Analysis of Microbial Consortia with High Cellulolytic Activities for Cassava Pulp Degradation

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Abstract. Biogas production is one of the means to manage the cassava pulp waste obtained from the cassava processing plants. The success of the process is determined by the hydrolysis in an anaerobic digester. When the digester failure is found, the new microbial consortium inoculum is introduced to the system with the long period of set up time. This research aimed to construct the endemic microbial consortium by re-cultivating the cellulolytic microbial consortia obtained from cassava pulp and digester wastewater with the expected shorter set up time. Modifications of enrichment and re-cultivation methods by varying the nutrients, pH and temperature improved the enzymatic hydrolysis yields, as reducing sugars, of CMC, rice straw and cassava pulp substrates approximately 9, 3, and 13 times, respectively. To analyze the enzymatic activities of the selected microbial consortia, the cellulase enzyme was extracted, partially purified and analyzed on CMC-zymogram. The ~130 kDa-sized cellulase enzyme was identified with endocellulase activity, and it was considered as a relatively large molecular size molecule compared to most bacterial endocellulases. The selected microbial consortia were tested for their biomass degradation capacities, and the optimal operational condition was obtained at pH 7.0 and 30 °C. This optimal condition showed the proof of the concept that this re-cultivated consortium could be applied in on-site digester with high efficiency.

1 Introduction

One of the major concerns of agricultural industries is involved in the waste management, especially processing by-products, for example, cassava pulp, corn cobs, sugarcane leaves. These sources of agricultural waste mostly have lignocellulosic biomass as common components. Lignocellulosic biomass mainly consists of cellulose, hemicellulose, and lignin that has potential to be utilized as raw materials for production of renewable fuels or valued added platform biochemicals [1-4]. In cassava processing industry, cassava pulp is currently used as feeds for dairy farms. However, it could be also used as raw materials in the biofuel production, mostly in the forms of biogas and bioethanol [5].

Cassava pulp contains high content of starch and lignocellulose. Due to high starch content and moisture content, large amounts of unused cassava pulps obtained from cassava processing plants cause adverse situation to surrounding areas and environments, especially the bad smell and water contamination [6]. Conventionally, cassava pulp is converted to biogas in anaerobic digester based on the functions of microorganism consortium in 4 steps reactions including hydrolysis, acidogenesis, acetogenesis and methanogenesis. The efficiency of biogas production is normally determined by the rates of hydrolysis and methanogenesis reactions.

In hydrolysis, microorganisms in a consortium secrete cellulolytic enzymes to convert the macromolecular compounds to micromolecules and monomers that subsequently converted to biogas or other products. In fact, the production rate and efficiency of cellulolytic enzymes depend on various factors, especially working condition of hydrolysis process, including temperature, pH, as well as nutritional value [7].

Many attempts have been done continuously to apply microbial consortia and to optimize the operational condition to maximize the efficiency of anaerobic digester. With addition of a mesophilic microbial consortium, for example, *Firmicutes* sp., *Bacteroidetes* sp., *Deferribacteres* sp., *Proteobacteria* sp., *Lentisphaerae* sp. and *Fibrobacteraceae* sp, the efficiency of biogas production from rice straw in anaerobic digester was increased by 9.3% when using the optimized operational condition [8]. Similarly, using a mesophilic microbial

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consortium consisting of 3 genera of bacteria, including *Bacillus, Providencia*, and *Ochrobactrum,* to decompose maize silage at 30°C and 37°C, it was found that the biogas production was increased by 38% [9]. Subcultivation and enrichment of microbial consortium obtained from swine manure were demonstrated to improve the biogas yield up to 40% produced from anaerobic digestion of lignocellulose [10].

However, on-site operation of anaerobic digester of cassava pulp usually faces with the problem of inconsistent efficiency and washing out of microbial consortium caused by inappropriate operational condition and overloading of cassava pulp, which subsequently resulted in the failure of digester systems. To solve the problem, the new microbial seeds could be re-inoculated in the digester, however it needs longer time for set up. Therefore, this research aimed to re-cultivate and enrich the cellulolytic microbial consortium obtained from cassava pulp and on-site digester to be used as inoculum seeds for using when the digester's failure occurs with the expected shorter set up time than an alien microbial consortium. The enzymatic hydrolysis efficiency of the re-cultivated microbial consortium enriched in different conditions were compared and characterized. Together with digestion capacity of cassava pulp and starch, the enriched microbial consortium was selected for further studies to apply in cassava processing plants.

2 Material and Methods

2.1 Culturing of microbial consortium

The microbial consortia in this study were collected from cassava pulp and digester wastewater in cassava processing plants located in Northeastern part of Thailand. 10 g of cassava pulp or 10 mL of wastewater were inoculated in 5% v/v of nutrient broth in a total volume of 50 mL. Each culture was incubated at 30°C and 50°C for 1 week. Next, each microbial consortium was taken in a sterilized micro-centrifuge tube that contained 15% glycerol and then kept at -40°C until use.

2.2 Compositional analysis of cassava pulp

The starch contents in cassava pulp samples were measured by using the Total Starch kit (Megazyme Ltd., Ireland), which followed the AOAC Official Method 996.11. Cellulose, hemicellulose, and lignin contents were determined according to Van Soest Method [11]. Total solid (TS) and volatile solid (VS) of cassava pulp samples were determined based on the methods in previous studies [12-14].

2.3 Preparation of cellulase enzyme from microbial consortium

100 μl of each microbial consortium from glycerol stocks were inoculated in 20 mL basal medium (containing 1% (w/v) rice straw or cassava pulp, 0.1%

$NaNo_3$, 0.1% K_2HPO_4, 0.1% KCl, 0.05% $MgSO_4$, 0.05% yeast extract, (pH 6.0) [15, 16]) or peptone cellulose solution medium (PCS, containing 0.5% (w/v) rice straw or cassava pulp, 0.5% peptone, 0.1% yeast extract, 0.5% NaCl, 0.2% $CaCO_3$, 0.5% filter paper, (pH 8.0) [17]) containing rice straw or cassava pulp as the carbon source. The mixture was incubated at 37°C and 50°C for 3 day in shaker incubator at 200 rpm and kept as a starter culture. Then 200 mL of basal medium or peptone cellulose solution medium was inoculated with 10% v/v of starting culture and incubated cultures again at 37°C and 50°C for 3 day in shaker incubator (200 rpm). Crude extracellular cellulase from each culture was collected by centrifuge at 6,000 rpm for 10 min at 4°C.

2.4 Purification of cellulase enzyme

The crude enzyme was precipitated by adding ammonium sulphate to 80% saturation and kept in 4°C for 24 h. The enzyme was collected by centrifuge at 6,000 rpm for 10 min at 4°C followed by dissolved in 5 mL of 50 mM Sodium Phosphate buffer (pH 7.0.0). Then, the sample was desalted in a 10 kDa MWCO dialysis membrane and dialysis was conducted at 4°C with 3 changes of buffer every 2 h. The dialyzed sample was concentrated protein by using Vivaspin™ spin columns with 10 kDa MWCO.

The molecular weight of the partially purified enzyme was analysed by using 12% gel SDS-PAGE analysis staining with Coomassie Brilliant Blue R-250 and by comparing with the standard protein marker (BLUeye Protein Ladder). The endoglucanase activity was examined by using CMC-zymogram. The concentrated enzyme was separated in 12% gel SDS-PAGE (containing 1% CMC). Then, SDS content in gel was washed out by soaking the gel in washing buffer (50 mM sodium phosphate buffer containing 40% isopropanol (pH 7.0.2)) for 1 h and transferred the gel into renaturing buffer (50 mM sodium phosphate buffer, 5 mM β-mercaptoethanol, 1 mM EDTA (pH 7.0.2)) at 4°C overnight. Then, the gel was stained with 1% Congo red solution for 15 min and washed with 1 M NaCl solution. The zymogram pattern of cellulase appeared as a clear zone band in red color background [15].

2.5 Enzyme activity assay

Hydrolysis reactions to test the enzyme activities were set up by mixing the tested enzyme samples with three types of substrates, including CMC, rice straw and cassava pulp with loading ratio at 5% w/v in 50 mM sodium phosphate buffer. The reactions were conducted in waterbath at 50°C for 60 min and the reactions were stopped by heating at 100°C for 5 min. Then, the supernatant of the reaction was separated by centrifugation at 12,000 rpm for 5 min [18]. The reducing sugar contents as the hydrolysis products were quantitated by using dinitrosalicylic acid (DNS) method [19], which the absorbance of each reaction was

measured at wavelength 540 nm. Three replications were conducted for each experiment.

2.6 Biomass digestion

The selected microbial consortia were grown in fresh broth media as described earlier. Then, two types of substrates, including cassava pulp and cassava starch were mixed with prepared microbial consortia at 10% w/v loading ratio. The reactions were set up in 50 mM sodium phosphate buffer at different pH and temperature. In this study, the pH was tested at 5 and 7, and temperature was tested at 30°C and 50°C. The anaerobic digestion tests were conducted for 7 days in waterbath. After digestion, the remaining solid biomass was collected by centrifugation at 6,000 rpm for 15 min, and the dried biomass weight of each sample obtained from drying in hot air oven at 60°C was recorded.

3 Results and discussion

3.1 Microbial consortium from cassava industry

Within the targeted processing plant in this work, there are two separated digesters which were operated at controlled temperature at 30°C and 50°C representing the mesophilic and thermophilic conditions, respectively. In general, mesophilic condition is operated in the range of temperature at 25-35°C [20], while thermophilic condition is conducted in the range of 50°C and above [21]. In this study, a total of 8 bacterial consortia was obtained from re-cultivation of cassava pulp and wastewater in anaerobic digester of cassava processing plant by re-cultivation in 30°C and 50°C (Table 1).

Table 1. Microbial consortium obtained from the cassava processing plant.

No.	Source	Re-cultivated temperature
1	wastewater in mesophilic digester	30°C
2	wastewater in mesophilic digester	50°C
3	cassava pulp lot.1	30°C
4	cassava pulp lot.1	50°C
5	wastewater in thermophilic digester	30°C
6	wastewater in thermophilic digester	50°C
7	cassava pulp lot.2	30°C
8	cassava pulp lot.2	50°C

3.2 Composition of cassava pulp

Cassava pulp composition was analysed and summarised in Table 2. Starch was the main polysaccharide in cassava pulp at 42.31%. Cellulose, hemicellulose, and lignin contents were 84.93%, 0.50%, and 10.83%, respectively. Total solid and volatile solid were 14.96% and 97.59%. A high starch content in solid waste reflected the low efficiency of starch extraction process in cassava processing factory.

Table 2. Compositional analysis of cassava pulp.

Component	% Content
Starch	42.31
Total solid (TS)	14.96
Volatile solid (VS)	97.59
Fiber content	
Neutral detergent fiber (NDF)	96.06
Acid detergent fiber (ADF)	95.56
Cellulose	84.93
Hemicellulose	0.50
Lignin	10.83

3.3 Enzyme activity

In this work, 8 re-cultivated microbial consortium, No.1-No.8, were cultured in basal media and PCS media supplemented with 2 types of carbon sources, including cassava pulp and rice straw. This experimental design resulted in 4 formulas of media, designated (i) Basal media + rice straw (Br), (ii) Basal media + cassava (Bc), (iii) PCS media + rice straw (Pr), and (iv) PCS media + cassava (Pc). A total of 32 crude enzyme samples from cultures of 8 microbial consortia was harvested from extracellular fraction, partially purified and concentrated. Each enzyme sample was tested for cellulolytic activity with 3 types of lignocellulosic substrate, including CMC (Fig. 1), rice straw (Fig. 2) and cassava pulp (Fig. 3). The efficiency of cellulase enzyme was compared based on the hydrolysis product in the form of reducing sugar [22-23].
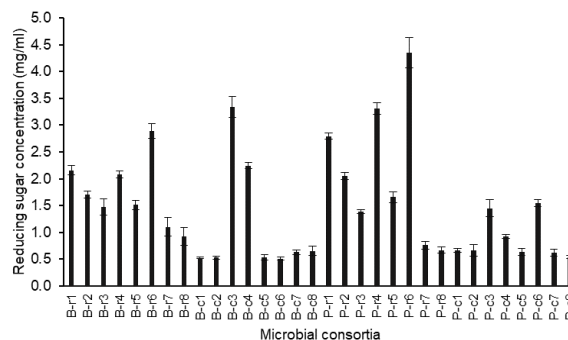

Fig. 1. Cellulolytic activities with CMC substrate of enzymes extracted from re-cultivated microbial consortia.

Testing with CMC substrate, the reducing sugars obtained from a total 32 samples were ranged from 0.51-4.35 mg/mL (Fig. 1). The highest and the lowest sugar yields were obtained from Pr-6 and Bc-6, respectively. Using rice straw as a carbon source in basal medium and PCS media, the cellulase activities were higher than using cassava pulp as a carbon source. This result suggested that the type of carbon source was the key parameter to determine the enzyme efficiency.

When the substrate was changed to rice straw, the trend of enzyme activities was also changed. The reducing sugars obtained from a total 32 samples were ranged from 0.71-2.61 mg/mL (Fig. 2). The highest and

the lowest sugar yields were obtained from Br-1 and Pc-8, respectively. Interestingly, the cellulase activities of all 8 microbial consortia were higher when the consortia were cultured in basal media supplement with rice straw as a main carbon source. The same pattern was also observed in PCS media that, in term of carbon source, rice straw induced the cellulase activity better than cassava pulp.
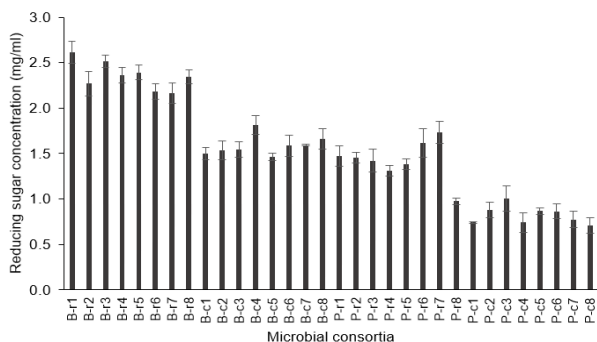

Fig. 2. Cellulolytic activities with rice straw substrate of enzymes extracted from re-cultivated microbial consortia.

Using cassava pulp as a substrate of hydrolysis reaction, the pattern of enzyme activity was similar to the other two substrates. The reducing sugars obtained from 32 samples were ranged from 0.92-12.88 mg/mL (Fig. 3). The highest and the lowest sugar yields were obtained from Pr-7 and Bc-5, respectively. Based on a previous study on the selection of suitable microbial consortium, isolated from swine manure, cattle manure and cassava pulp, for biogas production from cassava pulp, it was found that microbes obtained from cassava pulp produced the most biogas yield [24]. Similarly, another study reported that using of microbial strains isolated from original habitat enhanced the capacity of biogas production because these microbes were acquaintance to the environment and substrate [25].
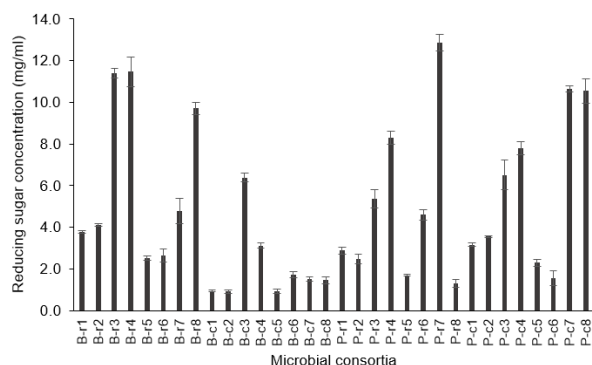

Fig. 3. Cellulolytic activities with cassava pulp substrate of enzymes extracted from re-cultivated microbial consortia.

3.4 SDS-PAGE and CMC-zymogram analysis

The molecular size of partially purified enzyme was analyzed by SDS-PAGE and CMC-zymogram analysis. In this experiment, Pr-6 sample was selected as a representative for endoglucanse activity because it had the highest activities in CMC substrate (Fig. 4). The

partially purified enzyme of Pr-6 was separated in CMC-zymogram gel to confirm CMCase activity *in situ* (Fig. 4). In SDS-PAGE, the result showed that a single protein band was 130 kDa (L1) and at the same position in Congo red background, the clear zone band of cellulase active was also detected (L2). This result suggested that a 130 kDa protein in concentrated enzyme has CMCase activity.
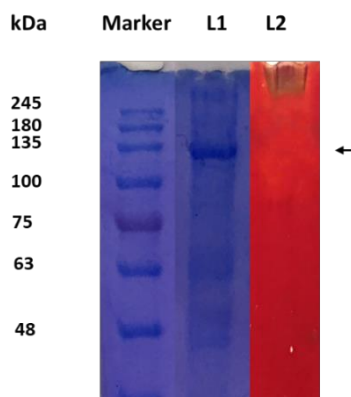

Fig. 4. Analysis of endoglucanase activity of partially purified enzyme isolated from Pr-6 by SDS-PAGE (L1, stained with Coomassie Blue R-250) and CMC-zymogram (L2, stained with Congo Red).

3.5 Cassava pulp degradation

Degradation capacities of cassava pulp and cassava starch by three selected microbial consortia including Br-3, Br-4, and Pr-7 , as the highest enzyme activities in cassava pulp (Fig. 3), were monitored at working condition at pH of 5.0 and 7.0 and temperature of 30°C and 50°C (Fig. 5-6) with anaerobic condition.
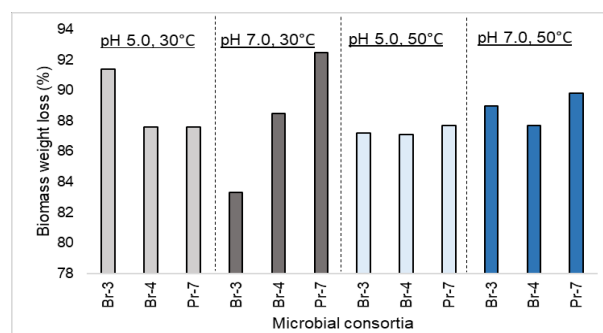

Fig. 5. Capacities of cassava pulp degradation by Br-3, Br-4, and Pr-7 within 7-day period at different pH and temperature.

Br-3, Br-4, and Pr-7 were originally obtained from cassava pulp samples and were re-cultivated at 30°C, 50°C and 30°C respectively. Using cassava pulp as a substrate, the biomass degradation capacities were ranged between 83.26%-92.54% (Fig. 5). Interestingly, the degradation capacities of three consortia in 4 different working condition were not really different, suggesting that these tested working condition could not clearly improve cassava pulp degradation. However, the best condition in this experiment was at pH of 7.0 and 30°C by using the Pr-7 consortium with

92.54% of biomass loss. This finding supported the idea of habitat adaptation and acquaintance because Pr-7 consortium was re-cultivated from original habitat at 30°C.

Likewise, cassava starch was also tested as a substrate for biomass degradation in anaerobic condition. The biomass degradation capacities were ranged between 9.83%-18.43% (Fig. 6). The best condition in this experiment was at pH of 7.0 and 30°C by using the Br-4 consortium with 18.43% of biomass loss. It is clear that the starch degradation capacities by selected microbial consortia were relatively less than cassava pulp degradation. It could be hypothesized that all microbial consortia used in this experiment, Br-3, Br-4, and Pr-7, were grown in media supplemented with lignocellulose, e.g. rice straw. During culture enrichment, they produced cellulolytic enzyme to digest lignocellulose and consumed those hydrolytic products to grow. Therefore, these consortia had better capability to degrade cassava pulp than cassava starch.
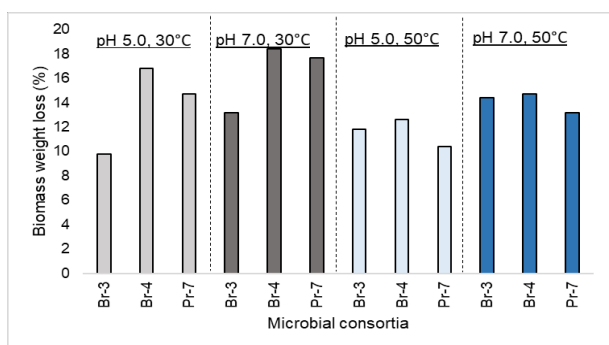

Fig.6. Capacities of cassava starch degradation by Br-3, Br-4, and Pr-7 within 7-day period at different pH and temperature.

4 Conclusion

In this study, a total of 8 microbial consortia isolated from cassava pulp and digester wastewater of cassava processing plant was re-cultivated and enriched in different culturing conditions, including types of carbon source, pH and temperature. The activities of cellulase enzymes obtained from these 8 cultivated microbial consortia were improved for approximately 9, 3, and 13 times when using CMC, rice straw and cassava pulp substrates, respectively. Additionally, the optimal condition for cassava pulp digestion was at pH 7.0 and 30°C, which was the original condition of habitat of tested consortium. Altogether, these results suggested that the working condition and culture enrichment parameters were important factors to determine the efficiency of re-cultivated microbial consortia in biomass degradation.

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