Screening of the cellulolytic microorganisms from the Cassava-growing land area

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Abstract

Cassava is one of Thailand most economic crops with its roots being used for human and animal feed. The residual material left in the field after harvesting can be used as green manures for another crop or crop rotation. This residue increases the nitrogen and remaining nutrient levels in the soil. However, the degradation of green manure takes long time which results in the delayed of the next plantation. One important factor of the delayed is that the cellulosic cell wall which is difficult to decompose under natural condition. The aim of this project is to screen for cellulolytic microorganisms from cassava growing land area for high cellulose activity. Fourteen isolateds were obtained and then tested for cellulose activity using Carboxyl Methyl Cellulose (CMC) and Dinitrosalicylic Acid (DNS) methods. Among these bacteria, CL-04 and CL-02 were the best producer strains The isolated bacteria will have to be further studied to identify the bacteria species by morphology and using 16S rRNA gene sequencing. The optimum of growth conditions such as pH, temperature, fermentation time will be investigated to obtain enzymatic function information.

Keywords: cassava, cellulase, cellulolytic microorganism, cellulose, soil

Introduction

Cassava is the one of the main economic plants of Thailand. The root of this plant processed as flour which is very important for the food industry. After harvesting, cassava residues are left in the field where they are used as green manures for another crop or crop rotation (McDonald MJ, 2011). However, degradation of these residues needs time to return the nutrient and mineral into the field. The lignocellulosic biomass (LB) which is composed of a heterogeneous complex of polymers (cellulose, hemicelluloses and lignin) is the major component of the green manures. Lignocellulosic wastes in the form of cellulose which is the most abundant renewable resources in the biosphere have been shown to be used in the production of valuable products by microorganisms (Devi and Kumar, 2012). These LBs are difficult to hydrolyze in nature. The cellulose molecule is very stable, with a half life of 5-8 million years for beta-glucosidic bond cleavage at 25 °C, whereas the much faster enzyme-driven cellulose biodegradation process is vital to return the carbon in sediments to the atmosphere (Zhang Y-HP et al., 2006). The conversion of cellulosic mass to fermentable sugars through biocatalyst cellulase derived from cellulolytic organisms has been suggested as a feasible process. It is potential to reduce environmental pollution and the use of fossil fuels (Devi and Kumar, 2012). Moreover, cellulases have been also utilized to improve the nutritional values of animal feed. Many microorganisms have been found and identified to degrade cellulose and other plant cell wall fibers (Dashtban et al., 2010). Most of them were isolated, screened and characterized the cellulolytic microorganisms from the soil or source which has the lignocellulosic biomass as a carbon source (Brito-Cunha et al., 2013; Mori et al., 2014; Nacke et al., 2012; Padaria et al., 2014). The aim of this study is to isolate and screen the cellulolytic microorganisms from the cassava growing land area by using the Carboxyl methyl Cellulose (CMC) method and DNS assay method.

Methodology

Isolation of cellulolytic bacteria

The cassava growing soil from Rayong province in Thailand was collected by Dr.Supajit Sraphet, Institute of Molecular Biosciences, Mahidol University on April 2014. Bacterial strains were isolated from cassava growing soil by using 10-fold serial dilutions in 0.1% peptone. Nutrients agar (0.5 (w/v) % peptone, 0.3 (w/v) % yeast extract, 0.5 (w/v) % NaCl and 1.5 (w/v) % agar) was used for bacteria isolation. After making serial dilutions, 100 µl of each serial dilution was spread on the agar plate and incubated at 30 ºC for 24 h to allow bacteria colonies to be formed.

Carboxyl Methyl Cellulose (CMC) method

Bacteria colonies from the nutrient agar plate were transferred to Carboxyl Methyl Cellulose (CMC) agar plate (0.5 (w/v) % CMC, 0.05 (w/v) % yeast extract, 0.1 (w/v) % glucose, 0.1 (w/v) % KCl, 0.05 (w/v) % $MgSO_4$, 0.1 (w/v) % K_2HPO_4, 0.12 (w/v) % KNO_3 and 1.5 (w/v) % agar. After incubation at 30 ºC for 24 hours, CMC agar plates were flooded with 1 (w/v) % Congo Red and allowed to stand for 15 min at room temperature then neutralized with 1M NaCl solution for counterstaining the plates. Formation of yellow color clear zone around the colony after confirmed the production of cellulase. The bacterial colonies displaying clear zones were selected for identification and cellulase production in submerged system.

Preparation of crude enzyme

One colony of a selected bacterial isolate was inoculated in 1 ml of CMC broth (CMC 1% (w/v) CMC; 0.2% (w/v) K_2HPO_4; 0.03% (w/v) $MgSO_4$; 1% (w/v) peptone; 0.25% (w/v) $(NH_4)_2SO_4$) and incubated agitation (140 rpm) at 35˚C for 24 h. The fermented broth was then centrifuged at 14000 × g for 10 min at 4˚C to remove the unwanted material. The clear supernatant obtained after centrifugation served as the crude enzyme source.

Enzyme activity assay

The cellulase activity assay was determined as described by Miller (1959) with some modifications. Two hundred microliter of crude enzyme was added to 0.5 ml of 1 % CMC in 0.05 M phosphate buffer (0.05 M phosphate without CMC served as the control) and incubated at 50˚C for 30 min. After incubation, reaction was stopped by the addition of 1.5 ml of DNS reagent and boiled for 10 min. An increase in reducing ends was determined by measuring the absorbance at 540 nm. One unit of activity was defied as the quantity of enzyme releasing 1 µmol of reducing sugar per min at 50˚C. Cellulase activity was expressed as units per milliliter of sample. Glucose (0-0.2 mg/ml) was used to prepare a standard curve.

$$\text{Enzyme Unit (Unit/mL)} = \frac{\text{mg of Glucose}}{\text{molecular weight of Glucose (g/mol)}} \times \frac{1}{30\ \text{min}} \times \frac{1}{\text{Volume of Enzyme (mL)}}$$

Results and Discussion

Soil microbial biomass is a common parameter used to assess the ecological role of microbes. The soil properties and the aboveground vegetation types are generally regarded as the main factors that contribute to the biomass and the diversity of soil microbes including cellulolytic microbes (Agnelli et al., 2004; Fujii et al., 2012; Ulrich et al., 2008). The specific environments and ecological systems exhibit distinct community compositions (Yang et al., 2014). The result showed that some of bacteria have the cellulase against CMC (Fig 1).

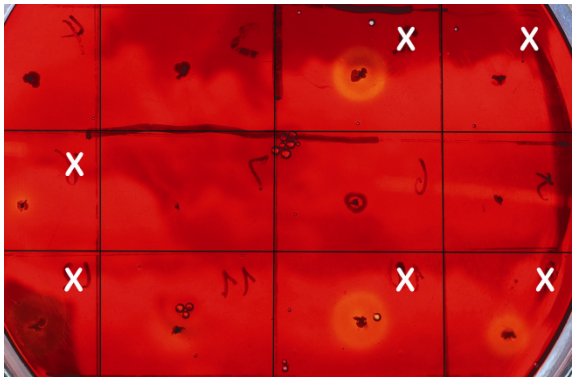

Figure 1: *Cellulolytic bacterial isolated on CMC plate.* The white labels showed the example of bacteria isolates producing clear hydrolytic zone after staining with Congo Red.

Congo red has been used as an indicator for beta-D-glucan degradation in an agar medium and provides the basis for a rapid and sensitive screening test for cellulolytic bacteria (Teather and Wood, 1982). Bacteria which produce the hydrolytic zone on CMC agar were selected to test further the enzymatic activity using DNS method. Carboxylmethyl cellulase (CMCase) activity is mostly determined by measuring reducing sugars released after 30 min. The activity of the isolated bacteria was shown in Figure 2 and Table 1. According to this data, four bacteria showed the higher than 0.05 enzyme units are CL-04, -02, -11 and 15. Among these bacteria, CL-04 and CL-02 were the best producer strains. The isolated bacteria will have to be further studied to identify the bacteria species by morphology and using 16S rRNA gene sequencing. The optimum of growth conditions such as pH, temperature, fermentation time will be investigated to obtain enzymatic function information.

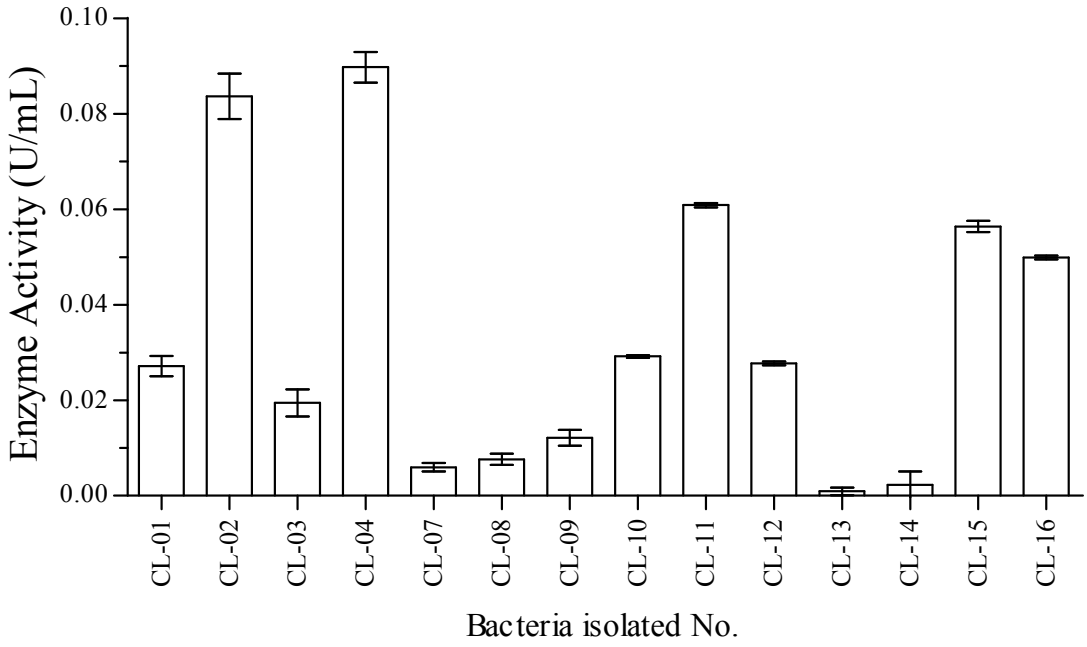

Figure 2 *Enzyme activities of the isolated bacteria from Cassava-growing land area.* The enzyme activity was determined by using the DNS assay method. The enzyme activities were performed in triplicate with the standard deviation.

Table 1: Enzyme activity of the isolated bacteria from Cassava-growing land area.

Bacteria isolated No.	Enzyme Activity (Unit/mL)	Bacteria isolated No.	Enzyme Activity (Unit/mL)
CL-01	0.0272 ± 0.0021	CL-10	0.0292 ± 0.0003
CL-02	0.0837 ± 0.0048	CL-11	0.0609 ± 0.0005
CL-03	0.0195 ± 0.0028	CL-12	0.0277 ±0.0004
CL-04	0.0897 ± 0.0032	CL-13	0.0009 ± 0.0007
CL-07	0.0060 ± 0.0009	CL-14	0.0023 ± 0.0029
CL-08	0.0077 ± 0.0012	CL-15	0.0564 ± 0.0012
CL-09	0.0121 ± 0.0017	CL-16	0.0499 ± 0.0004

Conclusion

This is the first report of the cellulolytic bacteria isolated from Cassava-growing land area.

Acknowledgements

This project was funded by the Ratchadapisek Sompoch Endowment Fund, Chulalongkorn University (Sci-Super 2014-024) and partially funded by Grants for Development of New Faculty Staff, Ratchadapisek Sompoch Endowment Fund, Chulalongkorn University.

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