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Isolation and Characterization of Cellulase Enzymes from Marine Endophytic Fungi

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ABSTRACT. Enzymes called cellulases can break down the β 1,4-glycosidic bonds in cellulose to produce glucose or simple sugars. In this study, marine endophytic fungi that were isolated from the seagrass Enhalus sp. were used to obtain cellulase enzymes, assess activity, and identify the ideal pH and temperature for cellulase enzymes. The research involved a number of steps for the isolation and characterization of cellulase enzymes from marine endophytic fungus EN isolated from Enhalus sp, including preparing the seaweed by soaking it in warm and room-temperature water, extracting the cellulase enzymes by cultivating the marine fungi on production media with pretreated seaweed, counting the total cellulase activity was assessed using the Mandels et al. (1976) technique, and purifying the cellulase enzymes by depositing it with ammonium sulfate at a saturation level of 30-80%. According to the findings, on day 9 with seaweed immersed in a warm water as a carbon source, cellulase enzyme a marine fungus isolated from a seagrass Enhalus sp. had the greatest crude extract activity of total cellulase enzyme activity (FPAse), amounting to 0.0276 U/mL. The cellulase enzyme precipitated at a 70% saturation level of ammonium sulfate with had an FPAse activity of 0.0381 U/mL and a specific activity of 1.85 U/mg. At pH 5 and 50 °C, cellulase enzyme deposition in this investigation was at its best.

Keywords: Cellulase enzyme, extraction, *Enhalus* sp., seaweed

INTRODUCTION

Cellulase enzymes play a critical role in breaking down the β-1,4-glycosidic linkages of cellulose into simpler sugars, including glucose. Cellulose itself is an unbranched polymer composed of D-glucose units linked by β-1,4-glycosidic bonds, which, when cleaved, can generate cellobiose as an intermediate (Sinjaroonsak et al., 2020). Optimization of cellulase and xylanase production has been successfully demonstrated in *Streptomyces thermocoprophilus* TC13W using low-cost, pretreated oil palm empty fruit bunch as a substrate. The cellulase enzyme complex comprises three primary forms: endoglucanase (CMCase or endo-1,4-β-D glucanase), exoglucanase (cellobiohydrolase or exo-1,4-β-D glucanase), and β-glucosidase, each contributing to cellulose hydrolysis (Sohail et al., 2016). Owing to their high efficiency in breaking down cellulose, cellulases are widely applied across various industries, including bioremediation, wastewater treatment, coffee processing, bioenergy, and biofuel production. Furthermore, they have gained prominence in the pharmaceutical and textile industries, particularly for recovering cellulose-related properties in textiles using detergents (Choudary et al., 2018).

The rising demand for cellulases across diverse industrial sectors has stimulated research into their production (Ezeilo et al., 2020). The choice of substrate is a crucial factor, as cellulose- or starch-based materials can stimulate enzyme synthesis (Maftukhah & Abdullah, 2018). Various agro-industrial residues such as paper processing waste (Nair et al., 2018), durian peel (Maftukhah & Abdullah, 2018), rice straw (Dutt & Kumar, 2014), banana peel (Mahmoud et al., 2014), pineapple skin (Tanamool et al., 2020), rice bran (Somchart et al., 2019), wheat bran, rice husk, and sugarcane bagasse (Gaur & Tiwari, 2015) have been effectively utilized for cellulase production. In this study, *Kappaphycus alvarezii* seaweed was employed as the substrate due to its significant cellulose content, reported at 12.21% (Tassakka, 2020), and its wide availability in natural environments.

Cellulase enzymes catalyze the hydrolysis of organic polymers, particularly cellulose, into glucose and other simple sugars. Within the cellulase complex, the three main enzymes—endo-β-1,4-glucanase, exo-β-1,4-glucanase, and β-1,4-glucosidase—work synergistically to degrade cellulose into glucose. Carboxymethyl cellulose (CMC) is commonly used as a selection medium for microorganisms capable of producing cellulase due to its linear cellulose structure. During cellulolytic activity assays, Congo red binds to the 1,4-β glycosidic bonds in cellulose, producing a red coloration. A clear zone around colonies indicates enzymatic hydrolysis, as the released cellulase prevents Congo red binding to glucose, revealing the enzymatic activity (Andriyono et al., 2015).

Cellulase production is generally attributed to both fungi and bacteria. Fungi are the most common producers of cellulase enzymes (Bekele et al., 2015), particularly terrestrial species such as *Trichoderma, Aspergillus, Hunicola*, and *Penicillium* (Salomao et al., 2019), including *Trichoderma viride* (Nathan et al., 2015), *Aspergillus niger* (Idiawati et al., 2014), and *Aspergillus carbonarius* (Ike, 2014). Bacterial producers include *Klebsiella ozeanae* (Kalaiselvi & Jayalaksmi, 2013), *Klebsiella variicola* (Gopinath et al., 2014), and *Bacillus vallismortis* (Gaur & Tiwari, 2015). Compared to terrestrial fungi, research on cellulase production by marine-derived fungi, particularly endophytic fungi, remains limited. Endophytic fungi maintain a symbiotic relationship with their hosts without causing disease (Ratnaweera & de Silva, 2017).

Marine endophytic fungi coded as EN, isolated from the seagrass Enhalus sp., have demonstrated substantial cellulase activity. Among endophytic fungi derived from seaweed, mangrove leaves, and sponges, *Enhalus* sp.-derived EN exhibits the highest cellulolytic index (Oktavia et al., 2014). Similarly, Andhikawati et al. (2014) reported that EN showed superior cellulolytic activity compared to fungi isolated from other marine sources, highlighting its potential as an effective cellulase producer.

EXPERIMENTAL SECTION

Isolation and characterization of cellulase enzymes from EN marine fungi involved several steps. The research stages include: (1) Seaweed preparation as a substrate which includes soaking seaweed with water at warm temperature and room temperature, (2) Cellulase enzyme extraction includes the stage of marine fungal culture on production media with pretreatment seaweed and (3) Cellulase enzyme purification includes the deposition of the cellulase enzyme with ammonium sulfate at a saturation level of 30-80%.

Kappaphycus alvarezii Seaweed Preparation as Substrate

Kappaphycus alvarezii was prepared by first rinsing the raw seaweed three times with clean water to remove surface impurities, followed by soaking in warm water (40–50 °C) and then in ambient temperature water for 30 minutes in a glass beaker. The softened biomass was subsequently homogenized with a blender until a uniform slurry was obtained. This pretreatment step facilitates the conversion of cellulose and improves its susceptibility to enzymatic action by altering structural components to enhance enzyme accessibility, as described by Rohmah et al. (2019). The resulting seaweed slurry served as the carbon substrate for the subsequent enzymatic processes.

Cellulase Enzymes Extraction

Cellulase enzyme extraction was carried out by transferring the marine fungi in the Potato Dextrose Broth (PDB) liquid medium into an Erlenmeyer containing 100 mL of production media. Transferring 10% of the marine fungal preculture to a 500 mL Erlenmeyer flask with 200 mL of 2 g/L KH_2PO_4 enzyme production media, urea, $MnSO_4.H_2SO_4$ 1.6 mg/L, $ZnSO_4.7H_2O$ 1.4 mg/L, $CoCl_2.6H_2O$ 2 mg/l, peptone 0.1%, tween 80 0.1%, with 1.5% carbon source, and $MgSO_4.7H_2O$ and 0.3 g/L $CaCl_2.2H_2O$, respectively. The fine seaweed was then incubated with a shaker at 120 rpm for 6 days, 9 and 12 days at room temperature. The culture results were then separated between the media and mycelia with filter paper. The enzyme's supernatant was prepared by centrifuging it at 5000 g for 20 minutes at 4 °C. The total cellulase activity of the centrifuged enzyme's crude extract was assessed using the Mandels et al. (1976) technique.

Cellulase Enzymes Purification

Purification of the cellulase enzyme was performed through ammonium sulfate precipitation following the general approach of Xia et al. (2017) with modifications. Crude enzyme extract was subjected to fractional precipitation by gradually adding ammonium sulfate to reach 30–80% saturation while continuously mixing until the salt was fully dissolved. The mixture was then stored at 4 °C overnight to allow protein precipitation. The precipitated fraction was collected by centrifugation at 8000 rpm for 15 min at 4 °C and subsequently resuspended in 50 mM citrate buffer (pH 5) at a 1:2 ratio. The activity of the resulting enzyme preparation was assessed using Whatman No. 1 filter paper as the substrate.

Cellulase Enzymes Characterization

The characterization of cellulase activity with respect to pH and temperature was conducted following the general approach of Behera and Ray (2016). Enzyme characterization was performed on the ammonium sulfate–precipitated fraction rather than on the crude extract, as fractionation allows partial enrichment of cellulase proteins and reduces interference from non-enzyme components that may affect activity measurements. Only the 30–80% saturation fraction was selected for characterization because preliminary tests indicated that this fraction contained the highest cellulase activity after precipitation. The optimal pH and temperature were subsequently determined by assaying this partially purified fraction across a range of pH and thermal conditions to assess its catalytic performance and stability under varying environments.

Calculation of the optimal pH

By combining 0.5 mL of enzyme and substrate in the form of Whatman No. 1 (1x6 cm^2) with citrate buffer (pH 3.0-6.0) and Tris-HCl, the ideal pH can be determined (pH 7.0-8.0). For 60 minutes, the enzyme combination was incubated at 50 °C.

Calculation of the optimal temperature

By combining 0.5 mL of enzyme with a substrate in the form of Whatman No. 1 (1x6 cm^2) and citrate or Tris-HCl buffer at the ideal pH, it is possible to determine the ideal temperature. For 60 minutes, the enzyme mixture was incubated at a temperature range of 30-80 °C with 10-degree intervals.

Analysis Procedure

The analysis carried out in this study was the analysis of reducing sugars using the DNS method, analysis of total cellulase enzyme activity (FPAse) and analysis of protein content. The analysis of the test is described as follows.

Enzyme activity analysis

Based on Mandels et al. (1976) about spectrophotometric approach, the cellulase enzyme activity was measured. The enzyme was treated with 1 mL of citrate buffer (pH 5) and Whatman No 1 filter paper in a total volume of 0.5 mL. 3 mL of DNS was then added after the mixture had been incubated at 50 °C for an hour. The test mixture was cooked for 5 minutes at 100 °C in boiling water. By combining the substrate with citrate buffer and incubating at 50 °C, a control was achieved. After adding DNS, the mixture was cooked for five minutes in boiling water. Citrate buffer and 3 mL DNS are combined to create the blank solution, which is then boiled in boiling water for 5 minutes. Spectrophotometry was used to assess activity at a wavelength of 540 nm. In order to quantify the absorbance, standard glucose (0.2–0.6 mg/mL) was measured. 1 U/mL of cellulase enzyme activity represents the quantity of enzyme required to convert 1 moL of cellulose into glucose in 1 mL of solution every minute of reaction. Cellulase enzyme activity is calculated by the formula:

$$\text{Activity(U/mL)} = \frac{(Xsample - Xcontrol)x1000xFP}{(txBMxV)}$$

Note

X sample: Concentration of glucose in sample solution
X control : Glucose concentration in control solution
FP : Dilution Factor
T : Incubation Time (minute)
BM : Glucose Molecular Weight (180)
V : Enzyme volume added (mL)

Protein content analysis

Protein concentration in the ammonium sulfate–precipitated cellulase fraction was quantified using the Bradford assay (Bradford, 1976). The dye reagent was prepared by dissolving 25 mg of Coomassie Brilliant Blue G-250 in a mixture of 12.5 mL of 95% ethanol and 25 mL of 85% phosphoric acid, after which the solution was brought to a final volume of 250 mL with distilled water. A bovine serum albumin (BSA) standard curve was generated using concentrations ranging from 0.03 to 0.05 mg/mL. For sample analysis, 0.1 mL of enzyme solution was combined with 5 mL of the prepared dye reagent and allowed to react for 5 minutes at room temperature. Absorbance was measured at 595 nm using a spectrophotometer, and protein content was calculated by interpolating the absorbance values against the BSA standard curve.

RESULTS AND DISCUSSIONS

Cellulose is a biopolymer that is found in nature. Cellulase enzymes in general can degrade cellulose into simple sugars (Bai et al., 2021). Cellulase is an enzyme that can break down cellulose's β-1,4 linkages. Cellulolytic microorganisms can produce cellulase enzymes in response to cellulose-containing substrates (Rohmah et al., 2019). One of the cellulolytic microorganisms that produce cellulase enzymes is a marine fungus isolated from a seagrass *Enhalus* sp.

Fungal Growth

The endophytic fungi EN isolate is the chosen and pure isolate with the highest cellulolytic index of 1.34 when compared to another isolates isolated from seaweed, mangrove leaves, and sponges, so it is employed as research material since it is a source of cellulase enzymes (**Figure 1**).

Figure 1. EN isolate in PDA medium

Fungi undergo several distinct phases during their growth, including the lag (adaptation) phase, exponential (logarithmic) phase, stationary phase, and death phase. During the lag phase, marine fungi adapt to their environment and modify their cellular machinery to produce the enzymes necessary for substrate degradation. The exponential phase is characterized by rapid cell division, which corresponds to increased metabolic activity and enzyme production. In the stationary phase, the growth rate slows as the number of living cells balances with the number of dead cells, whereas in the death phase, cell death exceeds cell proliferation (Rohmah et al., 2019). These growth phases can be effectively monitored by measuring the dry biomass of marine fungi cultured in GDP media, typically represented in the form of a growth curve.

The growth curve provides insight into the dynamic changes in cell population and enzyme production at each stage of development. Specifically, for EN marine fungi cultivated in GDP media, the growth curve demonstrates the progression through these phases and highlights the peak of biomass accumulation, which often coincides with maximal cellulase production (**Figure 2**). Such observations are crucial for optimizing enzyme harvest and improving the efficiency of industrial applications, as enzyme yield is closely linked to the physiological state of the fungal culture. The growth curve of EN marine fungi in Guanosine Diphosphate (GDP) media can be seen in **Figure 2.**

The growth phases of marine fungi have a significant impact on the production of enzymes. As noted by Bekele et al. (2015), cellulase enzymes produced by filamentous marine fungi play a crucial role in cellulolytic activity necessary for fungal growth. Based on the growth curve of EN marine fungi (**Figure 2**), the lag phase occurred from day 0 to day 3, during which the fungi adapted to the culture conditions and prepared the metabolic machinery for growth. From day 3 to day 12, a steady increase in the mycelial biomass of EN marine fungi was observed, indicating the onset of the exponential phase. During this phase, the fungi actively utilized the nutrients in the growth medium, resulting in a rapid increase in mycelial mass. The exponential phase is particularly important for cellulase production, as it corresponds to the period of maximum enzymatic activity and is therefore considered optimal for enzyme harvesting (Zhu et al., 2013).

Cellulase Enzyme Crude Extract Activity

Cellulase is composed of three main enzymes: endo-β-glucanase (CMCases), exo-β-glucanase (FPAse), and β-glucosidase (Bai et al., 2021). The total cellulase activity, often represented by FPAse, reflects the synergistic action of these enzymes in converting cellulose into glucose (Gupta et al., 2015). The enzymatic activity of cellulase is a key factor in its capacity to hydrolyze cellulose. In this study, cellulase activity was assessed after extracting enzymes from marine fungi cultivated in production media for 6, 9, and 12 days under different incubation temperatures. **Figure 3** demonstrates how the duration of incubation and temperature conditions, using seaweed as the carbon source, influence cellulase activity. To evaluate enzymatic activity, Whatman filter paper No. 1 was used, as it is a representative substrate containing both amorphous and crystalline cellulose (Idiawati et al., 2014). One unit of FPAse activity (U/mL) is defined as the amount of enzyme required to hydrolyze one mole of cellulose in filter paper into one milliliter of glucose (Roosheroe et al., 2014).

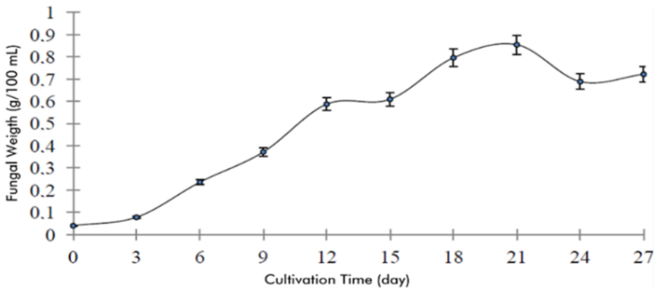

Figure 2. Growth curve of EN marine fungi on GDP medium

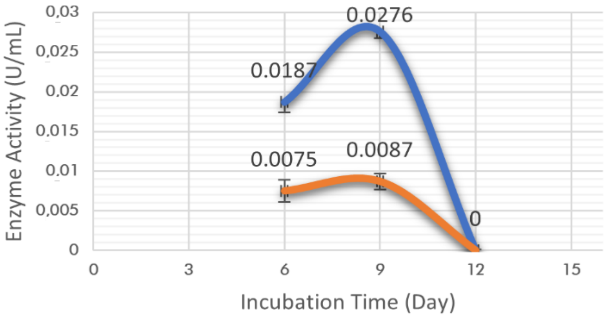

Figure 3 Activity of crude extract of cellulase enzyme (FPAse) with different immersion temperature and incubation time (🟦 warm temperature 🟧 room temperature)

The total cellulase activity observed in this study ranged from 0 to 0.0276 U/mL. When EN marine fungi were incubated in water at temperatures between 40 and 50 °C, the cellulase activity measured after 6 days was 0.0187 ± 0.0013 U/mL, while after 9 days it reached a maximum of 0.0276 ± 0.0008 U/mL. At 12 days, cellulase activity was negligible, indicating that the culture had entered the stationary phase. This aligns with previous reports stating that marine endophytic fungi such as *Aspergillus terreus* produce the highest cellulase levels during the exponential (log) phase of growth (Isti'anah *et al.* 2024). The peak cellulase activity in this study occurred after 9 days of incubation, corresponding with warm water pretreatment. Oktavia et al. (2014) reported a similar trend, where total cellulase activity increased up to the ninth day and declined thereafter. Likewise, Salomão et al. (2019) observed that *Trichoderma koningii* exhibited maximum filter paperase activity after more than 84 hours of incubation using sugarcane bagasse as a substrate. Commercial cellulase from *Aspergillus niger* also showed optimal activity at 9 days of incubation (Yoon et al., 2014), highlighting the importance of incubation duration in enzyme production.

The chosen incubation periods of 6, 9, and 12 days were based on the growth pattern of EN marine fungi, which enter the exponential phase between days 3 and 12 (Oktavia et al., 2014). The production medium in this study included seaweed as a carbon source. Cellulase biosynthesis requires substrates containing starch or cellulose, which stimulate enzyme synthesis (Ezeilo et al., 2020). Seaweed provides cellulose that marine fungi can metabolize during growth. The selection of shorter (6 days) and longer (12 days) incubation times was informed by the observed exponential phase at 9 days, allowing assessment of enzyme production before and after peak activity. Pretreatment of seaweed via water immersion at elevated temperatures aimed to reduce lignin content, which can hinder cellulase access to cellulose. This method avoids the use of harsh chemicals such as H_2SO_4 while enhancing enzyme accessibility. Pretreatment facilitates the breakdown of saccharide polymers into monomeric sugars, allowing cellulases to act efficiently (Sethi et al., 2013). Lignin, a complex phenylpropanoid polymer, forms resistant linkages that impede hydrolysis (Srivastava et al., 2019). By removing or reducing lignin, the interaction between enzymes and cellulose improves, thereby increasing glucose yield.

The total cellulase activity in this study surpassed that reported by Oktavia *et al.* (2014), who achieved 0.013 U/mL using waste agar as a carbon source. However, the activity remains lower than that of certain terrestrial microorganisms. For example, *Aspergillus niger* grown on banana peel exhibited 12.4 and 0.58 U/mL (Mahmoud et al., 2014), and *Bacillus amyloliquefaciens* AK9 produced substantial cellulase levels (Irfan et al., 2017). The type of carbon and nitrogen sources significantly affects cellulase synthesis, as highlighted by Choudhary *et al.* (2018), who reported maximal enzyme activity of 335.5 U/mL using glucose. These findings indicate that while marine fungi are promising sources of cellulase, substrate composition and incubation conditions remain critical factors for optimizing enzyme yield.

Semi Pure Cellulase Enzyme Activity

Cellulase enzyme purification was carried out to remove non-protein components that could interfere with enzyme activity. Purification begins with precipitating the crude extract of the enzyme with ammonium sulfate. Ammonium sulfate is a salt that is able to precipitate proteins and has the advantages of being easy to apply, inexpensive, has a very high solubility, is non-toxic to most enzymes and has a stabilizing effect on enzymes (Alviyulita et al., 2014). The addition of ammonium sulfate salt can remove non-protein components in the crude extract, so that the results obtained are protein deposits. Ammonium sulfate was added in increments of 30, 40, 50, 60, 70, and 80% to precipitate enzyme proteins. The high activity produced during the measurement of cellulase activity was used to establish the proper ammonium sulfate saturation concentration for precipitating the cellulase enzyme from EN marine fungi.

Ammonium sulfate is widely used for protein precipitation due to its ease of application, low cost, high solubility, enzyme-stabilizing properties, and general non-toxicity (Alviyulita et al., 2014). By adding ammonium sulfate, non-protein components in the crude extract are removed, resulting in enriched protein precipitates. In this study, ammonium sulfate was incrementally added at concentrations of 30, 40, 50, 60, 70, and 80% to selectively precipitate cellulase proteins. The optimal saturation level for cellulase precipitation was determined based on the highest activity observed during enzymatic assays. Figure 3 illustrates the cellulase activity profile corresponding to each ammonium sulfate concentration, highlighting the effectiveness of this approach in isolating the enzyme from EN marine fungi.

The precipitation of enzyme proteins induced by ammonium sulfate reduces the presence of impurities that could obstruct the enzyme's active site, thereby increasing its catalytic efficiency (Alviyulita et al., 2014). In this study, the highest cellulase activity was achieved at a 70% ammonium sulfate saturation, with a measured activity of 0.0381 U/mL and a specific activity of 0.7728 U/mg. However, increasing the saturation to 80% caused a decline in enzyme activity to 0.0129 U/mL. This reduction is attributed to excessive salting out, where the high concentration of salt ions in ammonium sulfate binds water molecules, diminishing protein solubility. Consequently, proteins aggregate and precipitate due to insufficient water to maintain them in solution, a phenomenon commonly referred to as salting out (Zahra et al., 2020).

Table 1. Enhancement of cellulase enzyme activity during the ammonium sulfate precipitation process

Stage	Volume / Mass	Enzyme Activity (U/mL)	Protein Concentration (mg/mL)	Specific Activity (U/mg)	Purification Fold
Crude Extract	200 mL	0.03	0.02	1.57	1.00
Precipitated Fraction	9 mg	0.05	0.03	1.85	1.18

The data in **Table 1** show that the ammonium sulfate precipitation step effectively improved the quality of the cellulase enzyme. The crude extract exhibited an enzyme activity of 0.03 U/mL with a specific activity of 1.57 U/mg. After precipitation, the enzyme activity increased to 0.05 U/mL, while the specific activity improved to 1.85 U/mg, corresponding to a 1.18-fold purification. This enhancement indicates that ammonium sulfate successfully removed non-protein impurities that could interfere with enzymatic activity, thereby increasing the proportion of active enzyme relative to total protein (Alviyulita et al., 2014). The observed increase in specific activity demonstrates that protein precipitation can enrich the functional enzyme fraction, which is crucial for subsequent biochemical applications and industrial processes (Zahra et al., 2020).

Protein molecules are composed of both hydrophilic and hydrophobic amino acid regions, which influence their behavior during precipitation. Hydrophilic regions interact with water molecules, whereas hydrophobic regions tend to aggregate and precipitate first in the presence of salt ions. High concentrations of ammonium sulfate can attract water molecules toward hydrophilic regions, promoting the precipitation of proteins containing these amino acids (Zohri et al., 2022). This principle explains why 70% ammonium sulfate is often effective in enriching enzyme fractions, as observed by Zhu et al. (2013), who reported increased specific activity of cellulase produced by *Bacillus amyloliquefaciens* XZ-173 under this condition. Similarly, Alviyulita *et al.* (2014) demonstrated that protease enzymes could be efficiently precipitated at 70% ammonium sulfate saturation, achieving activity levels of 116.13 U/mL. The efficiency of enzyme precipitation and resulting activity, however, can be influenced by multiple factors, including substrate concentration, enzyme amount, presence of inhibitors, and environmental conditions such as temperature and pH (Lu et al., 2020). Overall, ammonium sulfate precipitation is a reliable method for concentrating and partially purifying enzymes while enhancing their specific activity for downstream applications.

Cellulase Enzyme Characteristics

In this study, the optimal temperature and pH for the cellulase enzyme were determined through the characterisation of the enzyme at various variations in temperature and pH. The high or ideal activity of the cellulase enzyme generated is a sign of the ideal temperature and pH.

Optimal pH Level

The activity of cellulase enzymes is strongly influenced by the pH of the surrounding medium. Variations in acidity can significantly alter enzymatic performance, as each enzyme exhibits an optimal pH at which its catalytic function is maximized. Deviations from this optimal pH can lead to structural changes in the enzyme's protein, reducing its activity, whereas maintaining the ideal pH supports efficient substrate binding and catalysis. In this study, the effect of pH on the activity of the precipitated FPAse enzyme was evaluated, and the results, as shown in **Figure 4**, demonstrate how enzyme activity fluctuates with changes in acidity, highlighting the importance of pH control for maximizing cellulase performance.

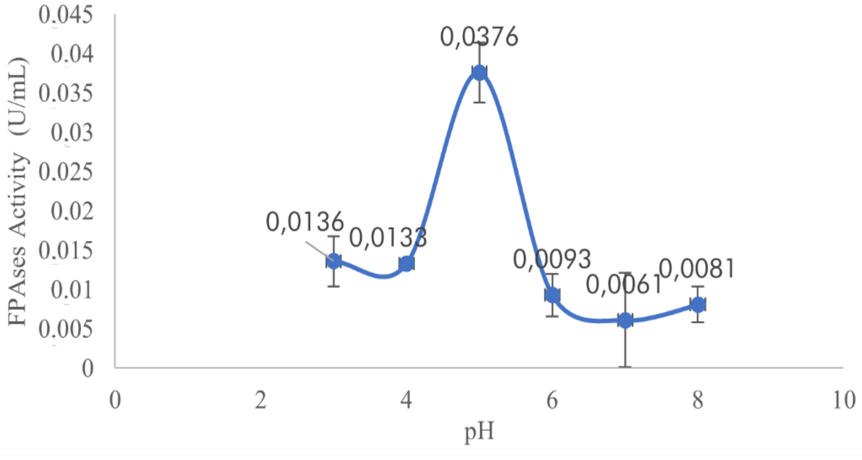

Figure 4. Cellulase (FPAse) activity at various pH.

In this study, cellulase from EN marine fungi exhibited maximum activity (0.0376 U/mL) at pH 5, with lower activity observed at both more acidic and more alkaline conditions, as shown in **Figure 5**. The enzyme's activity is strongly influenced by the ionic properties of its amino acid residues, particularly the carboxyl (-COOH) and amino (-NH_2) groups, which are sensitive to changes in pH. Deviations from the optimal pH can cause partial denaturation of enzyme proteins, leading to reduced catalytic function. As amphoteric molecules, enzymes carry both acidic and basic charges on their surfaces, and optimal activity occurs when these charges are balanced. In acidic conditions, the enzyme surface tends to be positively charged, whereas in alkaline conditions, it becomes negatively charged, reducing enzyme-substrate interaction and overall activity (Arnata et al., 2020). Cellulase catalyzes the hydrolysis of cellulose primarily at the glutamate residue in its active site. The carboxyl group (-COOH) of glutamate facilitates protonation of the glycosidic oxygen, promoting enzyme-substrate complex formation and efficient cellulose hydrolysis (Jadhav et al., 2013).

The pH also affects the sulfhydryl (-SH) groups of cysteine residues, which are critical for enzyme activity following ammonium sulfate fractionation. In alkaline conditions, excess hydroxyl ions (-OH) convert -SH groups into -S-, preventing proper protonation and inhibiting interaction with the substrate, thereby reducing glucose production and overall enzymatic activity (Putri et al., 2013). The optimal pH observed in this study aligns with prior research: cellulase from *Aspergillus niger* exhibited maximum activity at pH 5 (0.087 U/mL) (Idiawati et al., 2014), while crude cellulase extracts from seaweed-associated bacteria also reached peak activity at pH 5 (12.623×10^{-4} U/mL) (Dini and Munifah, 2014). Generally, cellulase enzymes are most active within a slightly acidic range, between pH 4 and 6, which supports efficient cellulose hydrolysis in various microbial systems (Lu et al., 2015).

Optimal Temperature Value

The activity of cellulase (FPAse) is influenced not only by pH but also by temperature, which plays a crucial role in maintaining enzyme structure and catalytic efficiency. Determining the optimal temperature is essential to achieve maximum enzyme activity, as temperatures exceeding this optimum can lead to denaturation, disrupting the enzyme's three-dimensional conformation and impairing substrate binding. In this study, the temperature characterization of EN marine fungi cellulase was conducted to identify the condition that supports the highest enzymatic activity, as illustrated in **Figure 5**. Understanding the temperature profile of cellulase is critical for optimizing its use in industrial or biotechnological applications where enzyme stability and activity are pivotal (Lu et al., 2020).

The optimal temperature for enzyme activity is defined as the temperature at which the rate of the catalyzed reaction is highest (Koç et al., 2015). In this study, the FPAse activity of EN marine fungi increased with temperature up to 50 °C, reaching a peak activity of 0.0515 U/mL, before declining at higher temperatures (**Figure 5**). The initial rise in enzyme activity is attributed to increased kinetic energy, which enhances molecular vibrations, rotations, and translations, allowing enzymes and substrates to collide more frequently and form more product. This effect accelerates the hydrolysis of cellulose by the cellulase enzyme. However, when the temperature exceeded 50 °C, the activity dropped, indicating enzyme denaturation. Elevated temperatures can disrupt the enzyme's tertiary and secondary structures, including hydrogen bonds, ionic interactions, and hydrophobic interactions, resulting in a loss of active site integrity and a reduction in catalytic efficiency (Alam et al., 2013; Hawar, 2022). Additionally, extreme temperatures can alter the substrate's structure, further limiting enzyme-substrate interactions (Bai et al., 2021).

The optimal temperature of 50 °C observed in this study aligns with previous reports on cellulase from various microorganisms. For instance, the cellulase enzyme from *Streptomyces transformant*, *Mucor* sp., and other cellulolytic fungi also exhibited peak activity at 50 °C (Sijaroonsak et al., 2020; Singh, 2020; Ezeilo et al., 2020). Similarly, Dutt and Kumar (2014) reported that cellulase enzymes generally function optimally within the temperature range of 50–60 °C. These findings highlight that the thermal stability and catalytic performance of cellulase enzymes from marine fungi are consistent with those from terrestrial sources, suggesting that EN marine fungi cellulase can be effectively applied in biotechnological processes requiring moderate to high temperatures.

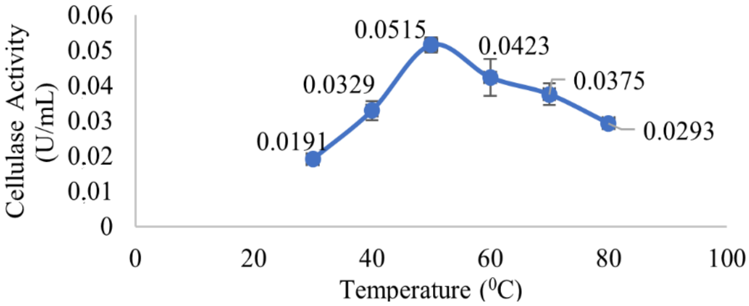

Figure 5. Cellulase (FPAse) activity at various temperatures.

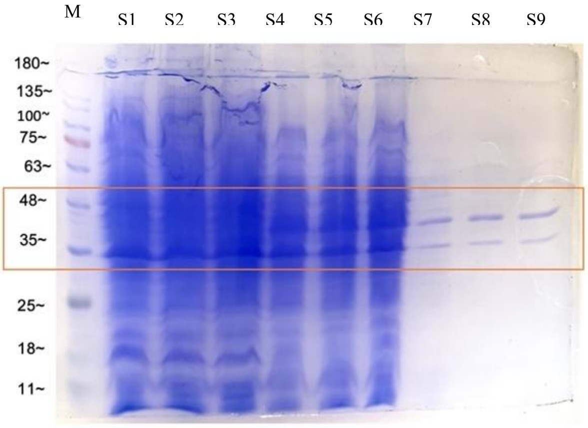

Figure 6. Molecular weight of extracellular enzyme with different saturation level.

SDS-PAGE analysis revealed that the extracellular cellulase produced by the EN isolate exhibited a molecular mass ranging from 35 to 48 kDa (**Figure 6**), which reflects the differences inherent in protein separation techniques. In addition to cellulase, endophytic marine fungi are capable of producing other hydrolytic enzymes, such as proteases. For example, extracellular protease from *X. psidii* KT30 has a molecular mass of 71 kDa, a specific activity of 0.091 IU/mg, and demonstrates optimal activity at 60 °C and pH 7 (Budiarto et al., 2015). Both cellulase and protease possess significant industrial and biotechnological potential, with applications spanning pharmaceuticals, food processing, biofuel production, agriculture, single-cell protein production, and use as probiotics in aquaculture. The coexistence of these extracellular enzymes highlights the multifunctional enzymatic capacity of marine endophytic fungi, making them promising candidates for diverse biotechnological applications.

CONCLUSIONS

The cellulase enzyme extracted from seagrass endophytic fungi (*Enhalus* sp.) exhibited its highest crude FPAse activity of 0.0276 U/mL on the ninth day of incubation using seaweed as a carbon source with warm water immersion. Subsequent ammonium sulfate precipitation at 70% saturation enhanced the enzyme activity to 0.0381 U/mL, with a specific activity of 0.7728 U/mg, indicating effective purification. The enzyme demonstrated optimal catalytic performance at pH 5 and a temperature of 50 °C, highlighting the suitable conditions for its activity and potential application. These findings emphasize the capability of marine endophytic fungi as a promising source of cellulase for biotechnological and industrial applications.

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