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Screening of Cellulolytic Actinomycetes for Decomposition of Agricultural Waste

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Each year, Viet Nam produces around 37 Mt of rice, 17-18 Mt of sugarcane and 4.5 Mt of maize and the total waste created by agriculture is estimated more than 50 Mt. Approximately nearly half of the straw is burned on the field, which is a common practice in intensive rice cultivation systems in this region and significantly impacts greenhouse gas emissions in the country. It has been a challenge for the government to reduce the burning of straw. Using microbials is a potential solution for the reason that fertilized straw, which contains essential nutrients as nitrogen, phosphorous and potassium, can be used as compost to enrich the soil. This study aims to screen for Actinomycetes with high cellulase activities for the decomposition of agricultural wastes. Among 21 isolated actinomycetes, 7 isolates including G1, G3, G11, D4, D5, DE2, X1 were selected with high enzymatic activities. The incubation of selected strains with rice straw and cane bagasse indicated that the ability of all these strains to degrade agricultural wastes. Rice straw showed better degradation results in comparison to sugarcane bagasse. The strains G3 and D4 showed the best degradation performance after 12 d of incubation. The remaining substrate including rice straw and sugarcane bagasse after incubation in turn with D4 and G3 were both 10.5 %.

1. Introduction

Cellulose, a linear polysaccharide of glucose residues with β-1, 4-glycosidic linkages, is the most abundant carbon source on earth and has been accumulating year by year. This raw material can be degraded into dextrin and glucose that are industrially important commodity products. The degradation can be proceeded with either acid or enzymes. Enzymatic method shows to be more efficient and environmentally friendly. In nature, complete hydrolysis of cellulose is mediated by a combination of three main types of cellulases: (1) endoglucanases (EC 3.2.1.4), (2) exoglucanases, including cellobiohydrolases (CBHs) (EC 3.2.1.91), and (3) β-glucosidase (BG) (EC 3.2.1.21) (Castañeda and Mallol, 2013). These enzymes are produced by a large number of micro-organisms including fungi, actinomycetes, facultative bacteria such as *Bacillus* and *Cellulomonas*, and strict anaerobes such as *Clostridium* (Fowler et al., 1999). Among them, Actinomycetes are widely distributed across different habitats and usually involve in important processes. Therefore, the evaluation of their distribution is important in understanding their ecological role (Prakash et al., 2013). Many previous research works revealed the ability of producing cellulase of Actinomycetes isolated from various habitats, nonetheless, little of them focused on the potential of these strains in degrading cellulose waste, especially from agriculture.

Vietnam is one of the five largest exporters of rice, cashew and many other items. However, this growth is also accompanied by pollution of land, water, and air especially burning straw in agricultural activities causes high air pollution in some parts of the region. Rice straw open burning in the suburban fields of Hanoi has had many adverse impacts to the air quality of the inner city. Based on the rice production data in 2015, the average proportion of rice straw burned in the field was around 44 %. This farming practice leads to the emission of several toxic gas such as CO_2 (419,889 t), CO (8,865 t) and NO_x (1,402 t) (Hoang et al., 2017). Therefore, agricultural waste in Vietnam has been a big problem which needs to be settled. The aims of this research are to identify and select Actinomycetes strains with high cellulase activities for bioprocess applications in agricultural waste treatment to improve the quality and value of agriculture. This potential ability of Actinomycetes, which has been overlooked in previous work, is demonstrated in this study.

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2. Materials and methods

2.1 Microorganisms

Actinomycetes strains were isolated from samples of soil, rotten wood, leaf and rice straw taken in June 2018 at different locations in Hanoi, Vietnam. The trains were cultivated and maintained on selective media, IPS-4 (Starch 10 g/L, K_2HPO_4 1 g/L, $MgSO_4.7H_2O$ 1 g/L, NaCl 1 g/L, $CaCO_3$ 2 g/L, $(NH_4)_2SO_4$ 2 g/L, $FeSO_4$ 0.001 g/L, $MgCl_2.7H_2O$ 0.001 g/L, $ZnSO_4.4H_2O$ 0.001 g/L; pH 7).

2.2 Sample collection

Rice straw sample was collected in Hanoi then cut into pieces of 3 cm long. Sugarcane bagasse sample was taken from Lam Son Sugar Factory. All samples were dried at 60 °C for 24 h. 50 g of each dried sample was then treated with 1L NaOH 1 % (w/v) for 24 h, followed by washing with sterile water before being dried at 100 °C to unchanged weights (Zhao et al., 2014).

2.3 Preparation of crude enzymes

Three actively growing colonies were selectively picked from 7-day-old cultures to incubate in 50 mL IPS-4 medium in 250 mL-Erlenmeyer flask. The fermentation was performed with 5 % (v/v) of 36 h - incubated inoculum in fermentation media containing different carbon sources for the production of different enzymes: CMC 1 % (w/v), Avicel 1 % (w/v) and rice bran 1 % (w/v) for the production of endoglucanase (Chellapandi and Jani, 2008), exoglucanase (Schlochtermeier et al., 1992) and β-glucosidase (Ahmed et al., 2017). The incubation was conducted under aerobic condition at 30 °C on a rotary shaker LSI-3016R Labtech (Korea) at 100 rpm for 72 h. Supernants containing crude enzymes were obtained by centrifugation at 11,000 rpm for 10 min. The crude enzymes were then stored at 4 °C until use.

2.4 Enzymatic assays

2.4.1 Endoglucanase assay

The reaction tube contaning 2 % CMC in 100 µL of 50 mM citrate buffer, pH 4.8 and 100 µL of crude enzyme solution is prepared followed by an incubation at 50 °C for 10 min in a water bath. The reaction is terminated by adding 400 µL of dinitrosalicylic acid (DNS) reagent to the mixture and subsequently placing the tube in a water bath at 100 °C for 5 min. The reaction solution is then well mixed with 400 µL of distilled water and cooled to room temperature before measuring absorbance at 540 nm on Spectrometer PD-303S APEL (Japan) (Zhang et al., 2009).

2.4.2 Exoglucanase assay

The reaction tube contaning 1.25 % Avicel in 400 µL of 50 mM citrate buffer, pH 4.8 and 100 µL of crude enzyme solution is prepared followed by an incubation at 50 °C for 2 h in a water bath. The reaction was terminated by submerging the tubes in ice-cooled water bath. After centrifuging the sample at 11,000 rpm for 10 min and withdrawing 150 µL of supernatant into microcentrifuge tubes, the reaction solution is then well mixed with 150 µL of 5 % phenol solution, 750 µL of concentrated sulfuric acid and cooled to room temperature before measuring absorbance at 490 nm (Zhang et al., 2009).

2.4.3 β-glucosidase assay

The reaction tube contaning pNPG 5 mM in 360 µL of 50 mM citrate buffer, pH 4.8 and 40 µL of crude enzyme solution is prepared followed by an incubation at 50 °C for 30 min in a water bath. The reaction is terminated by adding 800 µL of glycine buffer to the mixture. The reaction solution is then cooled to room temperature before measuring absorbance at 430 nm (Zhang et al., 2009).

2.4.4 Total cellulase assay

The reaction tube contaning a rolled filter paper strip (Whatman no.01, 1.0 cm x 6.0 cm) submerged in 1.0 mL of 50 mM citrate buffer (pH 4.8) and 0.5 mL of crude enzyme solution is prepared followed by an incubation at 50 °C for 1 h in a water bath. The reaction is terminated by adding 3 mL of dinitrosalicylic acid (DNS) reagent to the mixture and subsequently placing the tube in a water bath at 100 °C for 5 min. After withdrawing 0.3 mL of the colored solutions into microcentrifuge tubes and centrifuging at 11,000 rpm for 3 min, the reaction solution is then well mixed with 1.2 mL of distilled water and cooled to room temperature before measuring absorbance at 540 nm (Zhang et al., 2009).

2.5 Degradation of agricultural waste

Actinomycetes strains with high cellulase activities were selected for the screening for degradation of agricultural waste. All selected strains were incubated (seeding volume of 4 %) in broth culture with rice straw and cane bagasse as carbon source (rice straw/ sugarcane bagasse 10 g/L, K_2HPO_4 1 g/L, $MgSO_4.7H_2O$ 1 g/L, NaCl 1 g/L, $CaCO_3$ 2 g/L, $(NH_4)_2SO_4$ 2 g/L, $FeSO_4$ 0.001 g/L, $MgCl_2.7H_2O$ 0.001 g/L, $ZnSO_4.4H_2O$ 0.001 g/L; pH 7) at 30 °C on a rotary shaker at 100 rpm. After 4, 8 and 12 d of incubation, the contents of the flasks were centrifuged at 11,000 rpm for 10 min, 4 °C. The supernatant was used as crude enzyme extract and the precipitates were washed with an acetic acid/nitric acid solution and then with water twice and dried until their weight became stable. Non-inoculated medium served as a control (Zhao et al., 2014). The remaining substrate is calculated by Eq (1):

$$\%H = \frac{m - m_0}{m_0} \times 100\% \quad (1)$$

where m is the weight of sample (g) and m_0 is the weight of control sample (g).

2.6 Identification of selected strains

The bacterial 16S rDNA, full-length 1.5 kb, was amplified using universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT). The total reaction volume of 25 µL contained gDNA purified, 0.3 pmol of each primer, deoxynucleotides triphosphates (dNTPs, 400 µM each), 0.5 U DNA polymerase, supplied PCR buffer and water. The PCR was performed as follow: 1 cycle (94 °C for 2 min) for initial denaturation; 25 cycles (98 °C for 10 s; 53 °C for 30 s; 68 °C for 1 min) for annealing and extension of the amplified DNA. The PCR products were purified and directly sequenced with primers 785F (GGATTAGATACCCTGGTA) and 907R (CCGTCAATTCMTTTRAGTTT) using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, ThermoFisher Scientific, USA). The phylogenetic tree was constructed by neighbour-joining (NJ) method using MEGA-X program with bootstrap values based on the 1,000 replications.

3. Results and discussion

3.1 The production of cellulase

Twentyone isolates were cultured in ISP-4 medium where the carbon source was either CMC or Avicel. The production of cellulase was evaluated by measuring the enzymatic activities of endoglucanase and exoglucanase produced. Table 1 indicates the endoglucanase activity of the strains D4, G3, G1, DE2 and X1 appeared higher than the others with the highest activity of 0.202 U/mL belonging to the strain D4 after 4 d of incubation. The strain G11 presented the highest exoglucanase activity of 0.011 U/mL, however showed comparatively low endoglucanase production (0.089 U/mL).

Table 1: Endoglucanase and exoglucanase activity of isolated strains

Isolates	Endoglucanase Activity (U/mL)	Exoglucanase Activity (U/mL)	β-glucosidase OD_{430}	Isolates	Endoglucanase Activity (U/mL)	Exoglucanase Activity (U/mL)	β-glucosidase OD_{430}
K1	0.128±0.009	0.008±0.001	0.009±0.002	G4	0.058±0.012	0.010±0.001	0.024±0.001
K4	0.158±0.015	0.008±0.001	0.021±0.004	G7	0.047±0.000	0.006±0.001	0.011±0.002
DE2	0.177±0.001	0.005±0.000	0.020±0.002	G8	0.048±0.008	0.004±0.001	0.020±0.002
X1	0.175±0.017	0.007±0.001	0.027±0.002	G9	0.101±0.017	0.009±0.001	0.014±0.002
X5	0.155±0.016	0.009±0.000	0.019±0.002	G10	0.144±0.016	0.004±0.001	0.009±0.003
X6	0.046±0.004	0.006±0.000	0.023±0.002	G11	0.089±0.010	0.011±0.000	0.005±0.002
D1	0.124±0.004	0.008±0.001	0.012±0.002	G13	0.162±0.005	0.006±0.000	0.004±0.001
D4	0.202±0.001	0.007±0.000	0.019±0.003	G14	0.107±0.003	0.003±0.000	0.020±0.002
D5	0.163±0.004	0.007±0.000	0.050±0.002	G16	0.048±0.000	0.005±0.001	0.002±0.001
G1	0.181±0.008	0.004±0.000	0.031±0.001	XK3	0.046±0.002	0.005±0.000	0.011±0.001
G3	0.193±0.011	0.008±0.000	0.021±0.002				

The screening for Actinomycetes strain with high production of β-glucosidase was conducted on IPS-4 medium with rice bran as the carbon source. The production of β-glucosidase was assessed directly with absorbance value at 430 nm (OD_{430}) of the mixture of crude enzyme solution with pNPG 5 mM. The results were presented in Table 1 indicates the highest value of 0.05 belonging to the strain D5.

3.2 Degradation of agricultural waste

To assess the degradation capacity of the selected strains on rice straw and sugarcane bagasse, the two prevalent agricultural wastes in Vietnam, the Actinomycetes were incubated in a medium using rice straw and sugarcane bagasse as a sole carbon source. After 4, 8 and 12 d of incubation, the remains of carbon substrate were separated for determination of weight loss (Figure 1). The substrate showed a reduction trend during the whole course of incubation with relatively high reduction rate during the first 8 d and gradual rate during the next 4 d. By contrast, all strains exhibited increased enzyme production in the culturing medium. Except for the isolate G1, all the others provided better performances in terms of both enzyme production and degradation of agricultural wastes. It was demonstrated that these strains beside of being able to produce enzymes to degrade cellulose, they can enzymatically break down other components of plant cells, such as xylan in rice straw as well (data not shown).

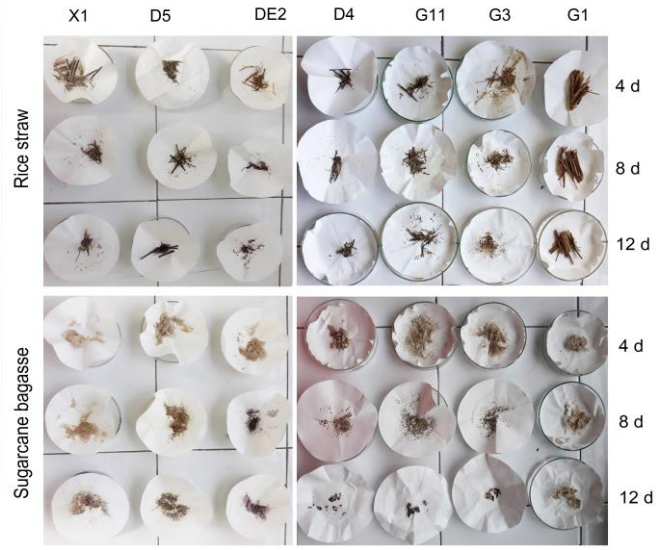

Figure 1: Remaining substrates after 4, 8, 12 d of incubation with the isolates D4, D5, DE2, G1, G3, G11, X1

SEM was used to investigate structural changes of the enzyme-treated rice straws and sugarcane bagasse (see Figure 2). The control sample of the two cellulose sources both displayed a rigid and well-ordered arrangement while the surface of rice straw after incubation with D4 isolate became porous and hollow. The fibrous surface of sugarcane bagasse incubated with G3 isolate showed loosen structure, which indicated the separation of fibrils.

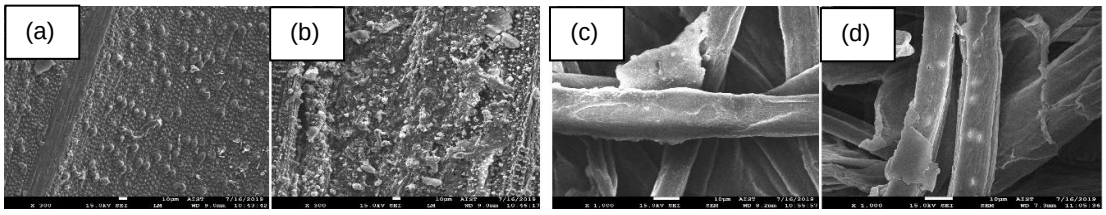

Figure 2: SEM of rice straw (at x300 magnification) and sugarcane bagasse (at x1,000 magnification) surface structures after 12 d of incubation: (a) control samples of rice straw, (b) rice straw after incubated with D4 isolate, (c) control samples of and sugarcane bagasse, (d) sugarcane bagasse after incubated with G3 isolate.

The total cellulase, endoglucanase and exoglucanase activities of 7 selected isolates (D4, D5, DE2, G1, G3, G11, X1) showed compatible to the results in the study of Mohamed et al. (2017). Results indicate that the highest exoglucanase activity of 0.024 U/ml was recorded for the strain D4 while using rice straw as a sole carbon source. The highest endoglucanase activity of 0.406 U/ml was recorded for the strain G3 while using sugarcane bagasse as a sole carbon source. The strain G1, on the other hand, hardly showed any degradation activity. D4 and G3 were the two strains with the best degradation activity among all strains. After 12 d of incubation, the remains of rice straw and sugarcane bagasse in both cases with D4 and G3 was 10.5 % (Figure 3).

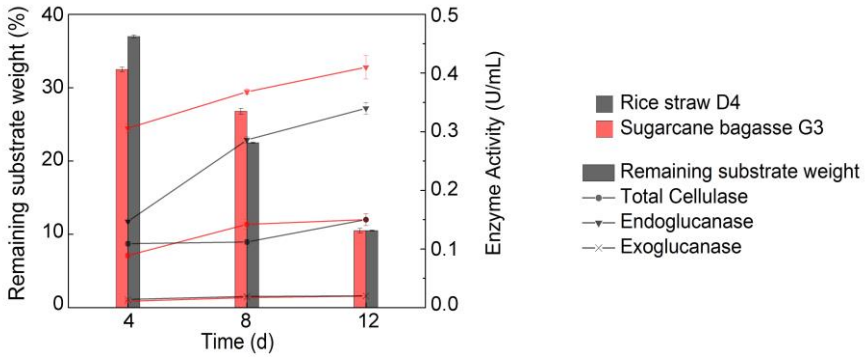

Figure 3: Degradation of the two agricultural wastes and cellulolytic activities of the isolates D4 and G3

3.3 Identification of selected strains

The identification and classification results with 16S rDNA of selected actinomycetes strains indicates that the strains D4 and G3 are quite close to the species Streptomyces thermocarboxydus and Streptomyces roseofulvus (Figure 4).

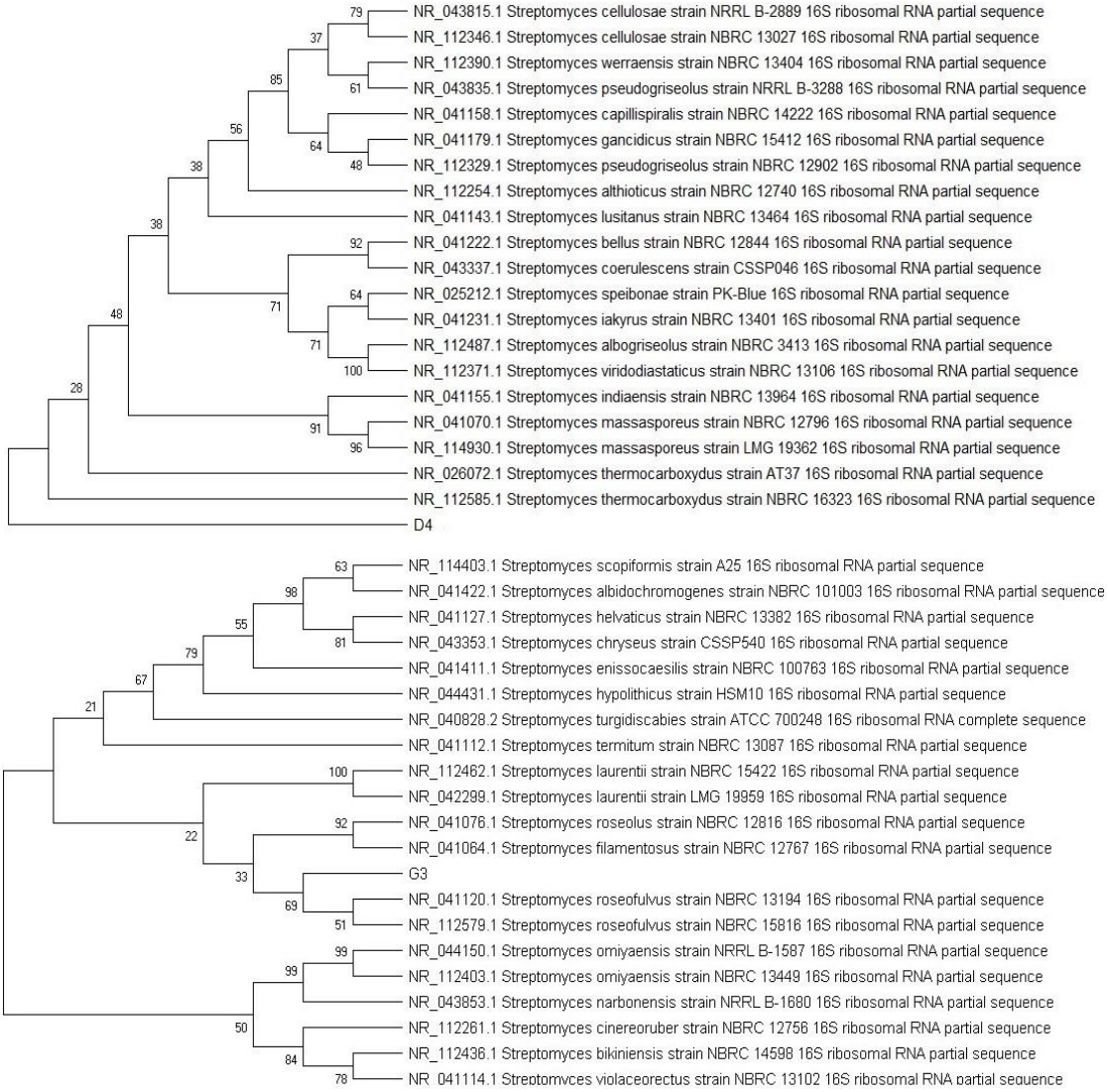

Figure 4: Phylogenetic tree of D4 and G3 isolates constructed with Bootstrap method

These two species are classified at ATCC Biosafety level 1. S. roseofulvus was used in the production of deoxyfrenolicin and frenolicin B (Iwai et al., 1978), while S. thermocarboxydus was known for its capacity of synthesizing pectinase (Priyanka, 2019) and ligninase (Kim et al., 1998). The strains G3 and D4, therefore, are considered as potential microorganisms for using in the treatment of agricultural wastes.

4. Conclusions

The two strains D4 and G3, identified to be *S. thermocarboxydus* and *S. roseofulvus* showed high cellulase activities and cellulose degrading abilities among the 21 isolates. Further investigations are required to make use of the full potential of these organisms for cellulase production to enhance agricultural wastes degradation process.

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