Proceedings of Science and Mathematics
Faculty of science, universiti teknologi malaysia
https://science.utm.my/procscimath/
Volume 27 (2024) 1-5

Molecular Weight Characterization of *Rhizomucor pusillus* Cellulase via Solid-State Fermentation

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Abstract
The growing demand for efficient lignocellulose-degrading enzymes has driven research into more cost-effective solutions for biofuel production. While the genus *Rhizomucor* has been studied primarily for pectinase, its cellulase potential remains underexplored. In this study, *Rhizomucor pusillus* AK2 was screened for cellulase activity using a carboxymethyl cellulose (CMC) agar plate assay, followed by solid-state fermentation (SSF) on oil palm mesocarp fibre (OPMF) with an inoculum size of 1×10^8 spores/g. The fermentation broth was quantitatively analyzed, and cellulase was characterized through SDS-PAGE and zymogram analysis, revealing molecular weights between 20 and 36 kDa. The cellulase activity index on the CMC plate was observed to be ≥ 1.5. This research highlights the potential of *Rhizomucor pusillus* as a promising source of cellulase enzymes for industrial applications.

Keywords: Cellulase; Fermentation; Molecular weight, *Rhizomucor pusillus*, CMC agar

Introduction

Lignocellulose biomass, such as agricultural and forestry residues, is the most bumper renewable carbon source with low cost worldwide. Production of biofuels from these residues has been widely accepted as a suitable strategy for reducing the dependence on non-renewable fossil fuel resources. It also mitigates the devastating effects of climate change as a result of the overconsumption of fossil fuels. The highest conversion of cellulosic biomass to biofuels commonly includes 3 primary paths: pretreatment to reduce the recalcitrance nature of the residue, enzymatic hydrolysis to release fermentable sugars, and the fermentation of the sugar to produce biofuels (Parisutham *et al*., 2014). Enzymatic hydrolysis is carried out by cellulolytic enzymes, exoglucanases, endoglucanases and β-glucanases, which break down cellulose into glucose monomers (Behera & Ray, 2016). However, the high cost of cellulase production remains one of the key barriers to the commercialization of biofuels from cellulosic biomass (Klein-Marcuschamer *et al.*, 2012). This reason remains the gearing factor for exploring different microorganisms for cellulase production. Many bacteria and fungi can produce cellulase; the most common ones exploited for industrial use are the filamentous fungi *Trichoderma*, *Penicillium* and *Aspergillus* (Behera & Ray, 2016). Other filamentous strains like *Rhizomucor* have not been recognized yet. Compared to the rest of the strains, Trichoderma reesei is ranked as the best cellulase producer; even with that, its use as a starting point requires performance modification (Behera & Ray, 2016; Zhang *et al.*, 2017). Performance modifications have been practicable using rational genetic modification and random mutagenesis. Nevertheless, the methods could have limitations in terms of stability and cost implications.

Carbon and nitrogen sources are also medium constituents that induce cellulase secretions from fungi. Carbon sources may include cellulose (pure), lactose, cellobiose, and sophorose, which are all

expensive for industrial use. Monosaccharides cellulase-inducers can easily be consumed by the strains amiss the fermentation process (Zhang *et al.*, 2017). Various authors have indicated that the fermentation strain, method, and cellulosic biomass can greatly induce enzyme production (Elisashvili *et al*., 2001; Hansen *et al*., 2015). Little information is known about the use of Oil palm mesocarp fibre (OPMF) as an inducer of cellulase. Our previous report (unpublished) has shown the quantitative cellulase secretions from Rhizomucor. This study further provided the SDS-page characterization of cellulase enzymes after zymogram analysis, on which there was no report to the best of our knowledge.

Materials and methods

Oil palm fibre preparation

The oil palm mesocarp fibre (OPMF) was obtained from the Mahamurnin Palm oil plantation in Sedenak, Johor Bahru, Malaysia. The fibre was washed with a detergent and dried for 48 hours, and then ground into particles by Toko Tenaga Keluarga Sdn. Bhd. The fibre was then sieved to 2000 µm and pretreated by soaking in NaOH (2 % (w/v)) at a ratio of 1:10 (w/v) for 4 hours and then autoclaved for 5 minutes at 121 °C. The fiber was rinsed several times with distilled water to neutrality (Iberahim *et al*., 2013).

Qualitative screening of *Rhizomucor pusillus for* cellulase production

Spores from 7 days late culture were harvested and diluted with tween 80 to an inoculum concentration of 1×10^7 spores/ml. The screening media were modified from Mendel media (Mandels & Weber, 1969). The CMC-agar plate served as the selective media for cellulase activity (Pointing, 1999). A 10 µL of the spore inoculum was transferred into a wire loop pre-cast hole at the center of the plate and incubated at 30 °C. The zones of hydrolysis were observed every 24 hours for two days by using standard staining methods, 1% (w/v) Congo red stain and 1M NaCl de-stainer on a CMC-agar plate. The diameter and extent of the clearing zones were measured and calculated.

Solid state culture of *Rhizomucor pusillus*

Spores from a 7-day culture on potato dextrose agar (PDA) were harvested with tween 80 and diluted to an inoculum size of 1×10^8 spores/g of OPMF. Prior to the inoculation, the OPMF was moistened with Mandels medium (Mandels and Weber, 1969) to generate a moisture of 80% using a moisture analyzer. The combination was autoclaved at 121°C for 15 minutes. The inoculated flasks were incubated at 30 °C inside an incubator and sampling was carried out every 24 hours for analytical assay. The fermented OPMF was mixed with 0.05 M Sodium acetate buffer (pH 5.0) and highly shaken by a vortex. The mixture was centrifuged at 4000 rpm, for 20 minutes at 4 °C. The supernatant was collected as the crude enzymes (Ang *et al*., 2015).

Analytical procedures

The Endoglucanase, exoglucanase and β-glucosidase activities were measured according to the standard method commended by IUPAC (Ghose, 1987) using the crude enzymes. Sodium Dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used using 12% (w/v) polyacrylamide gel. A 20 µL of crude enzyme was mixed with 5 µL of 5x SDS sample loading buffer and heated (100 °C) for 5 min. The electrophoresis was conducted on 200V for 50 min. The gel was plunged in 0.1% (w/v) coomassie blue solution for 30 min, then de-stained using 30 % (v/v) methanol. Zymogram analysis was conducted by incorporating 1% (w/v) CMC into polyacrylamide gel. The remaining gel with enzymes was washed 4x (8 min each) with 2% (v/v) isopropanol in 50 mM sodium acetate buffer at room temperature to wash SDS and re-nature the enzyme components. The enzymes were allowed to react with the CMC substrate incorporated into the gel by incubating in 50 mM sodium acetate buffer for 30 min at 50 °C. The gel was later stained using 0.1% (w/v) Congo red dye solution (30 min) and de-stained using 1 M NaCl for another 30 min. The active bands for the cellulase enzyme appeared as hydrolysis bands against the red-dark background (Ang *et al.*, 2013).

Results and discussion

The result obtained from the qualitative assay of *Rhizomucor pusillus* is shown in Figure 1. Figure 1a clearly shows the cellulase hydrolysis index of 1.5 mm after inoculating the *Rhizomucor pusillus* on a CMC agar plate for 48 hours and staining with congo red dye. The ratio of the halo region to the colony area represents the enzyme activities of a fungus. A higher ratio indicates higher activity and vice versa (Ang *et al*., 2011). The plate assay is helpful in screening large numbers of enzyme classes (Pointing, 1999). To Pointing (1999) the test gives a clue of the enzyme potential of strains. It is thus requisite that the result is supported by a quantitative experiment in SSF, as seen in Figure 2. Fourteen (14) days of solid-state culture of *Rhizomucor pusillus* cellulase enzyme production secreted maximum enzyme from day 7 to 10. CMCase 4.6 U/g (day 9), β-glucosidase 0.6 U/g (day 7) and FPase 3.4 U/g (day 8) were obtained.

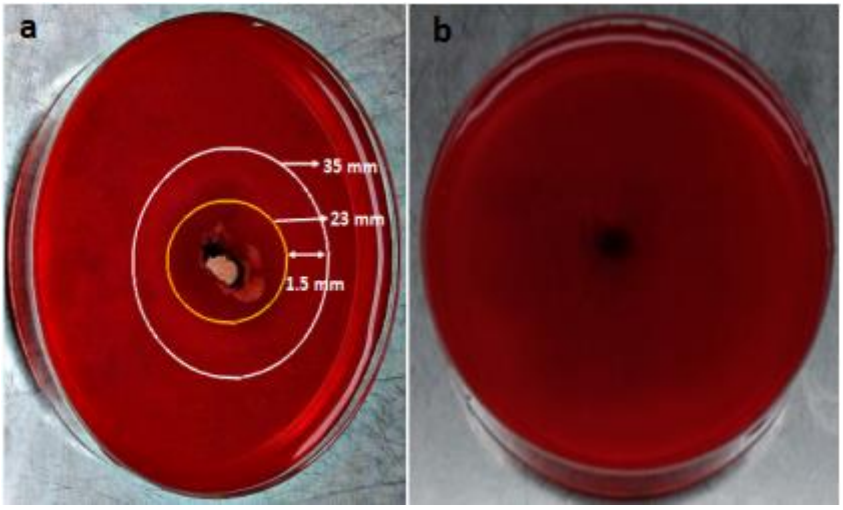

Figure 1 Qualitative assay of *Rhizomucor pusillus* cellulase potential on CMC–agar plates after Congo red staining. (a) experimental plate (b) control plate.

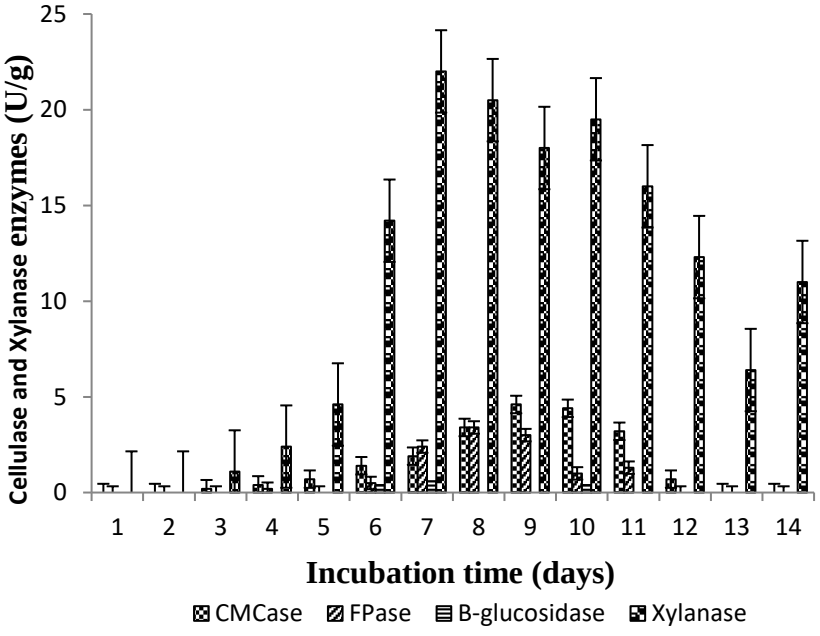

Figure 2 Cellulase and Xylanase enzymes production in 14 days solid state fermentation (SSF).

The molecular weight of the crude *Rhizomucor pusillus* cellulase is verified by SDS-Page and analyzed by zymogram assay, as shown in Figure 3. From the figure, Gel M represents the protein marker, while Gel 1 represents a negative control experiment of SSF (flask without inoculation). Lane 2 clearly shows the clear hydrolysis band of cellulase as it reacts to the incorporated CMC. Comparing gel 2 and gel M, it can be clearly seen that the hydrolysis zones fall around Mw 20 to 36. Comparing gel 2 and gel M, it can be clearly seen that the hydrolysis zones are seen around Mw 20 to 36. The revelation of Mw 20 to 36 in this study supported the postulation by Hoq and Deckwer (1995) who reported that *Rhizomucor pusillus* could have cellulase. It also supported Acosta Aranda et al. (2013), who also reported cellulase and protease activities from *Rhizomucor pusillus.*

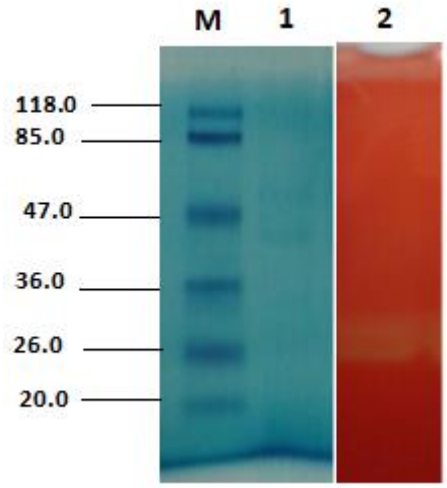

Figure 3 SDS-Page and Zymogram analysis of crude enzymes from *Rhizomucor pusillus* AK2. Lane M: 20 kDa to 118 kDa protein marker, Lane 1: negative control (SSF control experiment extract), Lane 2: crude cellulase of *Rhizomucor pusillus*.

Conclusion

This study ascertained *Rhizomucor pusillus* cellulase assertions. Further improvement in the fermentation process is required for improved cellulase production and for the future inclusion of *Rhizomucor pusillus* potent for industrial use.

Acknowledgement

Support from Tetfund Nigeria and Universiti Teknologi Malaysia is highly appreciated.

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