Biological evaluation of mangrove endophytic fungi *Aspergillus terreus* derived from *Sonneratia alba*

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Abstract. Mangroves represent highly diverse marine ecosystems, encompassing various vegetation such as shrubs, plants, trees, palms, and other plant forms that have adapted to thrive in both freshwater and saline environments. Within these mangrove ecosystems, secondary metabolites are present, contributing to various pharmacological functions and holding significant ecological importance. Endophytic fungi constitute a substantial and quantifiable portion of fungal biodiversity and are acknowledged for their impact on the composition and diversity of plant communities. This study aims to investigate the extracellular proteins and enzyme activity exhibited by endophytic fungi discovered in the leaves of *Sonneratia alba* mangroves collected in Buton, Southeast Sulawesi, Indonesia. The primary objective is to evaluate the compatibility of marine fungi with antibacterial and antioxidant activity. The research involves analyzing the biological activities of marine fungi containing cellulase enzyme through methods such as DNS for reducing sugar, protein concentration determination, antioxidant analysis, and total phenol content assessment. Sample A (shaker) has highest enzyme activity at Day 4 and Sample B has highest enzyme activity at Day 6 (static condition). The protein concentration in the purified cellulase from *Aspergillus terreus* surpassed that of crude enzyme extracts.

1 Introduction

Mangroves are essential to the ecology of tropical coastlines because of their habitat, which is located in the dynamic zone between land and sea. Mangroves can flourish in harsh aquatic settings and help produce metabolite chemicals [1]. Numerous endophytic marine

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fungus, such as *Aspergillus* sp., *Penicillium* sp., *Trichoderma* sp., and *Fusarium* sp., are supported by decomposing mangrove debris [2]. Interestingly, marine endophytic fungi isolated from the mangrove species *Sonneratia alba* show antibiotic action against pathogenic bacteria and fungi such *Candida albicans*, *Escherichia coli*, and *Staphylococcus aureus* [3].

Endophytic marine fungi engage in a mutualistic relationship with their hosts, involving protection against pathogens, sharing secondary metabolites, and possessing antibacterial and antiviral properties [4]. Aquatic microorganisms residing in water environments and sediments produce diverse enzymes [5]. Endophytic marine fungi can generate oligosaccharide-degrading enzymes, particularly cellulases, showcasing significant cellulolytic activity with potential applications in biotechnology [6]. Additionally, metagenomic analysis reveals that endophytic marine fungi can produce or metabolize bioactive compounds such as terpenoids [7].

Marine fungi isolated from various areas have shown excellent results for cellulase action [8]. Extracts from marine endophytic fungus can produce enzymes and also demonstrate anti-inflammatory action both in vitro and in vivo [9]. This suggests that there is potential for more research into its mode of action. Additionally, marine fungal extracts contain cellulase. Microbial cellulases have immense potential for various industrial and biotechnological applications, making them highly sought after. Cellulase is the world's third most utilized group of enzymes in the industrial sector, driven by its diverse applications in textiles, bioethanol production, detergents, food processing, and animal feed. Acting as a digestive aid that breaks down fibers, cellulase contributes to the alleviation of digestive issues such as malabsorption [10].

Aspergillus terreus, a species of marine fungi, has the potential to serve as a bioremediation agent [11] and exhibits antioxidant properties [12] as well as enzyme production [13]. Additionally, it can decompose carboxy-methyl-cellulose (CMC), xylan and pectin [14]. Cellulase activity testing of fungal isolates was carried out using Congo red staining, which showed a broad clearance zone that could aid the production process [15]. The study aims to analyze the biological activities of marine fungi containing cellulase enzyme by employing methods such as DNS for reducing sugar, protein concentration determination, antioxidant analysis, and assessment of total phenol content.

2 Methods

2.1 Extraction of bioactive compound

Extraction of marine endophytic fungi culture media using maceration method. The first stage involves mixing the sample with ethyl acetate solvent at a ratio of 1:1, followed by agitation for 3x24 hours without heating using a shaker. The agitation process is carried out for 3x24 hours, assuming that maceration is no longer effective in extracting the active components contained in the sample. The culture media and ethyl acetate extract are separated using a separating funnel and allowed to stand for a few moments until the phase between the culture media and ethyl acetate extract separates clearly. The obtained extract is

then concentrated using a vacuum rotary evaporator at 40°C. This temperature is used to prevent the extract from losing heat-sensitive active compounds [16].

2.2 Cellulase enzyme activity

The suspension of fermented *Aspergillus terreus* in a Carboxy Methyl Cellulose (CMC) substrate was centrifuged using a Universal 32 R Hettich Germany centrifuge at 5000 rpm (25 °C, 15 min). The Eppendorf tube was then filled with the suspension. The crude enzyme source was the collected supernatant, which was kept at −20 °C prior to the experiment in order to stop enzyme breakdown. It is interesting to note that the crude enzyme's cellulase activity was predicted by the activities of endoglucanase (also known as carboxymethylcellulase, or CMCase), exoglucanase (also known as filter paperase, or FPase), and β-glucosidase. The IUPAC Commission on Biotechnology's standard protocol was followed while analyzing the cellulase activity in triplicate. In order to measure endoglucanase activity, 0.5 mL of 2% (w/v) CMC (Sigma Chemical Co., St Louis, MO, USA) was incubated for 30 minutes with 0.5 mL of appropriately diluted crude enzyme in sodium acetate buffer (0.05 M, pH 5).

The concentration of enzyme that released 1 µmol of glucose per minute under assay conditions was defined as one unit of endoglucanase activity. The concentrations of released reducing sugars were determined for the assessment using the DNS technique [17]. Using a reaction combination made up of 1 milliliter of 1% CMC, 1 milliliter of the culture supernatant, and 3 milliliters of DNS reagent, the cellulolytic activity—expressed as carboxymethyl cellulase (CMCase) activity—was measured using the DNS method. The mixture was then incubated at 100°C for 30 minutes. Using a spectrophotometer, the color that formed after boiling was determined at 540 nm. Under assay conditions, one unit of the enzyme was defined as the amount that released one micromole of glucose per minute. The amount of generated enzyme was given in units per gram of dry substrate (U/gds) [18].

2.3 Protein concentration assay

The Bradford reagent was prepared by adding 25 mL of 85% (w/v) phosphoric acid after dissolving 25 mg of Coomassie Brilliant Blue G-250 in 12.5 mL of 95% ethanol. Distilled water was added to reach a final volume of 0.5 liters following full dissolution. Shortly before being used, the mixture was filtered through Whatman No. 1 filter paper. The Bradford technique was used to calculate the protein concentration. 0.1 mL of the sample was added to a reaction tube throughout this procedure. After that, the mixture was incubated for five minutes with the addition of five milliliters of Bradford reagent. The absorbance was determined with a spectrophotometer set at 595 nm in wavelength. The standard solution was generated using a BSA stock solution with a concentration of 2 mg/mL and concentrations ranging from 0.01-0.1 mg/mL. The same methodology was used to this solution. The protein concentration in the sample was then determined by plotting the acquired absorbance values on the Bradford standard curve [19].

2.4 FRAP method antioxidant assay

Making the FRAP reagent was the first step in performing the antioxidant assay using the FRAP method. Weighed separately at 187, 150, and 270 mg were sodium acetate trihydrate, TPTZ, and FeCl3.6H2O. 0.3 M acetate buffer at pH 3.6, 0.01 M TPTZ (2,4,6-tripyridyl-s-triazine) in 0.04 M HCl, and 0.02 M FeCl3.6H2O were combined to create the FRAP reagent. The proportions of TPTZ, FeCl3.6H2O, and acetate buffer were 10:1:1. Three milliliters of

the FRAP reagent, six milliliters of distilled water, and five milliliters of the sample were used to measure absorbance. After vortexing to homogenize the sample-reagent combination, it was incubated for 30 minutes at 37°C. At that point, the absorbance was measured at a wavelength of 593-595 nm. The standard was FeSO4.7H2O dissolved in distilled water. Because the FRAP approach can measure antioxidant capacity, which is quantified in μmol Fe (II) per gram of extract, it was chosen [21].

2.5 Total phenol content

The Folin-Ciocalteau reagent was used in conjunction with a spectrophotometer to quantify total phenols. Three duplicates of the total phenol content analysis were performed. One milliliter of the extract, one milliliter of 96% ethanol, and five milliliters of distilled water were combined to create the *S. polycystum* sample extract. After homogenizing 0.5 mL of the 50% Folin-Ciocalteau reagent into this mixture, it was left to stand for five minutes. After adding 1 mL of 5% Na2CO3 to the extract, the mixture was allowed to sit in the dark for 60 minutes. A wavelength of 725 nm was used to measure the absorbance. To calculate the phenol content, which was measured in milligrams of Gallic Acid Equivalent (GAE) per 100 milligrams of fresh sample, this process was performed three times. Gallic Acid Equivalent (GAE) per 100 g of the dry weight of the sample extract is the unit of measurement for the total phenolic content. The total phenolic content of the sample was determined by using the linear regression equation and plotting the absorbance values of the gallic acid standard against its concentration to create a linear curve [22].

3 Result and discussion

3.1 Cellulase enzyme activity

The β-1,4 glycosidic link found in cellulose, cellodextrin, cellobiose, and other cellulose derivatives is broken by the cellulase enzyme, which then turns the material into glucose. Cellulase is an induction enzyme that is produced in response to the presence of cellulose in the organism's growth environment. The enzymatic activity of cellulase is a collective result of three interconnected enzymes within the cellulase enzyme complex, namely (i) endoglucanase, (ii) exoglucanase, and (iii) β-glucosidase. Endoglucanase (EC 3.2.1.4) 1,4 (1,3;1,4)-β-D-glucan 4-glucanohydrolase breaks down β-glycoside linkages in cellulose at random, producing oligomers such as glucose, cellobiose, and cellodextrin [23].

Furthermore, it was discovered that oleaginous yeasts could assimilate carboxymethyl cellulose (CMC): *Candida intermedia* PLE6DP6, *Candida orthosilopsis* InaCC Y-302/Y09GS34, *Candida oleophila* InaCC Y-306/Y09GS48, *Crytococcus flavescent* PL3DP6, *Cryptococcus humicola* PLE3DP9, and *Yamadazyma aff. mexicana* PL1W2 [24]. The normal process for evaluating a yeast's ability to break down cell walls is analyzing the enzymatic characteristics of extracellular enzymes generated during aerobic development. The cellulases responsible for cellulose hydrolysis mainly consist of three types: (1) endoglucanases (EC. 3.2.1.4), (2) cellobiohydrolases (EC. 3.2.1.91), and β-glucosidases (EC.3.2.1.21). Endoglucanases (EG) can hydrolyze soluble, substituted celluloses like Carboxymethyl Cellulose (CMC) by internally attacking the carbohydrate chain (1-4, β glucosidic bond) [17]. Carboxy methyl cellulose (CMC) indicated by the formation of a clear zone. The divalent metal cations CuCl2, MgCl2, and ZnCl2 induced the enzyme activity, whereas NaCl and the divalent cation CoCl2 acted as inhibitors decreasing the enzyme activity. The optimum activities of cellulase isolated from the KRC 21.D strain were as

follows: 4.9 U/mL at an incubation duration of 5 days, 6.3 U/mL at pH 6.0, 7.3 U/mL at a temperature of 50 °C, and 6.8 U/mL at a CMC concentration. A glucose solution was selected as the solvent for constructing the standard curve since glucose, derived from substrate hydrolysis by cellulase enzymes, serves as a reducing sugar. To assess enzyme activity, the reduction in sugar concentration was determined by creating a glucose standard curve with concentrations of 0, 50, 100, 150, 200, 250, and 300 (**Table 1**).

The linear equation y = 0.0016x-0.0161 with a correlation value (R2) of 0.9911 is the outcome of the standard curve analysis (**Table 1**). The amount of glucose present in the sample enzyme fraction can be calculated using this equation. The synthesis of 1 μmol of reducing sugar per minute by enzymes hydrolyzing cellulose substrates is known as cellulase enzyme activity. Enzyme activity serves as an indicator of enzyme purity, where higher activity levels correspond to greater purity. The enzyme crude extract activity obtained from the fermentation of marine fungi *Aspergillus terreus* is illustrated in **Fig. 1**.

Table 1. Formulation of glucose solution for standard curve.

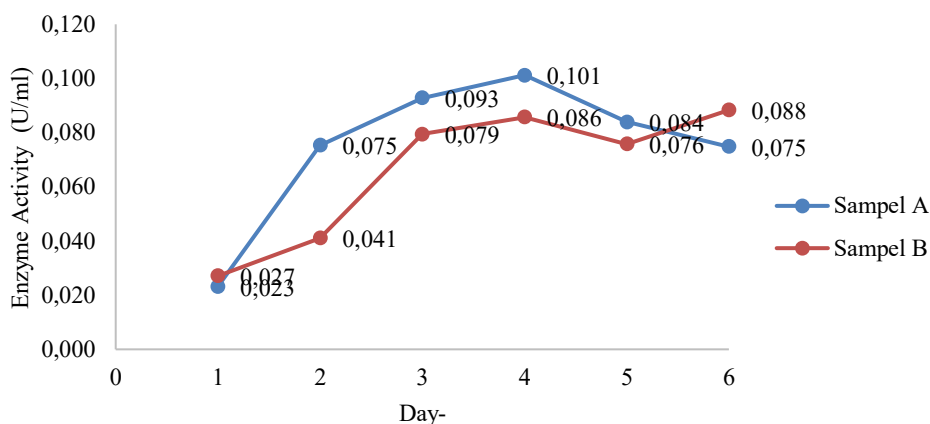| Concentration		DI water (ml)	Stock solution of standart glucose (ml)
ppm	mg/ml		
0	0.00	2.00	0.00
50	0.05	1.90	0.10
100	0.10	1.80	0.20
150	0.15	1.70	0.30
200	0.20	1.60	0.40
250	0,25	1.50	0.50
300	0.30	1.40	0.60

Fig 1. Enzyme activity curve of the enzyme crude extract.

The results showed that Sample A (shaker) has highest enzyme activity at Day 4 and Sample B has highest enzyme activity at Day 6 (static condition). This is due to the fact that shaking conditions can both accelerate the enzyme's activity and expand the mycelia's surface area in the culture. When it comes to soluble amorphous cellulose, this enzyme works well

on substituted cellulose, like carboxymethyl cellulose (CMC) and hydroxyethyl cellulose. It is inert when it comes to crystalline cellulose types, such as cotton and avicel (microcrystalline cellulose). Due to its notably high activity on CMC substrate, this enzyme is commonly referred to as CMCase [25].

3.2 Protein concentration assay (Bradford 1976)

Using the Bradford method [13], the protein content of the cellulase enzyme obtained from *Aspergillus terreus* was evaluated. The foundation of protein analysis is a chemical reaction and a color comparison with a reference protein. The standard protein in this procedure is frequently bovine serum albumin (BSA). Bradford analysis depends on the way the protein and dye interact, changing the dye's spectral characteristics as it binds to the protein [18]. For protein content testing, Coomassie Brilliant Blue (CBB) G250 dye was used, which produced a blue hue. Using a spectrophotometer [19], light intensity at a wavelength of 595 nm was measured.

The concentrations of the BSA standard solution varied from 0.2 g/L to 1 g/L. The protein concentration in the cellulase enzyme was then calculated by plotting the obtained absorbance values on a standard curve. The x-axis denoted the concentration of the standard solution, while the y-axis represented the absorbance values. The resulting regression equation was y = 0.0531x - 0.0163, with a coefficient of determination (R^2) of 0.9995 (**Fig. 2**).
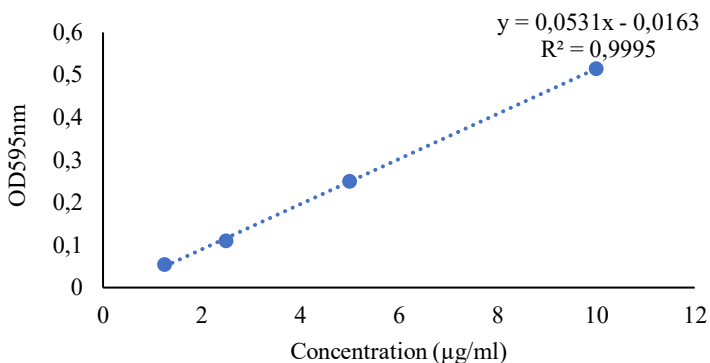

Fig 2. BSA calibration curve.

The recorded absorbance of the cellulase enzyme was 0.467, indicating a protein concentration of 9.1 mg/L. Evaluating the protein concentration is a crucial aspect of enzyme performance assessment. Commercial enzymes constitute intricate mixtures with diverse components. Factors such as stabilizers, preservatives, and remnants from the fermentation process contribute to the complexity of these enzymes, underscoring the significance of considering protein concentration before analysis [26]. In a study conducted by Rong *et al.* [27], cellulase enzymes (crude extract) derived from the marine fungus *Aureobasidium pullulans* 98 exhibited a soluble protein content of 4.7 mg/mL, while in a study by Pujiati et al. [28], a soluble protein content obtained from *Trichoderma viride* isolates measured at 0.147 mg/mL. The protein concentration in the purified cellulase from *Aspergillus terreus* surpassed that of crude enzyme extracts (**Fig. 3**).
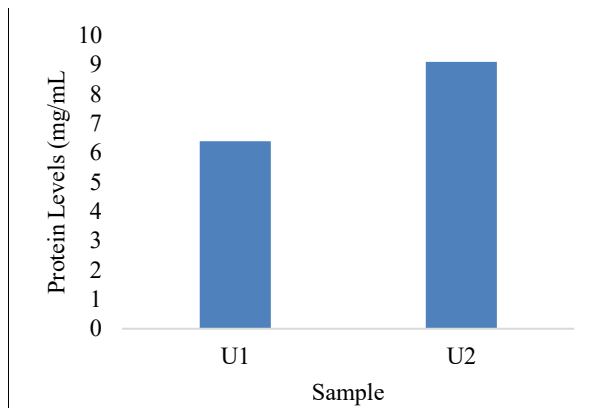
Fig 3. The protein content of the cellulase enzyme from *Aspergillus terreus.*

3.3 FRAP method antioxidant assay (Benzie and Strain 1996)

Fig. 4 illustrates the linear regression equation that was derived from the antioxidant activity testing data using the FRAP method. The equation reads y = 0.0014x + 0.0704 with a R² value of 0.99 at the maximal wavelength of 595 nm.
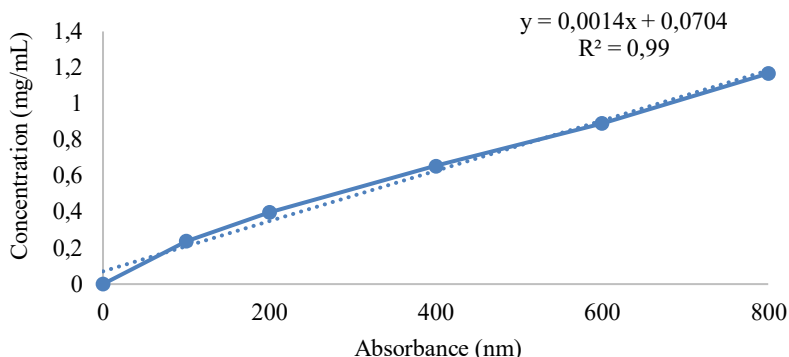

Fig 4. Standard curve of FRAP.

After plotting the sample concentrations against this linear regression equation, the antioxidant activity of both extracts was obtained, as presented in **Table 2**.

Table 2. Antioxidant activity test results.

No	Sample	Absorbance	Antioxidant Activity (mg/gram)
1	EAE (H9)	0.03	16.400
2	EAE (H12)	0.023	11.400

According to the test results, the extract produced on the ninth day had greater antioxidant activity than the extract produced on the twelfth day, with an equivalency value of 16.40 mg/g of the sample, compared to 11.40 mg/g of the sample for the extract produced on the

twelfth day. Electron transfer occurs between the antioxidant and the Fe^{3+}-TPTZ complex molecule when antioxidant activity is measured using the Ferric Reducing Antioxidant Power (FRAP) technique. The Fe3+-TPTZ molecule itself denotes potentially harmful oxidizing substances that may exist in the body and endanger cells. Under these circumstances, the Fe^{3+} ion is reduced to Fe^{2+}, as indicated by the solution becoming blue. To maintain the solubility of iron ions, the experiment was carried out in an acidic environment with a pH of 3.6. By comparing the changes in absorbance in the sample solution with a reference solution that has known concentrations of Fe2+ ions at λ 593 nm, antioxidant activity using the FRAP technique is ascertained [29].

3.4 Total phenol content

Using gallic acid as a reference component, the Folin-Ciocalteu technique was used to determine the total phenol concentration. Measurement of the gallic acid reference compound resulted in a linear regression with the equation $y = 0.0636x - 0.1057$ and an R^2 value of 0.9148, as seen in **Fig. 5.** From the measurement results, it is known that the phenolic compound content in both maceration and UAE extracts is 3.742 and 3.408 mg GAE/g, respectively (**Fig. 6**).
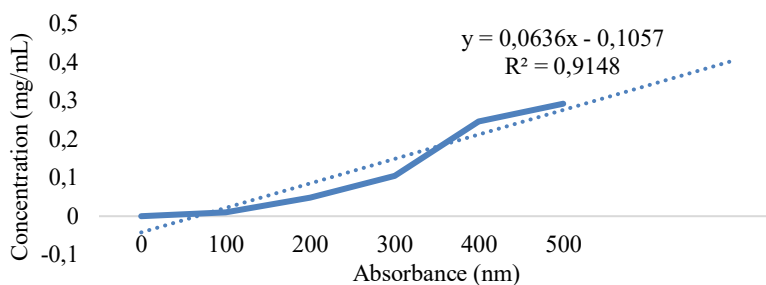
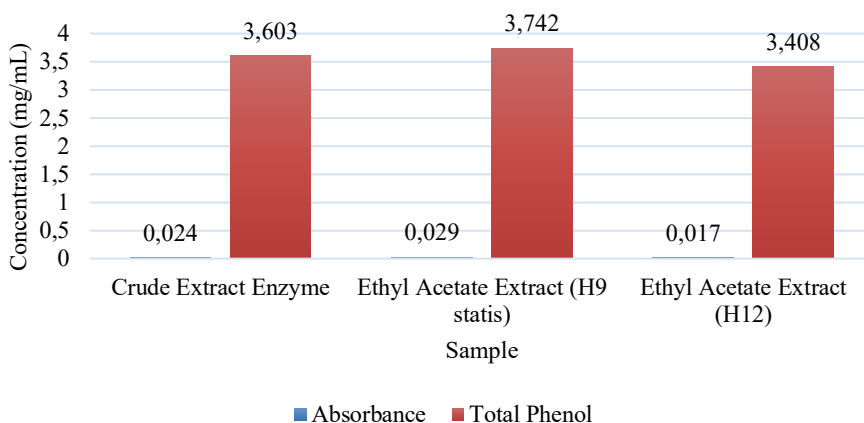

Fig 5. Standard curve of gallic acid.

Fig 6. Total phenol content from *Aspergillus terreus.*

Judging by these values, both methods yield approximately equivalent phenolic content. According to this study, the UAE extract's phenolic component level is actually lower than

the maceration extract's. This is suspected to be due to the increase in temperature in the UAE extraction system caused by continuous friction. It is known that some phenolic compounds are not stable when exposed to heat. For example, it has been established, according to Ibrahim and Kabede [30] that too high extraction temperatures and extraction periods that are longer than necessary might cause chemicals in the solution to evaporate.

Furthermore, bioactive components such as flavonoids are not heat-resistant, particularly at temperatures above 50 °C, which can cause structural changes and result in low extract activity [9]. Similar findings are also reported by Dzah et al. [31] who note that polyphenolic chemicals may be degraded by extraction temperatures higher than 50 °C. Additionally, it is stated that the ultrasonic frequency that can effectively increase phenol content should be below 40 kHz. If it rises above this threshold, it may produce free hydroxyl radicals, which have the potential to break down polyphenols, particularly when there is a lot of water present [32]. Consequently, the increased yield from the UAE approach in this study does not correspond directly with the quantity of phenolic compounds or antioxidant activity.

4 Conclusion

Mangrove endophytic fungi *Aspergillus terreus* produced an extracellular enzyme. Sample A (shaker) has highest enzyme activity at Day 4 and Sample B has highest enzyme activity at Day 6 (static condition). Evaluating the protein concentration is a crucial aspect of enzyme performance assessment. The protein concentration in the purified cellulase from *Aspergillus terreus* surpassed that of crude enzyme extracts. The extract produced on the ninth day had greater antioxidant activity than the extract produced on the twelfth day, with an equivalency value of 16.40 mg/g of the sample, compared to 11.40 mg/g of the sample for the extract produced on the twelfth day. The phenolic compound content in both maceration and UAE extracts is 7.72 and 7.25 mg GAE/g. This research show that marine endophytic fungi is prolific sources of cellulase enzymes. The diverse properties of marine endophytic fungal enzymes can be used in different biotechnological applications.

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