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Cellulose Degrading Ability of Bacterial Strains Isolated from Gut of Termites in Vinhlong Province - Vietnam

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Cellulose are the most abundant renewable biomass. The bioconversion of cellulose plays an important role for sustainable development and application in alcohol production, compost production and waste treatment. Termites were known as the most successful wood-degrading species on the earth, can degrade in large quantities of cellulosic biomass by activities of enzymes secreted by the termites and/or their gut. A total of 70 bacterial isolates were isolated from the gut of termites obtained from thirteen termite nests in TraOn district, VinhLong province, Mekong Delta, Vietnam. Of the 70 bacterial isolates, 60 (86%) are able to degrade CMC (Carboxyl Methyl Cellulose) after culturing in media containing CMC agar and halo formation after staining with Congo red. Among of them, 5 isolates having high CMC degradation efficiency are chosen to evaluate the potential ability to liberate glucose from cellulose of straw for 5, 10, 15 and 20 days respectively at room temperatures. The results showed that all of the 5 isolates have the cellulose degradation ability and produce the highest glucose contents after 15 days. Out of the 5 isolates, 2 produced glucose in large quantities are 2T1 isolate (0.73 g/L) and 5T6 isolate (0.79 g/L) in hydrolysis solutions. The 2T1, 5T6 strains were 98.99%, 99.01% of identity *Paenibacillus humicus* strain T20C, *Psychrobacter faecalis* strain AP3Ka respectively by 16S rRNA genes sequencing and could be potential sources of enzymes for cellulose hydrolysis from cellulosic biomass in future.

1. Introduction

Cellulose are the most abundant renewable biomass; formed by monomeric units of glucose (Santana Costa et al., 2020) is the primary structural material of the plant cell wall and the most abundant carbohydrate in nature. They are degraded by a biological process controlled and processed by the enzymes of the cellulase group including endoglucanase, exoglucanase or cellobiohydrolase, and ß-glucosidase.
The biomass residue of agriculture production contains large amounts of cellulose. The percentage of rice from the total biomass of rice plants is 65 - 72%. There are around 1149.6 million tons of rice straws in the world (Khoirunnisa et al, 2020). The rice industry generates the large quantities of solid waste such as straw, husk, ash, bran (Moraes et al., 2014). The degradation of biomass of rice straw is done by the collaboration of many microorganisms, including fungal and bacterial genera producing cellulolytic enzymes under aerobic or anaerobic conditions. Cellulose degrading bacteria was isolated from plant litter soil, the gut of termites (Shankar and Isaiarasu, 2011). Termite is a small tropical insect that eats wood. A typical colony of termites contains nymph alates, workers (pale-coloured heads), soldiers (red-coloured heads) and reproductive individuals of both sexes, containing several egg-laying queens (Wako, 2015). They can degrade cellulose to glucose. The intestinal microorganisms of these lignocellulose-degrading termites are considered to be essential for cellulose digestion (Hu et al., 2014). Several species were isolated from the gut of termites belonged to *Acinetobacter* (Nguyen et al., 2015), *Lysinibacillus*, *Stenotrophomonas, Bacillus, Paenibacillus, Cellulomonas* and *Aspergillus* (Huda et al., 2019). Vietnam is an agricultural country and export rice and produce a large amount of rice straw waste amounting to 55-60 million tons annually. Rice straw with a moisture content of 15 wt% contains glucan 34.4 wt%, xylan 13.6 wt%, lignin 24.1 wt%, and ash 17.7 wt% (on a dry basis) (Nhu et al., 2015).

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However, there have been no studies about the potential cellulose degrading ability to glucose from the rice straw powder of bacterial isolates isolated from the gut of the termite in Mekong Delta, Vietnam and may be applied in alcohol production in future.
The aim of this study was to evaluate the potential cellulose degradation ability to glucose for the rice straw powder of bacterial strains in the gut of termite and identify the isolates having the high cellulose degradation ability by 16S rRNA genes sequencing.

2. Materials and methods

2.1 Isolation of bacterial isolates and colony characteristic examination

A total of 13 termite nests were collected randomly TraOn district, VinhLong province, Mekong Delta, Vietnam and transferred to the laboratory of College of Natural Sciences, CanTho University. A total of 10 termites of each the termite nest were selected randomly to collect the guts from them. For collecting the guts of termites and isolation of bacterial isolates in mineral salt medium were carried out according to the method of Gupta et al. (2012) with minor modifications. First, the obtained termites were sterilized by 70% ethanol for 30 sec; washed with sterilized distilled water. After that, the termites were hold and fixed by using a stainless steel sterile tweezers for removing the heads out the body and collecting the guts. Finally, the collected guts which separated from of the termite's anus by using the other tweezers were used to touch gently to the surfaces of isolation agar medium plates (K_2HPO_4 1 g; $(NH_4)_2SO_4$ 2 g; $MgSO_4.7H_2O$ 1 g; KCl 0.5 g; $CaCl_2$ 0.02 g, $MnSO_4.4H_2O$ 0.001 g, $FeSO_4.7H_2O$ 0.01 g, Filter paper powder 0.5 g, agar 14 g in 1 litter water). The plates were incubated at 32 °C for 2 – 3 days; the cultures were streaked on the media to obtain single colonies as described by Luong et al. (2013). The pure isolates were subcultured onto the isolation agar plates and incubated at 32 °C for 48 h prior to testing. Morphological characterization of the bacterial colonies were carried out according to on the basis of their shape, size, color, margin, elevation on the media. Cell morphology (Gram staining, shape, motility and size) of the isolate were observed using optical microscopes (Olympus BX51 Microscope 100x) (Luong et al., 2003).

2.2 CMC degradation ability and cellulase activity assay of bacterial isolates isolated

This test aimed to evaluate the capability of degrading cellulose of the isolates isolated by observing the clear zone in CMC agar media. The method for assessment of the CMC degradation ability of bacterial isolates were performed following as: All of the bacterial isolates were in cultured in 3 mL TSB broth (Merck) (Casein peptone (pancreatic) 17 g/L; Soya peptone (papain digest) 3 g/L; Sodium chloride 5 g/L; Dipotassium hydrogen phosphate 2.5 g/L; Glucose 2.5 g/L; Final pH 7.3 +/- 0.2 at 25°C) with shaking at 120 rpm for 24 h. After that, 5 µL of each the isolate broth was dropped onto the CMC agar plate (K_2HPO_4 1 g; $(NH_4)_2SO_4$ 1 g; $MgSO_4.7H_2O$ 0.5 g; NaCl 0.001 g; agar 2 g, CMC 10 g, in 500 mL water) at 32 °C for 3 days. Halo-forming activities of the colonies were detected by Congo red (1 g/L) staining for 15 min and washing out by 1M NaCl solution. The CMC degradation ability was calculated according to the ratio of diameter of clearing zone and colony (Gupta et al., 2012). Of the bacterial isolates isolated, the isolates that had the high CMC degradation ability (the ratio > 2) were selected for the next experiments.
Cellulase activity assay of the selected bacterial isolate: The isolates were inoculated in enzyme production media, incubated at 30° C for 5 days on shaker at 150 rpm. The obtained culture was centrifuged at 5000 rpm for 15 min at 4° C. The obtained supernatant was stored at 4° C as crude cellulase enzyme for further assay. The cellulase (endoglucanase) activity of bacterial isolate was determined by measuring the amount of released glucose sugar according to the method of (Gupta et al., 2012). One unit of enzymatic activity is defined as the amount of enzyme that releases 1 µmol reducing sugars (measured as glucose) per mL per minute. All experiments were performed with three replicates and calculated by Excel Software 2010.

2.3 Cellulose degradation ability from the rice straws of selected bacterial isolates

In this study, the bacterial isolates had the high CMC degrading ability on the CMC agar were selected to test their ability to hydrolyze cellulose from the rice straw powder through the determination of the glucose concentration released after the cellulose hydrolysis (Tran et al., 2011). The glucose concentration in the hydrolysis solution were measured by the dinitrosalicylic acid (DNS) method of Miller (1959).
The bacterial isolate was suspended in 5 mL of sterile TSB broth (Merck) and incubated with shaking at 200 rpm for 24 h. After the culturing, for the isolate, the initial bacteria concentration of the obtained culture broth (10^8 CFU/mL) was determined and adjusted by measurement of the OD value at 600 nm in UV/VIS spectrophotometer (Multiskan Go). 1 g the rice straws powder (The biggest size of the rice straw powder is 1mm; the rice straw were dried to a moisture content of 12% and grinded by a Goodfor Q9 2300W Blender of Korea) were added into a flask 250 ml containing 25 mL of sterile distilled water and 5 mL of the obtained culture broth.

The flask was placed at room, away from sunlight to hydrolyze the cellulose from the rice straws powder. The samples were measured on 5, 10, 15 and 20 days respectively. The glucose concentration of the sample was determined by plotting of the standard curve.
The means and standard deviations were calculated by Excel Software 2010 for determination of the released glucose concentration, at least three replicates.

2.4 Identification of the good cellulose degradation ability isolates by 16S rRNA genes sequencing

The isolates having the good cellulose degradation ability from the rice straw powder were chosen to sequence 16S rRNA gene. The 16S rDNA gene was amplified from chromosomal DNA by polymerase chain reaction (PCR). Bacterial DNA was extracted from a bacterial suspension (1 mL from a TSB medium at 30 °C and 120 rpm for 24h) to DNA following published protocols according to Neumann *et al.* (1992). The amplification of the gene from isolates was performed using 27F (5'-AGAGTTTGATCTGGCTCAG-3') and 1492R (5'- TACGGYTACCTTGTTACGACTT-3') primers according to Frank *et al.* (2008). Amplification was performed in a total volume of 25 µL in 0.2 mL Eppendorf tubes using a DNA thermocycle (BioRAD). The reaction mix was prepared using the following: 12.5 µL master mix, 0.25 µL primer 27F (0.25 µM); 0.25 µL primer 1492R (0.25 µmol), 5 µL of DNA and 7 µL biH_2O. The standard thermal profile used for amplification of the 16S rRNA sequence was as follows: 6 min at 95 °C; then 40 cycles consisting of 30 s at 95 °C, 30 s at 55 °C , 30 s at 72 °C and a final cycle of consisting of 5 min at 72 °C. Aliquots (10 µL) of PCR products were electrophoresed and visualized in 1% agarose gels using standard electrophoresis procedures. Partial 16S rRNA gene of selected isolates were sequenced by MACROGEN, Republic of Korea (dna.macrogen.com). Finally, 16S rRNA sequence of the isolate was compared with that of other microorganisms by using the Basic Local Alignment Search Tool (BLAST) with a similarity cut-off of 98%.

3. Materials and methods

3.1 Bacterial isolation

In this study, a total of 70 bacterial isolates were isolated from the guts of termites from thirteen termite nests collected randomly in TraOn district, VinhLong province, Mekong Delta, Vietnam. The shape of bacterial colony varied from round to irregular, entire or lobate margin, raised, convex and flat elevation. The color were milky white, clear white, opaque white, light yellow and yellow (Figure 1). The sizes of colony ranged from 0.2 – 5.0 mm.

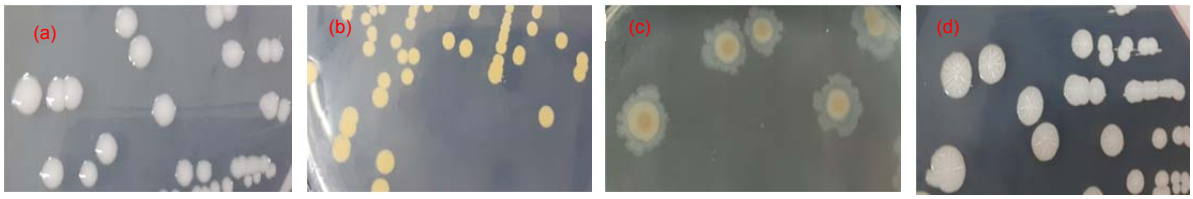

Figure 1: Shape of bacterial colonies on the isolation agar after 48 h: 4L1 isolate (a), 2T6 isolate (b), 4T5 isolate (d) and 1T3 isolate (d) respectively
Note: 4L1 isolate: Bacterial isolate no 4 isolated from soldier worker termite (L) of termite nests no 1
2T6, 1T3 and 4T5 isolates: Bacterial isolates no 2, 1 and 4 isolated from worker termites (T) of termite nests no 6, 3 and 5

3.2 The CMC degradation ability and cellulase activity of the bacterial isolates isolated

All of the 70 bacterial isolates isolated were cultured on the CMC agar at 32 °C for 3 days and detected halo formation detected by the Congo red staining. The results showed that only the 60 (68%) isolates which showed the formation of clear zones. The value of the clear zone ratio formed by the 60 isolates ranged from 1.10 to 2.83. The Congo red can be used as an indicator to detect the appearance of extracellular cellulolytic enzymes secreted by cellulose degrading bacterial isolates in the media, due to Congo red strongly interacted with polysaccharides containing continuous β-1,4-linked D-glucopyranosyl units and β-1,3-D-glucans and some hemicellulosic galactoglucomannans possibly (Nguyen et al,, 2016).
Out of the 60 isolates, only 5 isolates that had the ratio higher than 2 were 1T1 (2.24), 2T1 (2.73), 2T2 (2.73), 2T6 (2.65) and 5T6 (2.83) respectively (Figure 2). The results showed that the cellulose degrading capacity of the five isolates were stronger than cellulolytic aerobic bacterial isolates isolated from forest and farming soils in the previous study (Hatami et al., 2008) with the mean ratio of clear zone diameter to colony were 1.6 and 2.1 for forest and farming soil, respectively. Colonies and cells characteristics of the 5 bacterial isolates had the high CMC degradation ability was presented in the Table 1.

Morphological characteristics of the cells in Olympus BX51 Microscope (100x) (Japan) were rod-shaped or circular (Table 1). All of them were motility. Cell sizes ranged from 0.5 to 2.0 µm.

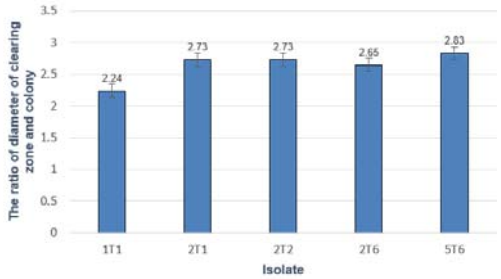

Figure 2: The ratio of diameter of clearing zone and colony of the 5 bacterial colonies that had the high CMC degradation ability on the CMC agar after culturing at 32 ^{0}C for 3 days
Note: 1T1, 2T1, 2T2, 2T6 and 5T6 isolates: Bacterial isolates no 1, 2, 2, 2 and 5 isolated from worker termites (T) of termite nests no 1, 2, 2, 2 and 5

Table 1: Colony and cell morphology of the 5 bacterial isolates had the high CMC degradation ability

Bacterial isolates	Colonies morphology				Cell morphology				
	Form	Color	Margin	Elevation	Dimension (mm)	Shape	Gram	Motility	Size (µm)
1T1	Circular	Opaque white	Entire	Flat	2.1	Circular	-	+	0.5
2T1	Circular	Opaque white	Lobate	Raised	1.8	Rod	+	+	2.0
2T2	Circular	Opaque white	Entire	Raised	0.5	Circular	-	+	1.0
2T6	Circular	Dark Yellow	Entire	Flat	0.3	Circular	-	+	1.0
5T6	Circular	Opaque white	Entire	Raised	1.0	Circular	-	+	1.0

Note: 1T1, 2T1, 2T2, 2T6 and 5T6 isolates: Bacterial isolates no 1, 2, 2, 2 and 5 isolated from worker termites (T) of termite nests no 1, 2, 2, 2 and 5

Results in Figure 3 shows the cellulase activity for the five isolates were 0.19 U/mL (1T1), 0.29 U/mL (2T1), 0.24 U/mL (2T2), 0.21 U/mL (2T6) and 0.31 U/mL (5T6) respectively.

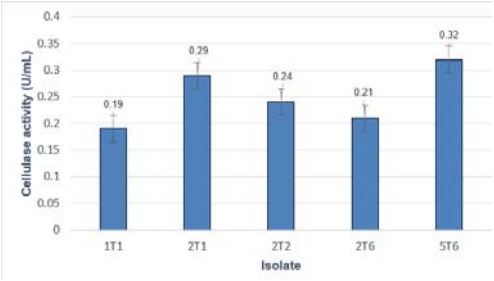

Figure 3: Cellulase activity (U/mL) of the 5 bacterial isolates had the high CMC degradation ability

The cellulase activity of the 5T6 isolate was the highest, and lower than the cellulase activity of *Paenibacillus lactis* AFC1 (1.47 U/mL) found by Huda et al (2019). In the comparision with the commercial enzyme, Yishui with enzyme activity of 7.0 (U/ mL), the cellulose activity of the bacterial isolates isolated from the gut of the termite were lower than hydrolysis of filter paper (Yu et al. 2016). The possible reason might be that the obtained cellulase activity was not optimal under the cultural conditions such as temperature, pH, substrate, etc (Nguyen et al., 2015).

3.3 The cellulose degradation ability from the rice straw powder of selected bacterial isolates

A total of the 5 isolates (1T1, 2T1, 2T2, 2T6 and 5T6) were selected to evaluate the cellulose degradation ability from rice straw powder. The cellulose degradation ability of the 5 isolates were calculated based on the quantity of produced glucose in the hydrolysis solutions for 5, 10, 15 and 20 days. The results in Figure 4 showed that all of them had the cellulose degradation capacities.

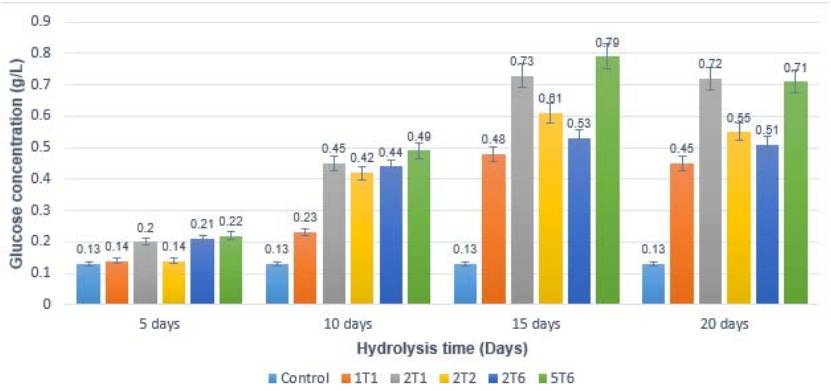

Figure 4: The quantity of produced glucose in the hydrolysis solutions for 5, 10, 15 and 20 days
Note: 1T1, 2T1, 2T2, 2T6 and 5T6 isolates: Bacterial isolates no 1, 2, 2, 2 and 5 isolated from worker termites (T) of termite nests no 1, 2, 2, 2 and 5

The glucose concentration in the hydrolyze solution increased during the hydrolysis period from 5 days to 15 days, peaked on day 15 and then decreased, for the all 5 isolates. The order of hydrolysis capacities increased from: 1T1, 2T6, 2T2, 2T1 and 5T6 respectively. The 5T6 isolate produced the highest glucose concentration after 15 days hydrolysis (0.79 g/L) whereas the lowest glucose concentration was obtained by the 1T1 isolate (0.48 g/L). An earlier study (Tran et al., 2011) had reported that cellulose hydrolysis separated from rice stubble/rice into glucose by *Aspergillus terrius* AF67 reached a peak on day 4. The glucose concentration in hydrolysis solution was 5.61 mg/mL. Out of 5 the isolates, the isolate 5T6 (0.79 g/L) and the isolate 2T1 (0.73 /L) had the high cellulose degradation ability, outperformed the others and were selected to identify by 16S rRNA genes sequencing.

3.4 The identification of the 2 isolates had the high cellulose degradation ability

In this study, the sequences of the 16S rRNA gene of the 2 isolates (2T1 and 5T6) had the high cellulose degradation ability were analysed. Results of homology search of 16S rRNA gene sequence of selected isolate in GenBank by BLAST showed that the strain 2T1 showed the closest sequence identity (98.99%) with *Paenibacillus humicus* strain T20C and the strain 5T6 showed the closest sequence identity (99.01%) with *Psychrobacter faecalis* strain AP3Ka.
In the previous study of Huda et al. (2019), AFC1 isolate belonged to the genus *Paenibacillus* was isolated the gut of the subterranean termite *Psammotermes hypostoma* Desneux. Cellulose (endoglucanase) activity of the isolate AFC1 was 1.47 U/mL. The genus *Paenibacillus* is characterized as rod-shaped Gram - positive or Gram - variable endospore forming aerobic or facultative anaerobic bacteria. *Paenibacillus* sp. produce multiple enzymes for lignocellulolytic degradation, have the cellulose degradation activity (Chiena et al, 2015).
The genus *Psychrobacter* was proposed for a group of mainly psychrophilic, Gram-negative coccobacilli found associated with fish, processed meat and poultry. *Psychrobacters* are widespread in Antarctic ecosystems, were isolated in ornithogenic soils, anchore and grease ice, and ice algae biomass. The genus *Psychrobacter* is classified into the family Moraxellaceae within the γ-subclass of *Proteobacteria*. This family includes also the genera *Moraxella* and *Acinetobacter*. *Psychrobacter faecalis* was a new species, isolated from pigeon feces and from human samples; was Gram (-), oxidase (+), catalase (+) (Kämpfer et al, 2002).

4. Conclusions

The 5 bacterial isolates isolated from the gut of termites had the cellulose degradation ability from the rice straw powder to produce the high glucose concentration in hydrolysis solution after 15 days. Out of the 5 bacterial isolates, 2 isolated from the gut of worker termites produced glucose in large quantities in the hydrolysis solutions were the isolate 2T1 (0.73 g/L) and the isolate 5T6 (0.79 g/L). The 2T1, 5T6 strains were 98.99%, 99.01% of identity *Paenibacillus humicus* strain T20C, *Psychrobacter faecalis* strain AP3Ka

respectively in which the 5T6 strain belonged to the genus *Psychrobacter* had not been found in the gut of termites from previous studies. These bacterial strains showed a potential to convert cellulose of the rice straw powder into glucose which could be readily used in many fields such as fermentation of cellulose into ethanol, agricultural areas, cellulosic waste of cow milk farms in future.

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