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Isolation and Characterization of a Cellulase Producer from the Gut Microbiota of Earthworm

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Abstract

Lignocellulosic biorefinery has been regarded as the promising alternative for fossil refineries towards environmentally friendly and sustainable production. However, the high cost of cellulase suppressed its development. The gut microbiota of earthworms can provide abundant microbial resources and efficient cellulase producers can be isolated from it. Hence, in this work, cellulase producers were isolated and characterized from the gut microbiota of *Metaphire vulgaris*. Twelve colonies with hydrolysis halo on the microcrystalline-agar plate were isolated, and their cellulase activity was evaluated in the submerged fermentation with microcrystalline cellulose as the carbon source. Among them, *P. oxalicum* exhibited the highest cellulase activity of 0.11 U/mL when microcrystalline cellulose was used as the carbon source. Pretreated corn stalk proved to be an effective inducer for cellulase production by *P. oxalicum*, resulting in an increased cellulase activity of 0.22 U/mL. The optimal conditions for cellulase production and hydrolysis by *P. oxalicum* were found to be a pH of 5. However, a significant reduction in hydrolysis performance (59%) was observed at 70°C in comparison with the one under optimal 50 °C. The strain isolated in this work could not only contribute to the lignocellulosic biorefinery but also provide insights into the gut microbiota of earthworms.

Keywords: Biorefinery, Cellulase, Earthworm, Gut microbiota, Penicillium oxalicum

1. Introduction

Lignocellulosic biomass is the most abundant renewable resource on the earth, and it mainly consists of cellulose, hemicellulose, and lignin (Singh et al., 2022). Lignocellulosic biomass, mainly agricultural and forest wastes, can be valorized to produce biofuel and biochemicals through biorefinery (Sun et al., 2023). The core of lignocellulose biorefinery is to produce cellulosic sugar from lignocellulosic biomass, while the recalcitrant nature of lignocellulosic biomass burdens this process as cellulose, hemicellulose, and lignin bond together tightly as a complex. Pretreatment should be applied to release the cellulose and hemicellulose from the complex, thereby facilitating the enzymatic hydrolysis (Huang et al., 2024). Cellulase is a mixed enzyme system that can degrade cellulose into glucose to support microbial fermentation for target products, and hence it is crucial in lignocellulosic biorefinery. However, the high price of cellulase makes the lignocellulosic biorefinery economically inefficient compared to conventional starch-based biorefinery and fossil refinery, despite the cheaper feedstock (Saini et al., 2022).

Cellulase mainly consists of endo-1,4-β-D-glucanase, which breaks the long chain of cellulose randomly, exo-1,4-β-D-glucanase producing disaccharides from the reducing end of cellulose and the hydrolysate of endo-1,4-β-D-glucanase, and finally β-glucosidase hydrolyzing disaccharides into glucose (Bischof et al., 2016). Producers of cellulase in nature include fungi, bacteria, and actinomycetes. Among these, *T. reesei* is popular in industrial cellulase production with a remarkable ability for extracellular protein secretion and a complete cellulase system. However, the cellulase production by *T. reesei* is impaired by its high-viscous fermentation broth and the requirement for an inducer. Vigorous agitation and aeration are required to address the issue of fluid behavior and oxygen demand of *T. reesei*.

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On the other hand, the inducer is expensive but mandatory to remove the cellulase production by *T. reesei* (Li et al., 2016). The high operation cost resulting from these two factors is the main reason for the high price of cellulase. Hence, the search for other cellulase producers may be the solution to reduce the cost of cellulase.

Microorganisms that decompose cellulose are widely present in soil and the intestines of herbivores. *Acremonium cellulolyticus* was isolated from Japanese soil and applied to cellulose decomposition (Fang et al., 2009). In screening forest soil samples from the subtropical regions of China, 22 strains with cellulase production activity were found, with three strains exhibiting high CMCase activity (Liang et al., 2014). *Bacillus subtilis* Bc1, isolated from the gut of the leopard moth, produces stable cellulase across a wide range of temperatures and pH levels (Dehghanikhah et al., 2020). Cellulase-producing bacteria with salt tolerance were found in the intestines of marine omnivorous bony fish and mangrove oysters (An et al., 2015). Additionally, a new *Chryseobacterium* strain producing bifunctional cellulase-xylanase was screened from cow dung, exhibiting high activity levels and thermostability among known cellulases and xylanases (Tan et al., 2018). The discovery of such tolerant cellulases is significant for meeting production needs.

Earthworm gut microbiota is an abundant resource for functional microorganisms (Ding et al., 2019; Yang et al., 2023). It has been reported that cellulase-producing and plastic-degrading microorganisms can be isolated from the earthworm gut microbiota (Carpena-Istan et al., 2024; Fujii et al., 2012). The purpose of this work was to expand the microbial source of cellulase producer, thereby contributing to cost-effective cellulase. Cellulase-producing microorganisms were screened and isolated from the gut microbiota of earthworms. Microcrystalline cellulose agar plates were used for high throughput screening. Twelve colonies with the ability to hydrolyze cellulose on the plate were isolated and identified. Among them, *Penicillium oxalicum*, with the highest cellulase production performance, was selected for further analysis. The cellulase production conditions, including carbon source, working volume, pH, and ammonium sulfate concentration, were optimized. This work could provide insight into lignocellulosic biorefinery and resource exploration in earthworm gut microbiota.

2. Materials and Methods

2.1 Medium

Utilizing a microcrystalline agar plate is a promising high throughput screen method for cellulase-producing microorganisms as cellulase producers can degrade the cellulose and present a halo in the plates (Peng et al., 2021). Specifically, 4 g microcrystalline cellulose was mixed with 100 g glass beads (3 mm diameter) and 200 mL water in a 500 mL flask. The mixture was shaken at 180 rpm and 28 ºC for grinding the microcrystalline, and hence the hydrolysis halo would be clearer during the screening. The microcrystalline agar plate contained 2.0 g/ L KH_2PO_4, 1.4 g/L $(NH_4)_2SO_4$, 0.75 g/L peptone, 0.3 g/L urea, 0.3 g/ L $CaCl_2$, 0.3 g/L $MgSO_4•7H_2O$, 0.25 g/L yeast extract, 20 g/L agar, 5 g/L ground microcrystalline cellulose, 0.00016 g/L $MnSO_4•H_2O$, 0.0005 g/L $FeSO_4•7H_2O$, 0.00014 g/L $ZnSO_4•7H_2O$, 0.0002 g/L $CoCl_2$, and 2.0 mL/L Triton X-100.

PDA plate contained 20 g/L glucose, 6 g/L potato steep powder, and 26 g/L agar. Seed medium contained 20 g/L glucose, 17.91 g/L $Na_2HPO_4•12H_2O$, 2 g/L KH_2PO_4, 1.4 g/L $(NH_4)_2SO_4$, 0.3 g/L urea, 1 mL/L Tween-80, 0.6 g/L $MgSO_4•7H_2O$, 0.6 g/L $CaCl_2$, 0.00016 g/L $MnSO_4•H_2O$, 0.0005 g/L $FeSO_4•7H_2O$, 0.00014 g/L $ZnSO_4•7H_2O$, 0.0002 g/L $CoCl_2$. Cellulase-producing medium contained 20 g/L microcrystalline cellulose, 17.91 g/L $Na_2HPO_4•12H_2O$, 2 g/L KH_2PO_4, 1.4 g/L $(NH_4)_2SO_4$, 0.3 g/L urea, 1 mL/L Tween-80, 0.6 g/L $MgSO_4•7H_2O$, 0.6 g/L $CaCl_2$, 0.00016 g/L $MnSO_4•H_2O$, 0.0005 g/L $FeSO_4•7H_2O$, 0.00014 g/L $ZnSO_4•7H_2O$, 0.0002 g/L $CoCl_2$.

2.2 Screening for Cellulase Producer

The gut extracted from the earthworm *M. vulgaris* was transferred into 1 mL distilled water and mixed by vortex. Then, the solution was spread on microcrystalline agar plates and incubated for 7 days at 30 ºC. The colony with a clear hydrolysis halo was regarded as a cellulase producer and selected for further analysis. The 16s and 26s rDNA of isolates was amplified and sequenced. The read sequence were aligned to the NCBI database by BLAST for the sequence identification and isolate annotation.

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2.3 Cellulase Production

The cellulase producer selected on the microcrystalline agar plate was then transferred into the PDA plate and incubated for 5 days at 30 ºC for enrichment. The spore was then washed out by distilled water and 100 µL spore solution was transferred into seed medium. After 48 hours of incubation at 30 ºC and 150 rpm, 5 mL broth from the seed medium was transferred into a cellulase-producing medium and incubated at 30 ºC and 150 rpm. After 5 days, the phenotype of the fermentation broth was recorded, and a 2 mL sample for testing cellulase activity. The crude enzyme was obtained by centrifuging fermentation broth at 10000 rpm for 5 minutes.

2.4 Filter Paper Activity Assay

Cellulase activity was defined as the amount of enzyme needed for releasing 1 µM sugar from filter paper in 1 minute at 50 ºC as shown in Eq. 1. For the experimental group, the Whatman No.1 filter paper was cut into small circles that have a diameter of 5 mm and add into the 120 µL mixture of citric buffer and diluted crude enzyme for maintaining reaction pH at 5.0. The reaction lasted for 30 minutes at 50 ℃. Finally, the reduced sugar content was quantified by the 3,5-dinitrosalicylic acid (DNS) method. The DNS method can be applied to measure the reducing sugar content in the solution by aligning the absorbance of the reacted solution with the one calibrated by 1 g/L glucose solution (Table 1). The hydrolysate was then mixed with 120 µL DNS and boiled for 10 minutes. The absorbance of the reactant was measured at OD_{540} and the cellulase activity was calculated as shown in Eq. 1. For the control group, conditions were in accordance with the experimental group except for the absence of the filter paper.

$$\text{Cellulase activity (U/mL)} = (OD_{experiment}-OD_{control})\times\text{Calibration Factor}\times\text{Dilution Rate}\div 180\ (\mu g/\mu mol)\div 30(\text{mins})\div 0.06(\text{mL}) \quad \text{Eq. 1}$$

Table 1. Standard glucose solutions of different concentrations for DNS calibration curve.

Tube	1	2	3	4	5	6	7	8
Glucose standard solution（µL）	0	10	30	40	50	60	70	80
Citric acid buffer（µL）	120	110	90	80	70	60	50	40
Glucose content (µg)	0	10	30	40	50	60	70	80

2.5 Cellulase Production Optimization

Induction is required for cellulase production by *Trichoderma* sp. and *Penicillium* sp., while the induction ability of inducers varies across different strains. Hence, the inducer should be chosen for cellulase high production. Glucose, lactose, catalyzed transglycosylation inducer (MGD), pretreated corn stalk, unpretreated corn stalk, and microcrystalline cellulose were used as inducers for cellulase production in this study. Glucose, lactose, and MGD were introduced into the cellulase-producing medium as the sole carbon source at 20 (g reducing sugar)/L. Pretreated corn stalk, unpretreated corn stalk, and microcrystalline cellulose were introduced at 20 g/L. MGD was prepared followed by Li *et al* (Li et al., 2016).The steam explosion pretreated corn stalk as was as potential inducer and carbon source (provided by SDIC Biotech Investment Co., Ltd.).

Nitrogen source concentration, aeration, and pH are key factors for cellulase production by filamentous fungi cultivation. Response surface was conducted to optimize the fermentation conditions, thereby supporting cellulase production. Analysis of variance (ANOVA) was performed by Design Expert V12 (Stat-Ease).

2.6 Cellulase Characterization

To characterize cellulase produced by *P. oxalicum* and evaluate its hydrolysis performance under various conditions, cellulase activity was quantified under three temperatures (30 ºC, 50 ºC, and 70 ºC) and two pH conditions (pH 5 and pH 7). Specifically, citrate buffer with pH at 5 and 7 was used while the temperature of the water broth was set at 30 ºC, 50 ºC, and 70 ºC during the cellulase activity quantification.

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3. Results and Discussion

3.1 Cellulase Producer Isolation and Identification

There are 12 strains with cellulase-producing ability screened as they could produce hydrolysis halo on the microcrystalline agar plates, and these strains were then isolated and identified, with P. oxalicum showing the highest cellulase activity (Table 2). These strains were from the *Talaromyces*, *Penicillium*, and *Aspergillus* genus. The isolated strains are all filamentous fungi and can use microcrystalline cellulose as the sole carbon source. The color of the fermentation medium for isolated strains cultivation during the solid state fermentation (plate) and submerged fermentation (flask) was listed in Table 2. It is noteworthy that Colony 1 (*T. flavus*) and Colony 12 (*T. angelicae*) could produce red pigment while producing cellulase (Table 2).

Table 2 Characteristics of fermentation broth and agar plates with the identification results of isolated strains.

Colony	Plate	Flask	Identification
1	Yellowish green	Pink	*Talaromyces flavus*
2	Yellow	White	*Talaromyces flavus*
3	White	White	*Talaromyces flavus*
4	Green	Yellow	*Penicillium sp.*
5	White	Yellow	*Aspergillus neoniveus*
6	Black	Yellow	*Penicillium oxalicum*
7	White	Yellow	*Penicillium citrinum*
8	White	Dark yellow	*Aspergillus flavipes*
9	White	Black	*Aspergillus flavipes*
10	Green	Dark yellow	*Talaromyces pinophilus*
11	Yellowish green	Light yellow	*Talaromyces flavus*
12	Yellowish green	Dark pink	*Talaromyces angelicae*

Cellulase production by *Talaromyces spp.*, *Penicillium spp.*, and *Aspergillus spp.* has been reported before (Infanzón-Rodríguez et al., 2022; Maeda et al., 2013; Sun et al., 2018). Colony 6 possessed the highest cellulase activity at 0.11 U/mL, which was then identified as *P. oxalicum* (Figure 1). Cellulase produced by *T. reesei*, a widely used industrial cellulase-producing strain, had low beta-glucosidase (BGL) activity, impairing their hydrolysis performance, especially in glucose production. In contrast, it has been reported that the cellulase system of *P. oxalicum* is more complete with abundant hemicellulases and pectinases in comparison to *T. reesei* (Liu et al., 2013). *P. oxalicum* has been regarded as the promising alternative for *T. reesei* in industrial cellulase production (Han et al., 2017), and hence optimizing the cellulase production conditions for the isolated *P. oxalicum* is worthwhile.

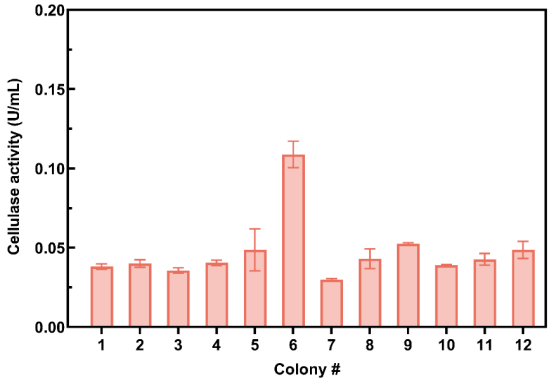

Figure 1 Cellulase activity produced by screened strains during the submerged fermentation in a flask with microcrystalline cellulose as the inducer and carbon source.

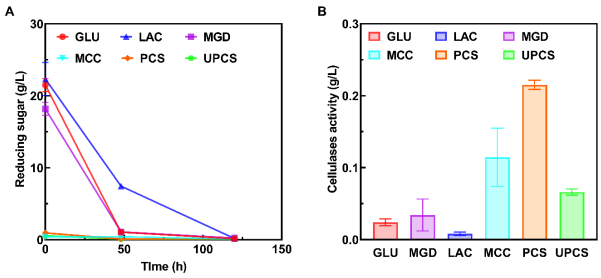

Figure 2. Cellulase production by *P. oxalicum* with different inducers. A: Reducing sugar profile; B: Cellulase activity at 120 h. GLU, glucose; LAC, lactose; MGD: enzyme-catalyzed transglycosylation inducer; MCC, microcrystalline cellulose; PCS, steam explosion pretreated corn stalk; UPCS, unpretreated corn stalk.

3.2. Cellulase-producing Conditions Optimization

Induction is important for cellulase production for *P. oxalicum*, while microcrystalline cellulose (MCC), MGD, lignocellulosic biomass, lactose are potential inducer and carbon source. MCC is widely used for both *P. oxalicum* and *T. reesei*. MGD has been reported as an efficient inducer involved in *T. reesei*. Corn stalks are the natural feedstock. Glucose, lactose, and MGD are soluble sugar-based (monosaccharide and disaccharide) inducers. In contrast, MCC, pretreated corn stalk and unpretreated corn stalk are insoluble cellulose-based inducers. The mechanisms for soluble sugar-based inducers and insoluble

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cellulose-based inducers were different. As *P. oxalicum* can not utilize insoluble cellulose-based inducers directly, base cellulase is required to hydrolyze them at first for sugar production to support strain growth and enzyme production.

During cellulase production, the uptake rate of glucose and MGD was faster than lactose (Figure 2A). Moreover, the reduced sugar accumulated during the cultivation was negligible, suggesting the sugar production rate by secreted cellulase is lower than the one uptake by *P. oxalicum* (Figure 2A). The cellulase activity induced by insoluble cellulose-based inducer (microcrystalline cellulose, pretreated corn stalk, and unpretreated corn stalk) was higher than the one induced by soluble sugar-based inducer (glucose, lactose and MGD). As shown in Figure 2B, cellulase production induced by pretreated corn stalk led to the highest cellulase activity at 0.22 U/mL followed by the one induced by microcrystalline cellulose (0.11 U/mL) and unpretreated corn stalk (0.07 U/mL). On the other hand, the cellulase activity conducted by soluble sugar-based inducers was all lower than 0.04 U/mL. Taking advantage of cheap and high cellulase induction performance, pretreated corn stalk is recommended for cellulase production by *P. oxalicum*.

Table 3. Response surface for optimizing cellulase production.

Run	pH	Working volume (mL)	Ammonium sulfate (g/L)	Cellulase activity (U/mL)
1	6	100	3	0.10
2	6	100	3	0.09
3	6	100	3	0.09
4	6	100	3	0.10
5	6	50	2	0.14
6	5	150	3	0.16
7	7	150	3	0.16
8	6	150	4	0.05
9	6	50	4	0.10
10	5	50	3	0.19
11	7	100	4	0.16
12	7	50	3	0.10
13	7	100	2	0.17
14	6	150	2	0.10
15	6	100	3	0.10
16	5	100	4	0.25
17	5	100	2	0.16

Table 4. Analysis of variance (ANOVA) of operation variable on cellulase production.

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	0.0323	9	0.0036	5.5	0.0175
A-pH	0.0036	1	0.0036	5.47	0.0519
B-Working volume	0.0006	1	0.0006	0.9133	0.3711
C-Ammonium sulfate	3.70E-08	1	3.70E-08	0.0001	0.9942
AB	0.0019	1	0.0019	2.87	0.134
AC	0.0027	1	0.0027	4.08	0.0832
BC	0	1	0	0.0285	0.8708
A^2	0.0214	1	0.0214	32.83	0.0007
B^2	0.0011	1	0.0011	1.75	0.2276
C^2	0.001	1	0.001	1.61	0.2457
Residual	0.0046	7	0.0007		
Lack of Fit	0.0045	3	0.0015	182.2	< 0.0001
Pure Error	0	4	8.30E-06		
Cor Total	0.0369	16			

The result of ANOVA suggested that the cellulase production was sensitive to pH ($p<0.05$ for pH^2 in Table 4). On the other hand, the effects of ammonium sulfate concentration were insignificant in this work ($p>0.05$). It may be caused by the fact that the lower bound of ammonium sulfate in this work (2 g/L) was sufficient for cellulase production with 20 g/L pretreated corn stalk. Changing the working volume during the flask cultivation can adjust the aeration performance with minimal effect on the shearing effect. Hence, the ANOVA results suggested that cellulase production by *P. oxalicum* was insensitive to aeration (Table 4).

3.3. Cellulase Characterization

As shown in Figure 3, cellulase produced by the isolated *P. oxalicum* possessed the highest hydrolysis performance at 0.22 U/mL under 50 ºC and pH 5. Under the given temperature, cellulase has a higher activity under pH 5. Moreover, under pH 5, hydrolysis at 50 ºC resulted in the highest cellulase activity followed by the one under 70 ºC (0.09 U/mL). Hence, it can be postulated that the produced cellulase favored mild conditions around 50 ºC, while it can also be used under a higher temperature, but not the ambient temperature. The cellulase activity truncated significantly (59%) during the hydrolysis under pH 7 in comparison to the one conducted under pH 5, suggesting that the cellulase produced by the isolated *P. oxalicum* can be characterized as acid cellulase.

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4. Conclusion

In this work, 12 cellulase-producing colonies were screened and identified from the gut microbiota of earthworms. Among them, *P. oxalicum* possessed the highest cellulase activity at 0.11 U/mL when using microcrystalline cellulose as the carbon source. Pretreated corn stalk was an efficient inducer for cellulase products by *P. oxalicum*, which can lead to 0.22 U/mL cellulase activity. The optimal pH for cellulase production by *P. oxalicum* and cellulase hydrolysis was 5. The optimal temperature for the produced cellulase was 50 ºC, while a 59% reduction of hydrolysis performance was found under 70 ºC. This work provides insights into lignocellulosic biorefinery and microbiota of earthworm gut.

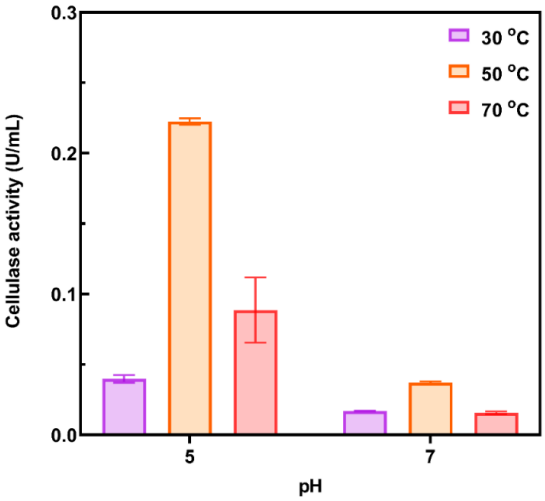

Figure 3. Cellulase activity under various hydrolysis conditions.

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