Lignocellulolytic degradation using secondary metabolites of *Trichoderma reesei* UMK04

Laila Naher¹,², Mahfuzah Mahmud¹, Nur Fatihah¹, Norhafizah Md Zain¹,², Huck Ywih Ch'ng¹ and Shafiquzzam Siddic³

¹Faculty of Agro-Based Industry, Universiti Malaysia Kelantan Jeli Campus, 17600 Jeli, Kelantan, Malaysia.
²Institute of Food Security and Sustainable Agriculture, Universiti Malaysia Kelantan Jeli Campus, 17600 Jeli, Kelantan, Malaysia.
³Biotechnology Research Institute, Universiti Malaysia Sabah, Jalan UMS, Kota Kinabalu 88400, Sabah, Malaysia.

Email: lailanaher@umk.edu.my

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ABSTRACT

Aims: Fungi species received much attention due to their numerical ability to manufacture various enzymes that can be used to break down cellulose, starch and lipids. However, the conventional way of mycelial incorporation in lignocellulolytic agriculture materials shows less quantity for the degradation of organic compounds. Therefore, fungal metabolites extract received much attention for large amounts of degradation. On the other hand, the effectiveness of fungal metabolite extracts depends on the solvent reaction process. Thus, this study compared degrading organic compounds such as lignin and cellulose in a plate assay experiment using *Trichoderma reesei* UMK04 secondary metabolites extract using ethyl acetate and hexene solvents.

Methodology and results: The *T. reesei* UMK04 was cultured in potato dextrose broth (PDB) media. Secondary metabolites from the broth culture of *T. reesei* were extracted using two different solvents of ethyl acetate and hexene separately. The degradation of organic compounds was determined in a plate assay experiment using Jensen media and Tannic acid media. Metabolite extract of *T. reesei* was used in concentrations of 5 mg/mL, 10 mg/mL, 15 mg/mL and 20 mg/mL. The hollow zone method was used to determine the degradation range. The ethyl acetate solvent extract showed the highest cellulose degradation-like pigmentation formation, which appeared at 6.6 cm on the plate, compared to lignin, which was 3.6 cm.

Conclusion, significance and impact of study: The secondary metabolites of *T. reesei* using ethyl acetate extracts showed higher cellulose degradation, which showed that solvents play the role of more effective compound extraction.

Keywords: Degradation, enzyme, industry, secondary metabolites, *Trichoderma reesei*

INTRODUCTION

Lignocellulose is widely available worldwide as a renewable organic material, and it is regarded as a valuable chemical compound and an essential alternative raw material to produce biofuels. The composition of lignocellulose is divided into three significant materials such as 30-50% cellulose, 25-30% hemicellulose and 15-20% lignin (Gupta and Verma, 2015; de Paula et al., 2019). Many industries have used value-added products from lignocellulose by converting its waste through a bioconversion process known as hydrolyses, such as fermentation, fiber material and the papermaking industry. The process begins with five and six simple carbon sugars produced by enzymatic hydrolysis right after the enzyme accesses the lignocellulose residue structure via cellulose. Then, it will be turned into a few products of biotechnological interest. For instance, biofuels, ethanol, methanol and animal feed are the most common sources produced by this method. Besides, in the past few decades, chemical, biological and physical pretreatment technologies have been established to maximize the lignocellulose waste’s benefit to gain high-yield biogas (Wang et al., 2020). However, the cost of enzyme synthesis remains a significant bottleneck. The generated enzymes have poor catalytic activity under industrial circumstances. The cost of commercial enzymes can account for 20-40% of the total operating expenses as enzyme generation for ethanol production on-site model (Liu et al., 2015). On the other hand, the ascomycete group of fungus, *T. reesei*, produces nearly all commercially available lignocellulolytic enzymes required

*Corresponding author

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for the hydrolysis stage, which can replace the cost of synthetic lignocellulolytic enzyme production (Bischof et al., 2016).

It is noted that fungi have a very effective enzymatic system compared to yeast and bacteria, allowing them to break down lignocellulose-containing source materials. The degradation system of fungi is performed by its two parts, which are intracellular and extracellular. The intracellular part includes the outer cell envelope layer, while the extracellular section has two kinds of enzymes: oxidative, which degrades lignin and hydrolytic and deteriorates polysaccharides (Andlar et al., 2018). Filamentous fungi are the most effective microorganisms for manufacturing lignocellulolytic enzymes (de Paula et al., 2019; Chen et al., 2020). This filamentous fungus can be found in various places, including lignocellulose wastes, live plants and ground. According to Kheil and Cheba (2014), T. reesei is able to manufacture hydrolytic enzymes and it has been widely applied in the biotechnology industry. The T. reesei is a primary workhorse in manufacturing cellulase, which hydrolyzes lignocellulosic biomass and turns it into bio-based goods. Many researchers have discovered T. reesei as an effective cellulase producer for degrading cellulose (Kantharaj et al., 2017). The present work evaluated cellulose and lignin degradation efficiency of local T. reesei UM04 strain using Tannic acid and Jensen media plate assay under halo zone conditions.

MATERIALS AND METHODS

Media preparation

Potato dextrose agar (PDA) media was used to culture T. reesei. For the preparation of PDA, 39.0 g of PDA powder was weighed and transferred into a 1 L media bottle that previously contained distilled water. Then, the media was mixed thoroughly to make a homogenate and then the water was topped to 1 L. Potato dextrose broth (PDB) was prepared by mixing 248 g of PDB powder with 2000 mL of distilled water in the media bottle. Then, the media was mixed thoroughly to make a homogeneity, and then the water was topped up to make volume 2 L. Then, pour 400 mL of the solution into a 500 mL conical flask and autoclave at 121 °C for 15 min. Next, both PDA and PDB media homogenate solution was autoclaved at 121 °C for 15 min. After the completion of the autoclave, the media was ready to use.

Culture of Trichoderma reesei in broth media

Trichoderma reesei was collected from the stock culture, which had been isolated from another study. The actively growing T. reesei’s mycelia were collected from the stock culture and then transferred to a plate containing potato dextrose agar media. The plate was left for five to six days to full grow the culture. Next, observe each subculture plate and select the plate media that grow and appear yellowish or greenish. In the aseptic condition, the grown pure culture was transferred into each 400 mL PDB using a 5 mm cork borer and the conical flask was covered with aluminum foil and kept at 25 ± 2 °C for 15 days. After 15 days of fermentation, the broth was entirely covered with the fungal mycelia on the top and was ready for metabolite extraction (Figure 1C).

Extraction of secondary metabolites using ethyl acetate and hexane solvent

For extraction, the fully covered broth culture mycelia were filtered using a muslin cloth and filter paper (Whatman filter paper No. 1). The extraction of secondary metabolites started with mixing 100 mL of ethyl acetate solvents and 400 mL of filtered fermentation broth culture into the separation funnel, followed by shaking it for 2 min. Then, the mixture was set in a separation funnel that was placed on the stand in reverse form, meaning the mouth was bottom and the bottom was up (Figure 1D). Once the solution mixture formed into two layers of solution, which were the organic phase and aqueous phase then, the separation funnel’s tap was opened to collect the waste (aqueous phase) in the bottom layer and the extract (organic phase) in the upper layer into the 250 mL conical flask. For Hexane extraction, the same procedure was followed.

Drying and collecting the secondary metabolites

The excess solvent in the organic phase was evaporated using a rotary evaporator machine (Figure 1E). The concentrated amount of extract was collected and poured into the Petri dish. Next, the extract was air-dried in the fume hood for 12 days. Meanwhile, the metabolites are observed at each three-day interval to collect the wet metabolites at one place, which is at the center of the Petri dish, in order to make it easier to harvest the dried metabolites on day 12. The collected dried metabolites were weighed at 3 g and kept in the chiller for further study.

Degradation activities using the extracts

Lignin activities test

Lignin degradation was determined using the extract of secondary metabolites of T. reesei based on the existence of the halo zone in the tannic acid medium. The ingredients were 50 g of malt extract and 5 g of tannic acid in 1000 mL of distilled water to prepare a Tannic Acid Media (TAM). The ingredients are mixed well into the media bottle and sterilized by autoclaving at 115 °C for 10 min. Next, the dried secondary metabolites were weighed at 5 mg/mL (T1), 10 mg/mL (T2), 15 mg/mL (T3) and 20 mg/mL (T4) in the falcon tube and 1 mL of sterile distilled water (T0) was added to dissolve the extract using a vortex mixer for about 10 sec to allow the metabolites to mix in the water shown in Table 1. The fluid of the extracts was poured into the glass Petri dish according to the concentration. The lignin degradation test was carried out using the paper diffusion incubation method. First, the
Figure 1: *Trichoderma reesei* culture and extraction of metabolites. Figure 1A shows 2 days of culture and 1B shows 7 days of a culture that appeared with green conidia with full growth. Figure 1C shows the broth culture of *T. reesei*. Figure 1D-1F shows the process of extraction of secondary metabolites using solvents, either hexane or ethyl acetate (1D), evaporation of the excess solvent (1E) and drying of the extraction in the flame hood (1F).

Table 1: The concentration of each treatment used for the lignin and cellulose enzymatic activities.

<table>
<thead>
<tr>
<th>Treatment (T)</th>
<th>Concentration of extract (mg/ mL)</th>
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<tbody>
<tr>
<td>T0 (Control)</td>
<td>0</td>
</tr>
<tr>
<td>T1</td>
<td>5</td>
</tr>
<tr>
<td>T2</td>
<td>10</td>
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<td>T3</td>
<td>15</td>
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<td>T4</td>
<td>20</td>
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A sterilized filter paper (Whatman filter paper No. 1) was cut like a disk paper. Next, by using forceps, the paper discs were dipped in the different concentrations of the extracts as 5 mg/mL, 10 mg/mL, 15 mg/mL and 20 mg/mL separately for 1 min to allow to absorb the desirable amount of metabolites. In contrast, water (0 mg/1 mL) was used in the plate as a negative control for tannic acid media. Then, the filter paper was mounted at the center of a TAM plate. The experiment was conducted in three replicates. The media plate was sealed with parafilm, covered with aluminum foil to make dark condition and incubated at 26-30°C for five days.

**Cellulose degradation test**

To determine the cellulolytic activity from the extract of secondary metabolites of *T. reesei* UMK04 based on the existence of the halo zone on the Jensen Media (JM). The ingredients of Jensen Media are 20 g agar, 20 g sucrose, 1.0 g dipotassium hydrogen phosphate, 2.0 g calcium carbonate, 2.0 CMC (carboxymethyl cellulose), 0.1 g iron (III) sulphate, 0.5 g sodium chloride, 0.5 g magnesium sulphate, 0.005 g sodium molybdate in 1000 mL distilled water. The mixture solution was shaken until all the ingredients were dissolved and then autoclaved for 15 min at 121 °C. Next, once sterilization was completed, the media was taken out from the autoclave and let it settle down to room temperature to pour into the Petri dishes. Meanwhile, different concentration of the extracts, such as 5 mg/mL (T1), 10 mg/mL (T2), 15 mg/mL (T3) and 20 mg/mL (T4) was prepared separately in Petri dish as shown in Table 1. Next, the sterile Whiteman filter paper disc was soaked for 1 min in each of the prepared concentrations. The forceps were pressed against the side of the plate to drain any excess fluid. Then, the filter paper disc was placed at the center of the Petri dish that contained Jensen media. Then, the plates were sealed using parafilm and incubated at 26-30°C for seven days. The water (T0) 0 mg/1 mL was used in the plate as a negative control (Table 1). The experiment was conducted in three replicates. After incubation for seven days, under sterile conditions, the plates were flooded with Congo red solution (dissolve 0.5 g of Congo red dye in 50 mL of distilled water and add 50 mL of ethanol) for 15 min and then effused. Next, 1 M of sodium chloride (NaCl) solution overflowed on the plate for 15 min and effused. The inoculated area appeared yellowish, evidenced by the diameter of a clear zone showing cellulose activity around the filter paper disc was recorded.
Figure 2: A) Lignin activities producing the clear halo zone using T. reesei extract. B) A graph showing the significance test by ANOVA. The Duncan’s homogeneity test was used to define the ranked class using alphabet letters. Error bars indicate standard errors.

**Statistical analysis**

The area of halo zone data was recorded and analysed using one-way ANOVA to establish the significant difference between the means where $p<0.05$. The calculations were done using statistical software, SPSS Statistics version 26.

**RESULTS AND DISCUSSION**

**Culture of Trichoderma reesei UMK04**

The culture of T. reesei UMK04 was performed from the stock culture. All the cultures showed good growth with morphological features such as the colour of the spore starting from whitish to yellowish and then changing to the dense dark yellow-greenish coloured colony (Figure 1A to 1B). The fungus’s morphological and anatomical confirmation was reported in our previous study (Naeher et al., 2021).

**Activities of lignin and cellulose degradation**

Lignocellulolytic degradation activities were conducted using tannic acid as the name tannic acid media and carboxymethyl cellulose as the name Jansen media (Sila et al., 2010). The result of lignin degradation showed the diameter of the dark brown halo zone on each
Lignin is a complex macromolecule in plant tissue and is used to protect the plant. This work was presented to produce ligninolytic degradation activities for the enzyme industry using secondary metabolites from the filamentous fungus of *T. reesei* using a tannic acid media plate. It showed that the fungal extract showed decent degradation production in the plate. The extracts of *T. reesei* showed that about 3.3 cm area was degraded by appearing in a dark, clear halo zone in the T4 (20 mg/mL) tannic acid media plate. It is also noted that as the concentration of extract was increased, the halo zone area was apparently increased in the plate (Figure 2A). A research study by Asis et al. (2020) found the dark halo zone was 0.5 cm under the tannic acid media using the mycelial disk. This work used secondary metabolite concentration of 5 mg/mL (T1), 10 mg/mL (T2), 15 mg/mL (T3) and 20 mg/mL (T4) metabolites of *T. reesei* in Figure 2A except 0 mg, which was water application. No halo zone in the water application (T0) plate means the extraction solution was free from contamination. The result of lignin degradation among the other treatments has found a significant difference between T2 (10 mg/mL), T3 (15 mg/mL) and T4 (20 mg/mL) (*p*<0.05). T1 did not show any activities in which no dark brown halo zone appeared, although 5 mg/mL concentration was applied, while in T2, T3 and T4, the diameter of the dark halo zone was measured as 1.33 cm, 2.5 cm and 3.3 cm, respectively. However, there are no significant differences between T3 and T4, while T2 significantly differed from T3 and T4.
extracts from fungal mycelia, which showed a higher amount of degradation than previous findings. The literature reported that there are few processes involved in degradation, such as oxidation, polymerization, complete mineralization and producing and accumulating metabolic intermediates, are involved during the lignin degradation process (Hernández-Péres et al., 1998; Sila et al., 2010). Therefore, it can be noted that the metabolite of fungal extracts worked as an elicitor to be faster for the degradation reaction mechanism than mycelial incorporation. Besides that, the effectiveness of fungal species is also important for the active degradation of organic compounds. As reported in the study of Sila et al. (2010) that Verticillum and Aspergillus sp. showed the best degradation of lignin compared to Trichoderma sp. However, the study of Asis et al. (2020) found T. reesei was best for lignin degradation, which is in accordance with this present work.

Activity of cellulose degradation was screened using Jensen media (JM). The lignocellulolytic activities of the extract that contained the metabolites were measured based on the diameters of the halo zones produced when degrading cellulose after staining with Congo red, incubated for 15 min, then excess strain drained off and 1 M NaCl solution was added for detaining. Optimization of the time course is of prime importance for cellulose biosynthesis by fungi (Sila et al., 2010). This diagnostic assay can identify that Trichoderma has polyphenol oxidase and endoglucanases when Jensen media is used for cellulose. It was found that there was a significant difference in the diameter of the halo zone of each treatment. Figure 3A shows the extract of T. reesei, which created degrading cellulose activities and produced a clear halo zone. All the treatments showed the degradation process as halo zone pigmentation except in T0 (applying water only), which found no pigmentation, which means extraction was not contaminated with any other particles. Figure 3B shows the significant differences in the activities among the implemented concentrations. T. reesei produces a minor diameter of the halo zone, which is 1.3 cm in T1 (5 mg/mL). However, T2 (10 mg/mL) showed 5 cm, T3 (15 mg/mL) showed 6 cm and T4 (20 mg/mL) showed 6.5 cm of forming larger clearing halo zones. A significant difference was observed between T1 and T2, while no significant difference was observed between T3 and T4.

The degradation of cellulose under Jensen media showed a larger area with a clear halo zone of 6.5 cm on the plate. As with lignin degradation, cellulose degradation also showed that as the concentration of metabolites increased, the halo zone also appeared larger. However, both enzymatic degradation in this study using fungal metabolite extracts showed larger halo zones compared to the studies of Asis et al. (2020), where they used mycelial discs for the degradation of cellulose.

CONCLUSION

The research findings suggest that the highest concentration of 20 mg had higher cellulose and lignin degradation activity. It observed that cellulose enzyme activity was highest at a 6.5 cm halo zone in ethyl acetate extraction compared to lignin in hexane extraction. This study also showed that the secondary metabolites extracted from T. reesei are more efficient than mycelia discs (from the literature). Based on research findings, it can be concluded that T. reesei metabolites extracts obtained using ethyl acetate solvent showed the most potentiality in lignocellulolytic, especially cellulose degradation.

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