera of Imperfect Fungi (Barnett and Hunter, 2002) and Introduction to Common Tropical Fungi (Gandjar *et al.*, 1999).

Test the $CaCO_3$ Precipitating Potential of Carbonoclastic Fungi

Three milliliters fungal suspension (25% T) was inoculated into 100 milliliters SDB U/Ca medium and incubated at 30°C for 15 days on a 150 rpm shaker. Examination was conducted at the end of the incubation period, which included the calculation of $CaCO_3$ precipitate mass, ammonia analysis, and carbonoclastic fungal cell biomass calculation.

Calculation of Precipitated CaCO3 and Dry Weight of Biomass Cells

The precipitate of $CaCO_3$ formed was collected by filtration using filter paper (Whattman No. 42) previously dried in an oven until a constant weight was obtained. The weight of the $CaCO_3$ precipitate was calculated using the formula (Krishnapriya and Venkatesh, 2015):

$$W_c = W_{fc} - W_f$$

Description: W_c = weight of precipitate (g)
W_{fc} = weight of filter paper and precipitate (g)
W_f = weight of filter paper (g)

Analysis of Ammonia Levels

A fungal culture was filtered through filter paper to measure ammonia levels. One milliliter of the filtrate was added to a 50 mL volumetric flask and then diluted with distilled water to obtain a volume of 50 mL. Next, 5 mL of the diluted solution was transferred to a reaction tube, followed by the addition of 0.5 mL each of Na-phenol and NaOCl solutions. The mixture was then homogenized by vortexing and allowed to stand for 5 minutes. Ammonia levels were measured using a UV-Vis spectrophotometer with a wavelength of 640 nm. The concentration of ammonia in a sample (x) can be calculated by regressing the absorbance values (y) against the constants (a, b) from the equation y=ax+b.

Calculation of Dry Weight Biomass of Cells

Cell biomass formed was collected by filtration using filter paper (Whatman No. 42) previously dried in an oven until a constant weight was obtained. The weight of cell biomass was then calculated using the formula (Krishnapriya and Venkatesh, 2015):

$$W_c = W_{fc} - W_f$$

Description: W_c = weight of precipitation/ cell biomass (g)
W_{fc} = weight of filter and precipitate/ cell biomass (g)
W_f = weight of filter paper/ cell biomass (g)

Carbonoclastic Fungi Selection

In this assay, genomic DNA is isolated using the Tiangen extraction kit, and the ITS region is amplified using the PCR method with 18S rRNA gene forward primers ITS-1 (5' TCCGTAGGTGAACCTGCGG 3') and reverse primers ITS-4 (5' TCCTCCGCTTATTGATATGC 3'). The amplified DNA product is then separated using electrophoresis on a 1.5% agarose gel. The results of electrophoresis are observed under UV light for DNA band detection. The sample yielding positive results was subsequently sequenced and the sequencing results were analyzed utilizing BLAST on the website www.ncbi.nlm.nih.gov for identification and analysis of nucleotide sequence homology. This method was employed to identify the closest fungal species based on percent identity.

Data Analysis

The quantitative analysis and presentation of the results obtained from the $CaCO_3$ precipitation test and the qualitative assay of the urease enzyme are shown in the form of a histogram. Furthermore, the identification of the fungal isolates was carried out through 18S rRNA gene sequencing analysis, and a phylogenetic tree was constructed using the MEGA-X program to visualize the relationships.

Results and Discussion

Sampling Locations

Carbonoclastic fungi responsible for damaging prehistoric cave paintings in this study were isolated from two sites: Parewe Cave and Bulu Sipong Cave, both in Pangkep Regency.

Figure 1. Sampling Location of Carbonoclastic Fungus Causing Damage to Paintings Prehistoric Paintings in Parewe Cave and Bulu Sipong Cave.

Parewe Cave is located in Borimasunggu, Labakkang District. Astronomically, it is situated at 4° 47' 40.1'' S and 119° 31' 7.25'' E. Similarly, Bulu Sipong Cave is located in Bontoa, Minasatene District, at coordinates of 4° 48' 23.81'' S and 119° 36' 36.9'' E. Based on observations during the sample collection, both locations exhibit numerous prehistoric handprint and babirusa paintings that are deteriorated. The colors of the painting have faded, and most of the artwork is covered in white sediment, making it difficult to distinguish the original image. According to Phang *et al.* (2018) and Gadd *et al.* (2014), the growth of carbonoclastic fungi on mural and cave paintings can cause structural damage such as peeling and discoloration of pigments due to the fungus, as well as the paintings being covered by calcite or calcium carbonate crystals.

Isolation of Carbonoclastic Fungi

The fungal isolations from both sites resulted in 24 pure isolates with different morphologies. Out of these isolates, 14 isolates were obtained from the Parewe cave and 10 isolates were obtained from the Bulu Sipong cave. Each isolate was then named according to the prefix letter or substrate source abbreviation, namely P (Parewe) and BS (Bulu Sipong). Isolates obtained are named Ps1-a, Ps1-b, Ps3, Ps4, Ps5, Ps7, Ps8-a, Ps8-b, Ps8-c, Ps8-d, Ps8-e, Ps11-a, Ps11-b, Ps11-c, Ps14, BSs2, BSs3, BSs5, BSs7, BSs8-a, BSs8-b, BSs8-c, BSs9, BSs10, and BSs11.

Carbonoclastic Fungi Selection

The isolated fungi were tested on Christensen Urea Agar (CUA) and incubated at 34^{o}C for 3x24 hours and fungal isolates were selected as shown in the figure below. Observations were made for color changes.

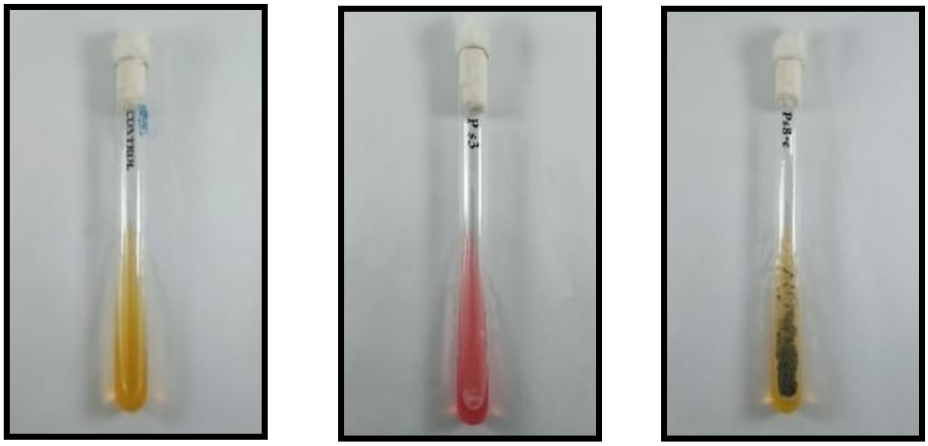

Figure 2. Fungal Growth in Christensen Urea Agar Medium

Fungal isolates capable of fermenting urea to produce calcium carbonate crystals in Christensen Urea Agar media are present in isolates Ps3, Ps8-c, Ps8-d, BSs3, BSs5, BSs7, and BSs11. The resulting change in color is closely tied to both the ability and amount of urea fermentation that increases the media's pH levels. The discoloration of Christensen Urea Agar media is caused by the phenol red content present in the media. Hammad *et al.* (2013) state that urease enzyme hydrolyzes urea, leading to ammonia accumulation in the media which in turn increases the pH of the media, resulting in an alkaline pH. As a result of this increase

in pH, the phenol red indicator changes color from yellow to bright pink, which confirms the capacity of carbonoclastic fungi to hydrolyze urea.

Characteristics of Carbonoclastic Fungi

Observation of Colony Morphology

Table 1. Morphological Observation of Carbonoclastic Fungus Colonies

Isolates	Observation Parameters				
	Color of Colony (3x24 h)	Colony Surface	Radial Line	Concentric Circles	Colony Color (7x24 h)
Ps3	White	Uneven like cotton	Absent	Absent	Greenish white
Ps8-c	Dark green	Flat like cotton	Absent	Absent	Dark green
Ps8-d	Greenish white	Velvety flat	Absent	Absent	Blackish green
BSs3	White	Velvety flat	Absent	Absent	Yellow-green
BSs5	White cream	Flat like flour and colonies spread upwards like cotton wool	Absent	Present	Light green
BSs7	White	Flat like flour and colonies spread upwards like cotton wool	Absent	Present	Yellow-green
BSs11	White	Uneven like cotton	Absent	Absent	Yellow

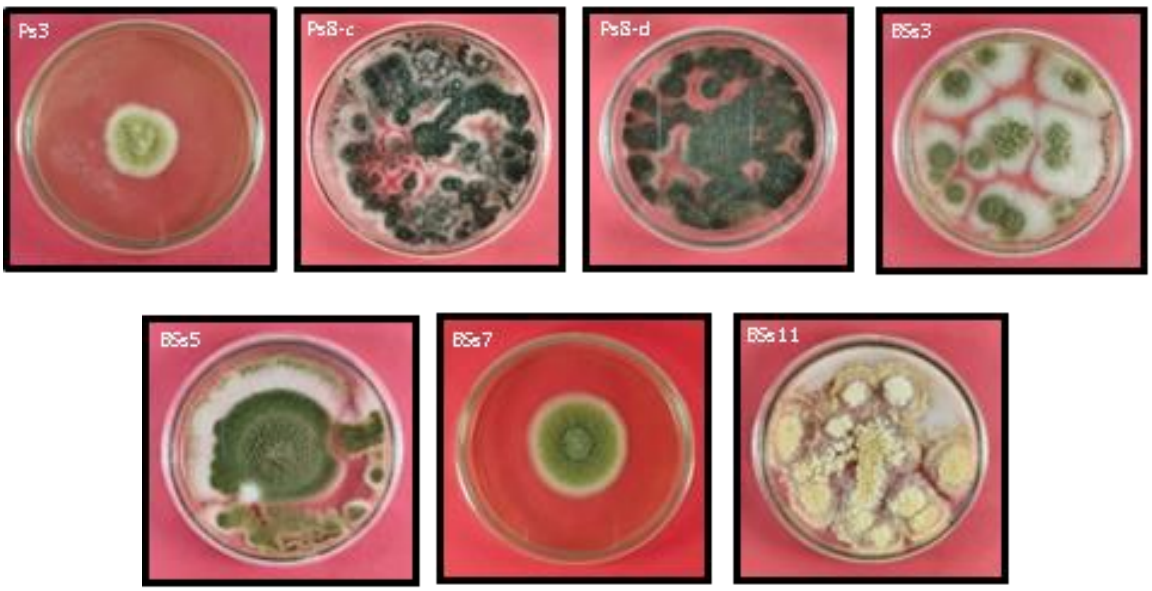

Figure 2. Fungal Growth in Christensen Urea Agar Medium

The same results were obtained in the research of Dylag *et al.* (2019) who isolated fungi from the Niedzwiedzia cave, Poland, where fungal growth on Malt Extract Agar (MEA) medium incubated for 7x24 hours showed colony characteristics including white which then became dark green at the end of incubation. Munawati *et al.* (2021) also grew mushrooms on the same medium and obtained the characteristics shown by yellow colonies, lacking radial lines and concentric circles, and rough surfaces.

Cell Morphology Observation

Based on the observation of cell morphology in Figure 4. with reference to the identification book *Illustrated Genera of Imperfect Fungi* by Barnett and Hunter (2002), isolates Ps3, Ps8-c, Ps8-d, BSs3, BSs5, BSs7, and BSs11 showed the same cell morphological characteristics, namely having intercepted hyphae, blue hyphal pigmentation, asexual spores in the form of conidiospores, round conidia, produced in chains and many cells. The same results were obtained in the research of Dylag *et al.* (2019) which obtained fungi with cell morphological characteristics, namely hyaline hyphal pigmentation (colorless), erect conidiophores, round vesicles, having asexual spores in the form of conidiospores with round conidia, produced in chains, and many-celled. The characteristics shown are like the characteristics of the fungus genus *Aspergillus*.

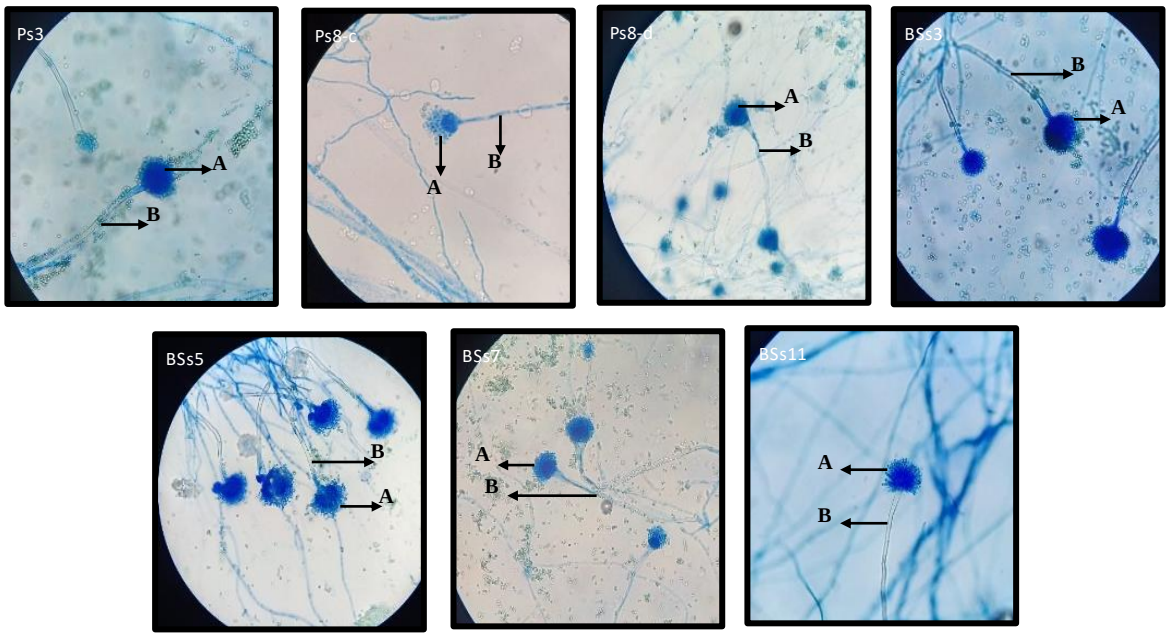

Figure 4. Observation of fungal cell morphology using a 400 magnification microscope, (A) Conidia and (B) Conidiophores

Test the CaCO3 Precipitating Potential of Carbonoclastic Fungi

Calculation of $CaCO_3$ Precipitate Weight

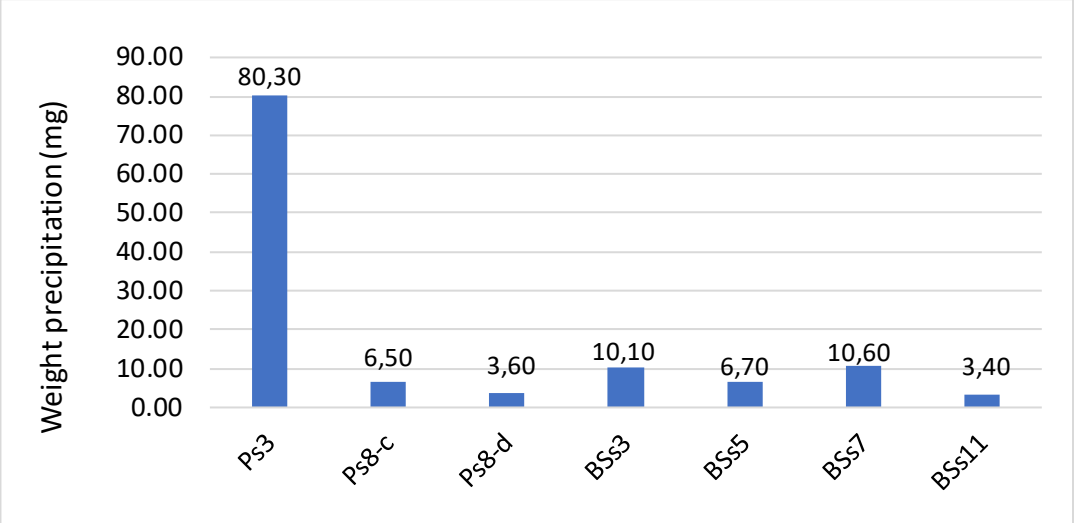

Figure 5. Calculation Result of CaCO3 Precipitate Weight

The Ps3 isolate demonstrated the highest CaCO3 precipitates (80.30 mg) as depicted in the histogram, while the BSs11 isolate showed the lowest amount (3.40 mg). The variation in $CaCO_3$ precipitates during the incubation process among fungi is attributed to distinct urease enzyme-facilitated urea hydrolysis capabilities of each isolate. According to Hassan (2020), various factors including differences in fungal species in producing urease enzymes cause differences in the amount of calcium carbonate precipitates produced by carbonoclastic fungal isolates. The production and activity of the urease enzyme can be influenced by factors such as incubation time, temperature, pH, appropriate urea concentration, carbon source, and concentration of the final product of urea hydrolysis in the form of ammonia.

Analysis of Ammonia Level

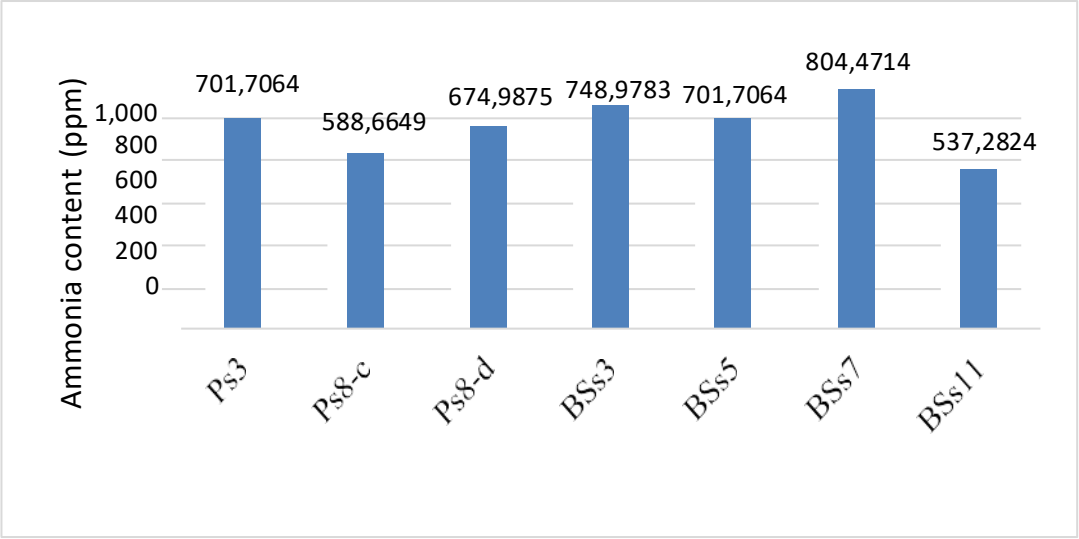

Figure 6. Calculation Results of Ammonia Content of Carbonoclastic Fungi

Based on the histogram presented, isolate BSs7 displayed the highest ammonia levels at 804.4714 ppm, while isolates PS3 and BSs5 exhibited the lowest levels at 701.7064 ppm. The discrepancy in ammonia levels produced can be attributed to the variation in urea enzyme production in each fungal species. One method to ascertain the presence of urease enzyme activity is to measure ammonia levels, which is the end-product of urea hydrolysis. Dhami *et al.* (2017) state that the urease enzyme hydrolyzes urea to form carbonate, which spontaneously decomposes into ammonia and carbonic acid. Calcium ions (Ca^{2+}) present in the environment bind with the carbonic acid (CO_3^{2-}) to produce calcium carbonate ($CaCO_3$). The ability of fungal isolates to hydrolyze urea determines the difference between the $CaCO_3$ and ammonia precipitates formed, as per Littman's (1957) findings. In their research, Maciejewska *et al.* (2017) found that the decrease was attributed to the production of organic acids, which are by-products of carbon metabolism from the degradation of dextrose (glucose) within the media. These organic acids include acetic acid, citric acid, and oxalic acid.

Calculation of Dry Weight Biomass Cells

Based on Figure 7, the isolate with the highest dry weight of cell biomass is indicated by BSs3 with 1,528.90 mg, while the lowest cell biomass is shown by Ps3 with 333.80 mg. The low production of cell biomass is influenced by the pH of the growth media. Fungi that grow in alkaline pH can inhibit the development of fungal mycelium, thus significantly decreasing biomass production due to strong alkalinity in the media (Zhao *et al.*, 2022). In addition to affecting cell biomass growth, pH, urea, and $CaCl_2$ act as major nutrient sources. Fungi that hydrolyze urea and $CaCl_2$ faster at the start of incubation will also form rapid cell growth, while fungi that take longer to adapt will begin to grow in the middle or end of the incubation period. Jamur that grows faster will experience a lack of nutrients during the incubation period, causing those unable to survive to enter a phase of death, while those that are still able to survive will enter a state of dormancy. According to Luo *et al.* (2018), when fungi grow under environmental pressure such as limited nutrients, most carbonoclastic fungi will produce inactive spores to withstand these conditions for an extended period until more favorable conditions arise. This mechanism is known as the fungal dormancy period, which is a form of adaptation for carbonoclastic fungi to thrive in any environmental conditions.

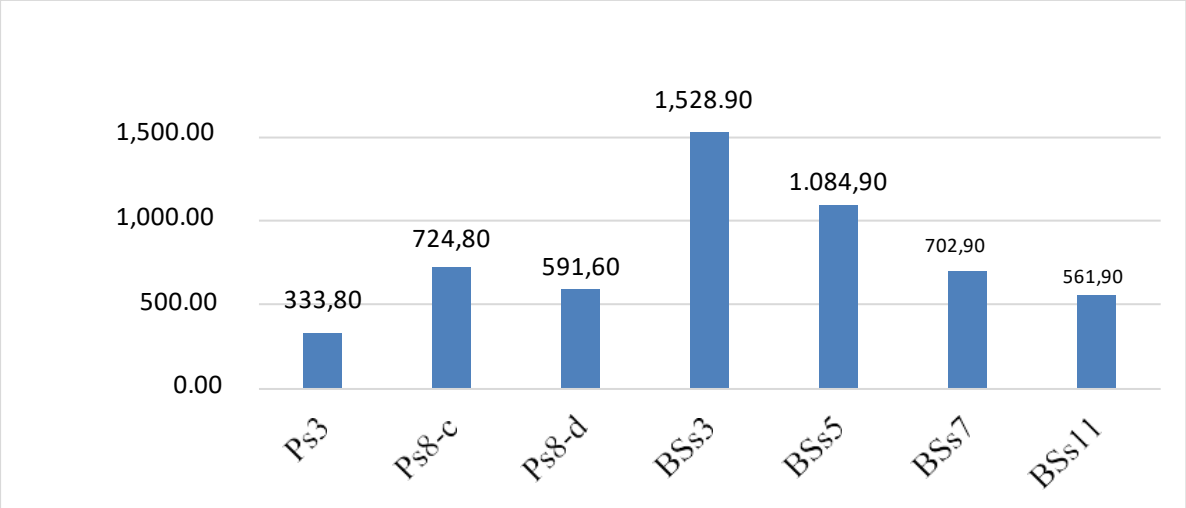

Figure 7. Dry weight biomass yield of carbonoclastic mushroom cells

Molecular Analysis Based on 18S rRNA Gene

The sequencing results obtained using the BLAST NCBI program and the Internal Transcribed Spacer region (ITS) database (Table 2) reveal that the Ps3 fungal isolate is most closely related to *Aspergillus* sp. strain BW1, with a DNA sequence similarity of 99.48% to the Gen Bank data and an error value of 0.0.

Tabel 2. BLAST Results of Ps3 Fungal Isolate

Isolate	Fungal Species	Query Cover	E Value	Per. Ident	Accession
Ps3	*Aspergillus* sp. strain BW1	98%	0,0	99,48%	MG372022.1

The next step was to construct a phylogenetic tree using the UPGMA method in the MEGA X program, which can be seen in the following figure.

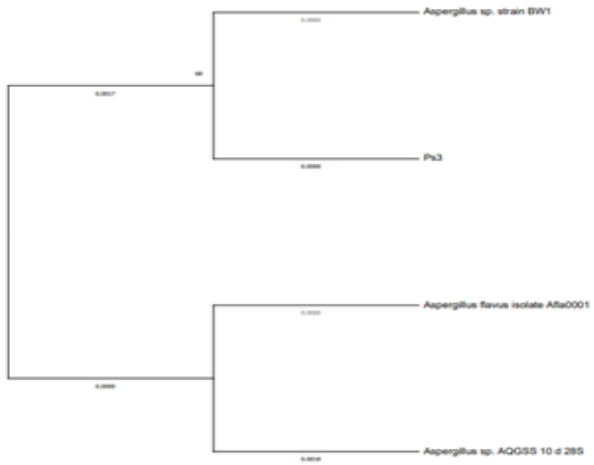

Figure 8. Phylogeny with the UPGMA Method for Isolate Ps3

The phylogeny analysis outcomes using the UPGMA method for Ps3 isolates indicate that fungi from the *Aspergillus* genus display the closest kinship with *Aspergillus* sp. strain BW1, with a genetic distance of 0.0017. These identification results unswervingly correspond with the research by Wasti *et al.* (2021), highlighting that the most frequently found carbonoclastic fungi type in karst caves is *Aspergillus* spp. at an estimated 36.4%. Dhami *et al.* (2017) provide evidence that fungi belonging to the *Aspergillus* genus have the ability to produce multiple types of calcium carbonate crystals, including calcite, vaterite, and aragonite.

Conclusion

Based on the research findings, it can be concluded that:

1. 24 fungal isolates were obtained from Karst Maros-Pangkep rocks, including 7 carbonoclastic fungus isolates, with 3 isolates from Parewe cave and 4 isolates from Bulu Sipong cave. One isolate, Ps3, showed the highest potential for $CaCO_3$ precipitation according to quantitative testing.
2. Based on genotypic identification using the DNA sequence of the 18S rRNA of isolate Ps3, it was determined that it belongs to the *Aspergillus* sp. strain BW1 fungus.

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