Production and characterization of cellulases derived from saprophytic fungi *Penicillium bilaiae* InaCC F16

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Abstract. Cellulases are the enzymes that hydrolyze β-1,4 glycosidic bonds in cellulose molecules into simpler molecules such as glucose. The cellulase complexes derived from microbes have been considered as a potential source for biochemical, biotechnology, and bioindustry. Thus, they are broadly used in industries for leather tanning, food, medicine, and detergent formulations. The important group of cellulase-producing fungi, such as *Penicillium*, is reportedly able to produce high levels of cellulase and hemicellulase complexes. This study aimed to determine the characterization and activity of cellulase enzymes produced by fungi *Penicillium* and analyze their cellulase activities, both qualitatively and quantitatively. The fungal strain used in this study was obtained from the Indonesian Culture Collection (InaCC) with the accession number of InaCC F15, InaCC F16, and InaCC F17. The parameters used for investigating the enzyme characteristic and activity were incubation time, index of substrate concentration on carboxymethyl cellulose (CMC), temperature, pH, and the effect of the addition of several metal ions as activators and/or inhibitors. The semi-qualitative analysis showed that the clear zone was formed in the surrounding colony of *Penicillium* InaCC F16 with a cellulolytic index was at 3.3. The optimum cellulase activity was obtained on two days incubation period, the temperature of 40ºC, pH 5, and substrate concentration at 2%. The addition of metal ions at a concentration of 1mM showed that enzymes were activated by cations CoCl\(_2\), CuCl\(_2\), MgCl\(_2\), and inhibited by NaCl and ZnCl\(_2\).

1. Introduction

A large number and variety of enzymes have been reportedly produced at large-scale used in the food and feed industries [1]. Potential enzymes can be used as an industrial enzyme to improve the nutritional quality of food for humans and animals. In the metabolic reactions, enzymes are efficient catalysts, offering much more competitive processes compared to the chemical catalysts [2].

Cellulases are the enzymes for hydrolyzing the cellulose biomass, and microorganisms produce these complex enzymes when grown on the over cellulosic medium [3]. There are many previous studies reported about high cellulolytic activity among the organisms. A cellulolytic enzyme was produced by microorganisms for degrading carbohydrates and produced intensively by strains, which were unable to utilize lipids and proteins as a source of energy for metabolism and growth [4]. Cellulase is a complex of three types of enzymatic complexes, namely, cellobiohydrolases (EC 3.2.1.91), endoglucanases or CMCases (EC 3.2.1.4), and β-glucosidases (EC 3.2.1.21). These complexes act synergistically to convert complex carbohydrates present in lignocellulosic (LC) biomass into glucose [5].
Many studies reported that *Penicillium* species secreted the cellulase enzyme activity [6-8]. *Penicillium* strains, such as *P. brasilianum*, *P. occitanis*, and *P. decumbens*, are capable of secreting a complex of cellulase enzymes. They could have practical application in the enzymatic hydrolysis of cellulose and have the capability to give high levels of extracellular cellulases [9-11]. According to [12], *Penicillium* species (i.e., *P. verruculosum/Talaromyces verruculosus*) is an efficient producer of highly active extracellular cellulase multienzyme system with the PvLPMO9A content up to 57% of the total secreted protein obtained [12-14]. Another study by [15] reported that cellulase enzymes from *P. decumbens* could be used for increasing the yield of apple juice, giving a clear yellow apple juice with negative pectin and starch contents. The study conducted by [16] showed the good performance of *P. funiculosum* cellulase with mixed industrial strains of *S. cerevisiae* on sugarcane bagasse cellulolignin during simultaneous saccharification and fermentation. It showed the production of second-generation ethanol on a production scale. Another study conducted by [8] reported for the first time the stable alkali cellulases from alkali tolerant fungus *P. citrinum*. Thermostable endoglucanase from *P. citrinum* may have potential effectiveness as additives to the laundry detergents.

The important group of cellulase-producing fungi, such as *Penicillium* species, was reportedly able to produce high levels of cellulase complexes (endoglucanase, exoglucanase, and β-glucosidase) and hemicellulase complexes [17-20]. Some of *Penicillium* species have a higher ability to produce cellulytic enzymes compared to fungi *Trichoderma reesei* [21-23]. This study was conducted to determine the characterization and activity of cellulase enzymes produced by fungi *Penicillium* strain InaCC F15, InaCC F16, and InaCC F17, and to analyze their activities, both qualitatively and quantitatively.

2. Materials and methods

2.1. Laboratory equipment and consumables
The materials and equipment required were water bath, oven, digital balance, incubator, centrifuge, vortex, shaker incubator, spectrophotometer, measuring pipette, measuring glass, test tube, Erlenmeyer flask, magnetic stirrer, magnetic stirrer bar, micropipette, micropipette tips, plastic heater, test tube rack, Petri dish, fungal strains, potato dextrose agar (PDA), paper disc, distilled water, yeast extract, bacteriological peptone, carboxymethyl cellulose (CMC), KH2PO4, (NH4)2SO4, MgSO4.7H2O, CaCl2, NaCl, bacto agar, 3,5-dinitro salicylic acid (DNS) reagent, and Bradford reagent.

2.2. Fungal strains
Fungal strains used in this study were obtained from the Indonesian Culture Collection (InaCC) Research Center for Biology – LIPI. They consisted of *Penicillium herquei* InaCC F15, *Penicillium bilaiae* InaCC F16, and *Penicillium paxilli* InaCC F17. Fungal strains were revived from inactive into active metabolism conditions by picking up the cryotubes containing fungal cultures from the deep freezer-80°C and then thawed immediately in the water bath at 37°C for three min. After being thawed, the bead or disc-shaped containing fungal cultures in the cryotube was three points inoculated onto PDA, and then incubated at 27°C for 5-7 days [24].

2.3. Medium preparation
Media were used for qualitative selection contained 1% (g/v) CMC and composed of 2.0 g of KH2PO4 (Merck), 1.4 g of (NH4)2SO4 (Merck), 0.3 g of MgSO4.7H2O (Merck), 0.1 g of CaCl2 (Merck), 1.0 g of peptone (Difco) and 2.0 g of agar (Bacto). All materials were dissolved in 100 mL distilled water and prepared in accordance with [25]. Fungal cultures showed the best growth with the widest clear zone. They were then selected and transferred into PDA slant, and then incubated in the incubator at 27°C for 3-4 days.

2.4. Cellulase production and activity assay
Fermentation media for cellulase production were prepared and composed similarly to the qualitative selection media, as previously described in [25]. The flasks were incubated in the shaker incubator with 120 rpm velocity, at 27°C for five days. After five days of incubation, they were centrifuged at
velocity 8000 rpm, the temperature of 4°C for ten minutes to separate supernatant (crude enzyme) from their substrate particles and fungal mycelia. The acquired supernatant was then stored in a freezer (-10°C) for further being measured its cellulase activity. The cellulase activity was observed and analyzed using a spectrophotometer at λ 540 nm, as stated by [16]. Furthermore, cellulase enzyme activity was quantitively tested according to the DNS method [26] by measuring the quantity of reducing sugar produced by hydrolyzed enzyme activity in 1% CMC substrate.

2.5. Qualitative examination of cellulase activity
Each of fungal strain was inoculated in selective agar media containing 1% (g/v) CMC. Each of the fungi strains was grown on the surface of media, then incubated for 72 hours. After 72 hours, the fungal colony was dropped with 0.1% (g/v) Congo red solution as an indicator and let it for 30-60 minutes, and then washed with 2% (g/v) NaCl solution. The clear zone was formed around the fungal colony, then measured by [27]. The formed clear zone around a colony was an indication of the existence of cellulase enzyme activity. The cellulolytic index was measured by dividing the value of the colony diameter and the diameter of the formed clear zone. Isolates showing the existence of cellulase activity with high relative value was then examined quantitatively.

\[
\text{Cellulolytic index} = \frac{\text{clear zone diameter}}{\text{colony diameter}}
\]  

2.6. Quantitative examination of cellulase activity
To measure the cellulase activity quantitatively, we prepared the reaction following [28]. An equal amount of enzyme supernatant (i.e., 0.125 mL) was added into 0.125 mL of substrate (i.e., phosphate buffer solution with pH 7.0). The reaction was incubated at 40°C for 30 minutes. To terminate the reaction, we added 0.25 mL of 3,5 dinitrosalicylic acid (DNS) into the reaction [28]. The reaction was then boiled for five minutes and cooled under flowing tap water. Furthermore, as much as 2.5 mL distilled water was added, and then the mixed solution was measured with a spectrophotometer at λ 540 nm. Calculation of cellulase activity was carried out with a formula:

\[
\text{Cellulase activity} = \frac{(\text{Glucose content} \times \text{dilution factor}) \times 1000}{(\text{Glucose molecular weight} \times \text{incubation time})}
\]

One unit of cellulase activity is defined as enzyme quantity producing 1 µmol of glucose in one minute at examination condition. It is multiplied by 1000 as a conversion from mmol to µmol.

2.7. Characterization of cellulase enzyme
The parameters used for investigating the cellulase characteristic and activity were: incubation time, index of substrate concentration on carboxymethyl cellulose (CMC), temperature, pH, and the effect of the addition several metal ions as activators and/or inhibitors. The effect of incubation time against enzyme activity was carried out by reacting enzyme supernatant in a period of time (i.e., one to five days). The effect of pH against enzyme activity was carried out by reacting enzyme solution with the concentration of CMC 1%, then incubated in acetic buffer 0.05 N at pH 5, and phosphate buffer 0.05 N at various pH. The tested pH was 5.0, 6.0, 7.0, 8.0, and 9.0. The effect of temperature against enzyme activity was carried out by reacting enzyme solution with the concentration of CMC 1% in a buffer solution with the obtained optimum pH from the previous analysis at various temperatures (i.e., 35, 40, 45, 50, 60 and 70°C). The effect of CMC substrate concentration against enzyme activity was carried out by reacting enzyme solution with a concentration of 0.5%, 0.75%, 1.0%, 1.25%, 1.5%, and 1.75% at the obtained optimum pH dan optimum temperature. The effect of the metal ion with final concentration 1 mM was carried out by reacting enzyme solution with the addition of various metal at the obtained optimum conditions pH, temperature, and CMC concentration from the previous analysis. The tested ions were Na+, Co2+, Cu2+, Mg2+, and Zn2+, which were available in the form of NaCl, CoCl2, CuCl2, MgCl2, and ZnCl2, respectively. The samples were also compared with the reacting enzyme without metal addition as a control.
3. Results and discussion

The cellulolytic enzymes system in many potential fungi was characterized following their ability in a cellulose-degrading potential [29]. The successful strategy to improve the cellulolytic enzymes produce consists of selecting the potential microbes, understanding the basic physiology of cellulolytic in microbes, and optimizing the fermentation process conditions [1].

Three fungal strains of *Penicillium* (i.e., InaCC F15, InaCC F16, and InaCC F17) were inoculated in selective media containing 1% CMC to screen their ability in producing cellulases (Figure 1). The results showed that only one strain of *Penicillium* (InaCC F16) was positive as a cellulase producer. Meanwhile, two strains (InaCC F15 and InaCC F17) were negatives. Fungal strain *Penicillium* InaCC F16 was then selected for further quantitative screening (Figure 2).

![Figure 1. Semi qualitative assay result of Penicillium strains InaCC F15 (A), InaCC F16 (B), and InaCC F17 (C) in selective media of 1% CMC.](image1)

![Figure 2. Cellulase production halo or clearing zone was formed around the colony of Penicillium InaCC F16.](image2)

Clearing zones were formed on the surrounding of fungal colonies after two days of incubation, and it indicated the ability for cellulase production. The cellulase activity of fungal strain was measured by measuring the diameter of a clear zone around the colony and hydrolytic value on cellulose Congo red agar media [13]. According to [30], the Congo red method was combined with the dinitro salicylic acid reagent method that could clearly be used as a rapid, sensitive, and reproducible way for screening cellulase-producing in fungi. However, plate screening methods using dyes are not quantitative methods due to the poor correlation between enzyme activity and halo size [31].

| Strain number | Fungal taxa          | Incubation period (h) | Colony diameter (mm) | Clear zone diameter (mm) | Cellulolytic index |
|---------------|----------------------|-----------------------|----------------------|--------------------------|-------------------|
| InaCC F15     | *Penicillium paxilli*| 48                    | -                    | -                        | -                 |
| InaCC F16     | *Penicillium bilaiæ* | 48                    | 1.3                  | 0.4                      | 3.3               |
| InaCC F17     | *Penicillium herquei*| 48                    | -                    | -                        | -                 |

Temperature, aeration, carbon sources, incubation period, medium additives pH of the medium, and presence of inducers are essential parameters for the optimized production of cellulase enzymes [32, 33]. According to [34], the addition of yeast extract for a suitable nitrogen source becomes crucial in cellulase production derived by *Penicillium janthinellum* during SSF using wheat bran and pure cellulose as the substrate. The ratio of clear zone diameter and colony diameter is expressed as a cellulase index (semi qualitative) of the screened *Penicillium* strain (Table 1).

Characteristics of the microbial cellulolytic enzyme could be carried out by measuring the optimum condition in several different parameters such as pH, temperature, substrate concentration, and the existence of metal ion cofactor as activator or inhibitor [35]. The effect of incubation against cellulase enzyme activity was determined by examining the cellulase activity for five days incubation period.
Enzyme activity against the incubation time of *Penicillium* InaCC F16 ranged between 2.2- 3.5 U/mL (Figure 3). The optimum time of incubation was obtained on the second day (3.5 U/mL), and it was slightly different from the fifth-day incubation (3.3 U/mL). On the first day, the cellulase activity was at 2.7 U/mL. On the third day, the lowest activity was at 2.2 U/mL, and on the fourth and fifth day, the cellulase activity was 2.7 U/mL.

![Figure 3](image1)

**Figure 3.** The effect of incubation time against the cellulase activity of *P. bilaiae* InaCC F16.

In general, microbial growth is effective at a temperature of 28 ± 2 °C; pH 6.0 ± 0.4; in aerobic condition; and on 2-5 days incubation [13]. According to [36], fungi *Penicillium echinulatum* has an optimum incubation time for cellulase production on seven days incubation. Meanwhile, [37] reported that fungal strain *Penicillium* sp. AKB-24 produced maximum β-glucosidase (4.80 IU/gds) on the ninth day of incubation and then decreased.

The cellulase activity against pH was carried out on crude cellulase enzyme from the supernatant, which was incubated for two days as an optimum incubation time (Figure 4).

![Figure 4](image2)

**Figure 4.** The effect of pH against the cellulase activity of *P. bilaiae* InaCC F16.

The supernatant containing crude enzyme had a varied activity at different pH. The optimum activity of cellulase was reached at pH 5.0 (3.5 U/mL), which was slightly different from pH 8.0 (3.2 U/mL). The lowest was reached at pH 6.0 (2.5 U/mL). Meanwhile, the enzyme activities at pH 7.0 and pH 9.0 were 3 U/mL and 2.9 U/mL, respectively. According to [38] and [39], the cellulase activity was influenced by pH variation and depended on the fungal strain.

The optimum condition of pH and temperature will support the enzyme to catalyze the reaction properly. Whereas the poor temperature and pH will damage the protein structure, causing...
denaturation, and thus it will decrease the function and activity of the enzyme. The cellulase activity against the temperature was carried out at crude cellulase enzyme from the supernatant, which was incubated for two days and at optimum pH 5.0.

**Figure 5.** The effect of temperature against the cellulase activity of *P. bilaiiae* InaCC F16.

The crude cellulase enzyme activity started increasing at 35ºC (2.2 U/mL) and reached the optimum activity at 40ºC (5.9 U/mL). The activity then started decreasing after temperature increased to 40ºC. As shown in Figure 5, enzyme activities at 45ºC and 50ºC were 3.7 U/mL and 2.2 U/mL, respectively. The lowest activity was obtained at 60ºC (1.6 U/mL), and it was not detected at 70ºC (Figure 5).

**Figure 6.** The effect of concentration of CMC against the cellulase activity of *P. bilaiiae* InaCC F16.

The optimum temperature for cellulase activity varies depending upon the organisms [29]. The crude extract obtained from PDC fermentation was then partially characterized. Optimal temperatures for cellulase action ranged from 52 to 58ºC, and pH values of around 4.9 contributed to maximum enzyme activity. At 37ºC, the cellulases were highly stable, losing less than 15% of their initial activity after 23 hours of incubation [40]. Fungi *Penicillium oxalicum* was used in fermenting banana agro-waste as a substrate, which was able to produce the highest crude cellulase at pH 6.0 and a temperature of 28ºC [41]. According to [42], *Penicillium funiculosum* had an optimum temperature of 60ºC and was stable in the acidic pH ranging between 2.5-6.0. Meanwhile, cellulase from *Penicillium echinulatum* showed maximum activity between pH 4 and 5, and the thermal stability at 55ºC [43].

The cellulase activity against the addition of the CMC substrate increased along with the increase of CMC concentration (Figure 6). The high rate of cellulase activity occurred in the addition of 1.25%
and 1.5% CMC with the rate of cellulase activity was 2.5 and 2.8 U/mL, respectively. Figure 6 also shows that cellulase activity continuously increases with increasing CMC at 1.75 and 2%. However, the rate of cellulase activity tended to decline gradually after the addition of CMC concentrations above 1.5%. Furthermore, the addition of CMC above 2% was not conducted due to its inefficiency.

Substrate concentration is one of the essential factors that can affect enzyme activity [44]. This reaction takes place because the abundance of CMC and its high viscosity can decrease the probability of substrate binding with the active side of the enzyme [38].

**Figure 6.** The effect of substrate concentration against cellulase activity of *P. bilaiae* InaCC F16.

As shown in Figure 7, among the various metal ions, the enzyme activated by Co$^{2+}$, Cu$^{2+}$ and Mg$^{2+}$ at a concentration of 1mM as compare to the control. According to [35], several metal ions could have a positive or negative effect against cellulase enzyme activity. The positive effect happens when enzymes can interact with metal ion as a cofactor (activator) so that the enzyme activity can increase. At the same time, the negative effect can decrease the enzyme activity since the metal ion acts as an inhibitor. Following the research of [45], many monovalent, divalent, and trivalent metal ions such as Na$^+$, K$^+$, Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, Fe$^{2+}$, Co$^{2+}$, Cu$^{2+}$, Ni$^{2+}$, Zn$^{2+}$, Hg$^{2+}$ and Fe$^{3+}$ were generally used to be examined as inhibitor or activator in cellulase characterization.

An assemblage of fungal strain from genera *Penicillium* was reported having potential cellulolytic abilities, such as *Penicillium italicum*; *P. brasiliianum*; *P. occitanis*; *P. decumbans*; *P. echinulatum*, mutant *P. janthinellum* (NCIM 1171; EMS-UV-8), *P. mallochii* LMB-HP37, *P. chrysogenum*, and *P. marneffei*. Furthermore, all those strains were considered as potential candidates for cellulolytic enzymes producer in the industry [8,13,46,47,48]. The previous study of cellulase producing activity of *P. griseofulvum* InaCC F14 showed that the average of cellulotic index at 2.67 and the optimum activity of cellulase were obtained on two days incubation at 50º C, pH 6, 1.25% substrate (CMC) concentration [49]. Cellulolytic enzymes are also used in detergents to aid in the removal of fiber encrusted dirt and soil and to increase the colour brightness of the washed garments and recovery of cellulosic textile waste [50]. Cellulase from *P. echinulatum* on denim fabric can display more colors than denim fabric and produce less indigo dye (back-staining) than commercial acid or neutral cellulase under test conditions [51]. According to [52] cellulolytic enzyme, in combination with other enzymes, has significant contribution and recognition in the food industry.

**4. Conclusions**

Cellulase complex enzymes derived from microbes provides an opportunity for achieving tremendous benefits in biomass utilization. A previous study showed that an assemblage of fungal strain from genera *Penicillium* had potential ability in producing cellulose. Three strains of *Penicillium* from InaCC were used in this study, and one strain (i.e., *Penicillium bilaiae* InaCC F16) showed a positive indicator as a cellulase producer. The semi-qualitative analysis showed that the clearing zone was
formed around the colony of *P. bilaiae* InaCC F16 with a cellulolytic index was at 3.3. The optimum cellulase activity was obtained on two days incubation period, the temperature of 40º C, pH 5, and substrate concentration at 2%. The addition of metal ions at a concentration of 1mM showed that enzymes were activated by cations CoCl$_2$, CuCl$_2$, MgCl$_2$, and inhibited by NaCl and ZnCl$_2$.

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