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Characterization of carboxymethyl cellulase produced by *Trichoderma asperellum*

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Abstract

Background: The present study characterized extracellular carboxymethyl cellulase (CMCase), an enzyme catalyzes the hydrolysis of the cellulose, from *Trichoderma asperellum* PQ34 that was isolated from agricultural cultivation soil in Thua Thien Hue, Vietnam.

Methods: CMCase was produced by culturing *T. asperellum* PQ34 on Czapek-Dox medium supplemented with carboxymethyl cellulose (CMC) at 28ºC for 96 h at a shaking speed of 150 rpm. Enzyme activity was spectrophotometerically determined with CMC used as a substrate and the absorbance was measured at 540 nm. The molecular weight of CMCase was determined by zymogram based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 0.2% CMC.

Results: CMCase achieved the highest activity after 4 days of culture with the optimal pH and temperature at 4 and 55°C. The enzyme maintains pH and thermal stability in the range of 4-5 and 10-40°C and the relative activity of both is more than 90%. The presence of metal ions at 5 mM such as Ca^{2+}, Al^{3+}, or Co^{2+} increased the enzyme activity up to 133%, 124%, and 217%, respectively. CMCase was stable with 1 mM ethylenediaminetetraacetic acid and 1 M urea, but inhibited by 5% sodium dodecyl sulfate (SDS) and 5% Triton-X100. Zymographic analysis showed that CMCase from *T. asperellum* PQ34 consists of two enzymes with a molecular weight of about 31 and 66 kDa.

Conclusion: CMCases from *T. asperellum* PQ34 exhibited characteristics of an acidic and thermostable enzyme. The interaction of Co^{2+} and CMCase resulted in a significant increase in enzyme activity, however they were strongly inhibited by 5% SDS.

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Introduction

Cellulose is an important structural component of the primary cell wall of green plants, many forms of algae and the oomycetes fungi [1]. Cellulose is made up of glucose monomers that are linked by β-1-4 glycosidic bonds. Carboxymethyl cellulase (CMCase), also known as endo-β-1,4-glucanase or endo-β-glucanase (EC. 3.2.1.4), is one of three enzymes of the cellulose hydrolytic enzyme system (cellulase) including exo-β-glucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) [2, 3]. Cellulases break down the β-1,4-glycosidic bonds of the cellulose molecule into monosaccharides such as β-glucose, or shorter polysaccharides and oligosaccharides [4]. Different source of cellulases usually affect to their substrate specificity, optimal pH and temperature [5]. Many fungal strains can secrete cellulase in larger amounts than bacterial strains and *Trichoderma* is known as the fungal genus with the most ability to secrete cellulase. Most commercial cellulases are produced by two genera *Trichoderma* and *Aspergillus*. This reflects the fact that mold is a powerful natural source of enzyme production and can be studied for applications on industrial scale [5, 6]. To date, there have been many reports on isolation and characterization of CMCase from *Trichoderma* species such as *T. reesei* [7-9], *T. viride* [5, 10] or *T. harzianum* [11]. In last few years, the study of cellulase production from *T. asperellum* has also become area of interest [12-17]. The present study conducted characterization of CMCase produced by *T. asperellum* PQ34 strain. The results of this study promise potential applications for large-scale in the future.

Methods

Fungal strain

T. asperellum PQ34 strain (GenBank accession number: HM545081.1) was isolated from the surface soil layer (15-20 cm in depth) under different agricultural lands in Thua Thien Hue province, Vietnam [18, 19].

Production of extracellular CMCase

T. asperellum PQ34 strain was cultured on Czapek-Dox medium at 28ºC for 48 h to induce spore germination. Fungal spores were then harvested in sterile distilled water and diluted to a density of 10^7 spores/mL. 2 mL of spore solution were subcultured in 100 mL of Czapek-Dox medium and incubated at 28ºC for 72 h at a shaking speed of 180 rpm for mycelial production. After washing with $MgCl_2$, mycelium biomass was cultured in the same medium, but glucose was changed by 1% (*w/v*) carboxymethyl cellulose (CMC) to induce CMCase production, at 28ºC for 96 h with a shaking speed of 150 rpm. The culture was centrifuged at 12,000 ×g for 10 min at 4ºC to discard biomass and obtain culture filtrate (crude enzyme). Crude enzyme was partially purified by 70% saturation ammonium sulfate at 4°C for 2 h and then centrifuged at 24,000 ×g for 10 min at 4ºC. The pellet was resuspended and dialyzed in 10 mM citrate buffer at an appropriate pH for further use.

The diffusion method in agar plate was used to evaluate extracellular CMCase production of *T. asperellum* PQ34 strain. 100 µL of the partial purified filtrates were loaded into pre-punched holes of equal sizes on the assay plate containing 1.5% agar and 0.5% CMC. The plate was then incubated at 50ºC for 24 h and subsequently stained with 1% Lugol solution for detection of substrate hydrolysis [20].

CMCase assay

CMCase activity was assayed according to Iqbal *et al* [5] with CMC was used as substrate. Reaction mixture consist of 300 µL of 1% CMC (in 0.05 M sodium acetate buffer, pH 5) and 150 µL of partial purified enzyme was incubated at 50ºC for 30 min. Reaction was then stopped by adding 600 µL of 3,5-dinitrosalicylic acid and boiling for 5 min to develop the colour. The absorbance was measured at a wavelength of 540 nm.

One unit of enzyme activity was defined as the amount of enzyme required to form 1 µmol of glucose per one minute under the assay conditions [9]. Total protein concentration was determined by the method of Bradford [21] with bovine serum albumin (Sigma-Aldrich) as the standard. The samples were read at a wavelength of 595 nm against the blank. The specific activity of CMCase was obtained by dividing units of the enzyme (U/mL) with the total protein content (mg/mL) in the sample.

Characterization of CMCase

Effects of pH and temperature on CMCase action were tested within the range of 3-8 and 10-90°C. 20 mM citrate buffer was used for pH 3-5, and 20 mM phosphate buffer for pH 6-8. Thermal and pH stabilities were determined by pre-incubating the enzyme at pH 3-8 and 10-90ºC for 30 min without substrate. The enzyme activity was measured at the optimal pH and temperature.

CMCase were also incubated with 5 mM of metal ion (Al^{3+}, Cu^{2+}, Co^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Fe^{3+}, or Hg^{2+}) or reagents (5% SDS, 5% Triton X-100, 1 mM ethylenediaminetetraacetic acid (EDTA), or 1 M urea) at room temperature and optimal pH for 30 min. Enzyme relative activity was then determined at the optimal temperature.

Zymography

The molecular weight of CMCase was determined by sodium dodecyl sulfate (SDS)-12% polyacrylamide gel electrophoresis (PAGE) with 0.2% CMC used as substrate. Electrophoresis was conducted at 4ºC for 3 h, the gel was then incubated in 20 mM citrate buffer at 50°C overnight with 1% (*v/v*) Triton X-100 to remove SDS. Finally, the gel was stained with Lugol's solution.

Statistics

Each experiment was repeated 3 times. The data were expressed as the means ± standard errors, followed by one-way ANOVA analysis with Duncan's test at p=0.05.

Results

Cellulase activity

Extracellular cellulase activity of different *T. asperellum* strains including PQ34 was tested by agar plate with CMC substrate. The results indicated that the *D-d* value of *T. asperellum* PQ34 was the highest (about 1.4 cm)

compared to that of other strains (CH2, SH16, and TN42) which were also isolated from the same place. Where, *D* is the diameter of the clear zone and *d* is the diameter of the pre-punched hole for enzyme loading (data not shown). After 4 days of culture, cellulase activity from PQ34 strain had peaked about 0.83 U/mL (equivalent to 3.62 U/mg protein) (Fig. 1). This strain was, therefore, used for characterizing CMCase.

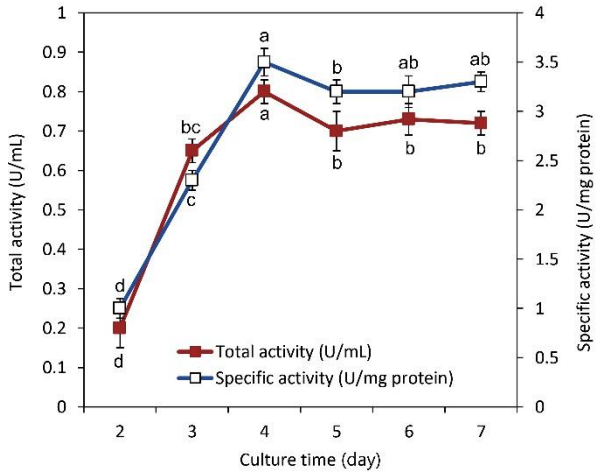

Figure 1: The profile of CMCase activity in *T. asperellum* PQ34. Different letters on a line represent significant differences at $p<0.05$ (Duncan's test).

Effect of temperature on CMCase activity

Data from Fig. 2 show that CMCase from *T. asperellum* PQ34 peaked at a relatively high temperature of 55°C with total activity of 1.7 U/mL (8.1 U/mg protein) and then drastically decreased at higher temperatures (60-90ºC). The activity of CMCase remained more than 90% when it was treated at temperatures below 40ºC. However, the enzyme activity decreased significantly at higher temperatures, especially it was only approximately 10% at temperatures from 70-90ºC (Fig. 2).

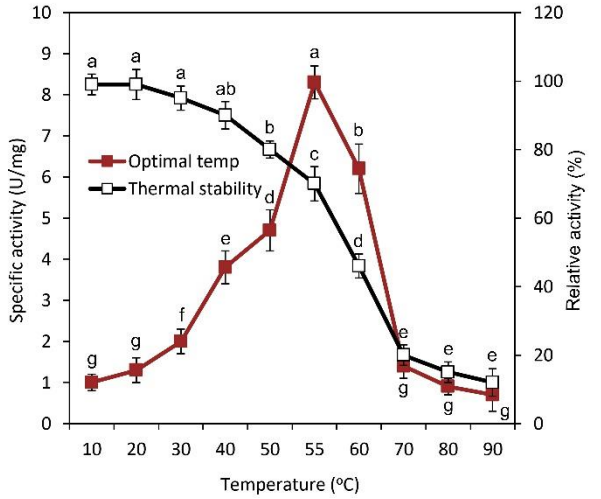

Figure 2: Effect of temperature on CMCase of *T. asperellum* PQ34. Different letters on a line represent significant differences at $p<0.05$ (Duncan's test).

Effect of pH on CMCase activity

The present work on *T. asperellum* PQ34 strain has shown that CMCase has an optimal pH of 4 with a specific activity of approximately 40 U/mg, enzyme activity was completely lost at pH greater than 7. The pH stability of enzyme ranged from 4-5 with the relative activity of over 90%. Generally, the CMCase from *T. asperellum* PQ34 only works within a very narrow pH range in the acidic environment (Fig. 3).

Similar to CMCase from PQ34, the pH stability of CMCase from *T. asperellum* UC1 is also at 4 [17].

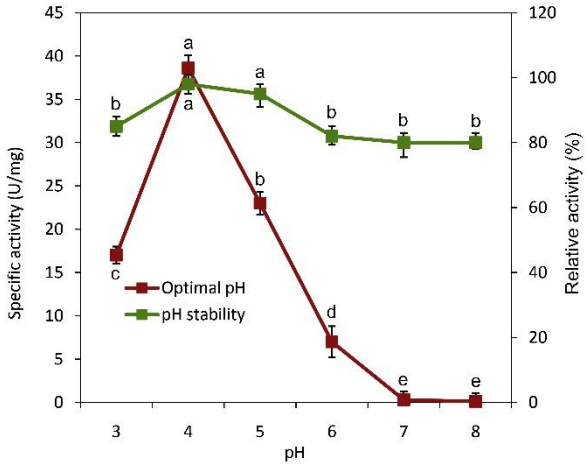

Figure 3: Effect of pH on CMCase of *T. asperellum* PQ34. Different letters on a line represent significant differences at $p<0.05$ (Duncan's test).

Effect of metal ions and reagents on CMCase activity

Investigation showed that some metal ions such as Zn^{2+} and Hg^{2+} slightly inhibited PQ34 CMCase, the relative activity was approximately 80%. While the other tested ions increased the enzyme activity such as Ca^{2+} (125%), Al^{3+} (124%) and Co^{2+} (128%). In the present work, all of the tested reagents inhibited enzyme activity, especially SDS (relative activity was about 25%) and Triton X-100 (relative activity was about 55%) (Fig. 4).

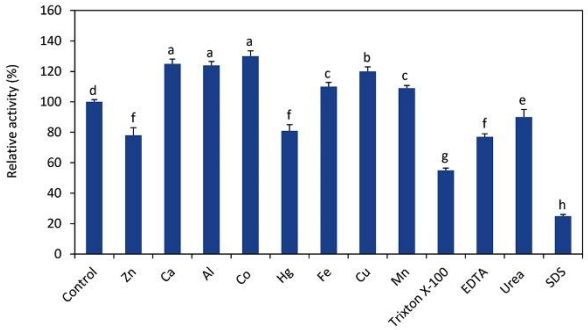

Figure 4: Effect of some metal ions and reagents on CMCase of *T. asperellum* PQ34. Different letters on columns represent significant differences at $p<0.05$ (Duncan's test).

Zymogram

Zymographic analysis of CMCase by SDS-PAGE with CMC used as the substrate has detected enzymatic activity on the gel. Two clear zones were found on the

gel which have the molecular weight of about 31 and 66 kDa (Fig. 5).

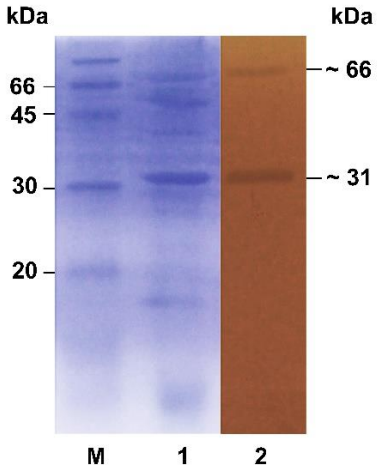

Figure 5: Zymographic analysis of CMCase from *T. asperellum* PQ34. M: protein marker (1610304, Bio-Rad), 1: partial purified CMCase on the polyacrylamide gel, 2: partial purified CMCase on the polyacrylamide gel with 0.1% CMC.

Up to now, there are no any reports related to the molecular weight of CMCases in *T. asperellum*. However, previous studies in *Trichoderma* species have shown that this enzyme has many types with different molecular weights. For example, cellulase III from *T. viride* has a molecular weight of 45 kDa [10], endoglucanase III from *T. reesei* QM 9414 is 48 kDa [7], CMCase from *T. viride* is 55 kDa [22], and cellulase from *T. viride* is 58 kDa [5].

Discussion

Baig [23] obtained CMCase from *T. lignorum* with total activity of 0.25 U/mL on medium supplemented with CMC as carbon sources after 8 days of culture. Recently, solid state fermentations were performed for the production of cellulase and endoglucanase from *T. asperellum*, the enzyme activity reached maximum values of 5.6 and 4.1 U/g with paper used as substrate, respectively [16].

Some other studies in *Trichoderma* genus also showed the enzyme activity reached maximum values at the 55ºC likes CMCase from PQ34 strain. For example, endoglucanse III from *T. reesei* QM 9414 [7], cellulase from *T. viride* [5], or CMCase from *T. reesei* NRRL 3652 [9]. However, several other cellulases had maximum activity at lower temperatures such as 50ºC for cellulase III from *T. viride* [10, 22], 45°C for cellulase from *Trichoderma* sp. [24], 40-50°C for cellulase from *T. reesei* [25], 30-40°C for CMCase from *T. harzianum* [26, 27], or 30ºC for CMCase from *T. reesei* and *T. harzianum* [8, 11]. According to Guoweia *et al* [28] the optimal temperature for production of CMCase from *T. reesei* HY07 was 30°C. Endoglucanase III from *T. reesei* QM 9414 can withstand the activity at 65ºC for 30 minutes [7]. CMCase from *T. asperellum* UC1 retained 50% activitiy at 60°C [17]. Zhekova *et al* [9] obtained CMCase from *T. reesei* NRRL 3652 with thermal stability at 50°C within 160 minutes.

Trichoderma asperellum RCK2011 strains including wild-type and mutant can induce cellulases in the range of pH 4-10 [13]. Some CMCases only work in the acidic environment such as endoglucanase III from *T. reesei* QM 9414 at pH 4-5 [7], CMCase from *T. reesei* NRRL 3652 at pH 5 [9], cellulase III from *T. viride* at pH 4.5-5 [10], CMCase from *T. viride* at pH 5 [22] , cellulase from a mutation of *T. reesei* 2414 at pH 4.5 [25], even at pH 3 as CMCase from *T. reesei* [8]. Iqbal *et al* [5] and Nawaz *et al* [11] also found cellulases from *T. viride* and *T. harzianum* had higher optimal pH of 6.5 and 6, respectively. Li *et al* [29] showed CMCase from *T. viride* reached a maximum activity after 60 h of solid-state fermentation at 50ºC and pH 5. Attitalla and Salleh [26] found the optimum initial pH was 5-7 for CMCase from *T. harzianum*. Whereas cellulase from *T. hazianum* and *Trichoderma* sp. have the optimal pH at 4-6 and 6.5, respectively [24, 27].

The use of metal ions to enhance the cellulase activity has also been studied [30]. For example, Co^{2+} and Mn^{2+} enhance the activity of cellulase from *T. viride* [5], Ca^{2+} and Co^{2+} enhance CMCase activity from *T. harzianum* nearly two-fold [11]. Okada [10] found that cellulase III from *T. viride* was inhibited by Hg^{2+} but not affected by EDTA. While Iqbal *et al* [5] reported Hg^{2+}, SDS and EDTA inhibited cellulase activity from *T. viride*. Nawaz *et al* [11] also showed that Hg^{2+} inhibited CMCase activity from *T. harzianum*.

CMCase of *T. asperellum* PQ34 strain has the optimum pH and temperature of 4 and 55°C, respectively. The pH and thermal stability of CMCase are in the range of 4-5 and 10-40°C with relative activities in both are more than 90%. The presence of metal ions at 5 mM concentration such as Ca^{2+}, Al^{3+}, or Co^{2+} increased the enzyme activity up to 133%, 124%, and 217%, respectively. Enzyme is stable with 1 mM EDTA and 1 M urea, but inactivated by 5% SDS and 5% Triton-X100. Zymographic analysis showed that CMCase from *T. asperellum* PQ34 strain consists of two enzymes which have the molecular weight of about 31 and 66 kDa.

Author Contributions

Loc NH: Designed study, processed experimental data and analyzed the results, wrote the manuscript.
Van NH and Quang HT: Conducted the research work.

Conflict of Interest Statement

The authors declare that there is no conflict of interest regarding the publication of this paper.

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