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Short Communication

Efficient saccharification for non-treated cassava pulp by supplementation of *Clostridium thermocellum* cellulosome and *Thermoanaerobacter brockii* β-glucosidase

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ABSTRACT

Cassava pulp containing 60% starch and 20% cellulose is a promising renewable source for bioethanol. The starch granule was observed to tightly bind cellulose fiber. To achieve an efficient degradation for cassava pulp, saccharification tests without pre-gelatinization treatment were carried out using combination of commercial α-amylase with cellulosome from *Clostridium thermocellum* S14 and β-glucosidase (rCglT) from *Thermoanaerobacter brockii*. The saccharification rate for cassava pulp was shown 59% of dry matter. To obtain maximum saccharification rate, glucoamylase (GA) from *C. thermocellum* S14 was supplemented to the combination. The result showed gradual increase in the saccharification rate to 74% (dry matter). Supplementation of GA to the combination of commercial α-amylase, cellulosome and rCglT is powerful method for efficient saccharification of cassava pulp without pretreatment.

1. Introduction

The production of fuel bioethanol from a renewable photosynthetic biomass has currently drawn an attention. There are many advantages for such bioethanol: (1) a variety of raw materials can be used, (2) it is non-toxic and (3) it is easily to introduce into the existing infrastructures (Maki et al., 2009). However, the sustainable and economically feasible path is still hindered. Cassava pulp, produced in large amounts as a by-product of starch manufacturing, is a major biomass resource in Southeast Asian countries and therefore has potential as a feedstock for fuel ethanol production. It was reported to contain up to 60% (on a dry weight basis) starch together with cellulose (20% on a dry weight basis), which both can be used as substrates for ethanol conversion (Kosugi et al., 2009). Until now, all reported conventional processes to convert cassava pulp into ethanol involve pre-gelatinization to liberate the trapped starch, saccharification of starch with amylolytic enzymes and fermentation to ethanol by yeast (Rattanachomsri et al., 2009; Kosugi et al., 2009; Hermiati et al., 2012). Even though, this process works efficiently, it consumes high energy and cost, and does not release sugars from cellulose fiber. Only one reported the simultaneous starch saccharification and cellulose hydrolysis in cassava pulp by the enzymes combination as a pretreatment prior to ethanol fermentation was by Rattanachomsri et al. (2009). They suggested the application of multi-enzyme system containing cellulase, pectinase, β-glucosidase, hemicellulase, glucoamylase and α-amylase for saccharification of 4% cassava pulp to obtain fermentable sugars equivalent 91.2% of the theoretical yield of available glucose. This research showed possibility of using both starch and cellulose fiber in cassava pulp for ethanol fermentation.

In attempts to further improve the cassava pulp saccharification efficiency, bacterial cellulosomes has been thought as an alternative of typical fungal cellulase for cellulose fiber hydrolysis. There are several reasons: it is expressed in multi-enzyme complexes providing increased function and synergy, its carbohydrate-binding domain increases binding stability to cellulose and sometimes catalyzes the disruption of non-covalent interactions between cellulose chains (Maki et al., 2009). Cellulosomes, found in several anaerobic microorganisms, are large, extracellular enzyme

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complexes consisting of non-catalytic scaffolding and catalytic subunits. Among the cellulosomes-producing microorganisms, *Clostridium thermocellum* has the most potent cellulose-degrading ability. *C. thermocellum* S14, having high cellulose-degrading ability, was isolated from bagasse paper sludge (Tachaapaikoon et al., 2012). Its cellulosome was reported to possess faster degradation of microcrystalline cellulose and 3.4- and 5.6-fold greater Avicelase activity than those from *C. thermocellum* ATCC27405 and JW20 (ATCC31449), respectively, and resistant to the inhibition by cellobiose, a major end product of cellulose hydrolysis. Therefore, it could be of advantageous to investigate whether the combination of *C. thermocellum* S14 cellulosome with other relevant enzymes would improve the efficiency of cassava pulp saccharification. In order to avoid the feedback inhibition of cellulosome by cellobiose, its major end product, the recombinant β-glucosidase from *Thermoanaerobacter brockii* was also added. This recombinant enzyme has been shown to remarkably improve the cellulose hydrolysis activity (Waeonukul et al., 2012). Moreover, recombinant glucoamylase from *C. thermocellum* S14 was used instead of commercial glucoamylase to hydrolyze maltose into glucose.

In this report, we describe an improvement of dry cassava pulp saccharification through *T. brockii* β-glucosidase, *C. thermocellum* S14 glucoamylase and commercial α-amylase in combination with *C. thermocellum* S14 cellulosome compared with commercial fungal cellulase. The ethanol fermentation from saccharified liquid was also performed.

2. Methods

2.1. Substrates, strains, media, growth conditions and commercial enzymes

Cassava (*Manihot esculenta* Crantz) pulp was obtained from Sanguan Wongse Starch Industries (Thailand). Dry pulp was prepared by oven-drying wet cassava pulp at 50 °C for 3 days. Then, it was ground, sieved through a 40 mesh screen and stored in plastic bags at 4 °C. The hyper cellulolytic strain, *C. thermocellum* S14 has been deposited with the National Institute of Technology and Evaluation Patent Microorganisms Depositary (NPMD; Chiba, Japan) as NITEP-627. *T. brockii* ATCC33075 was obtained from the American Type Culture Collection (Manassas, Virginia, USA). *Escherichia coli* DH5α (TAKARA BIO, Shiga, Japan), BL21 (DE3), and plasmids pET19b (Merck KGaA, Darmstadt, Germany) served as the cloning host, expression host, and vector, respectively. *E. coli* cells were grown at 37 °C in Luria–Bertani (LB) medium containing ampicillin (50 μg/ml) prepared according to Waeonukul et al. (2012). The ethanol fermenting yeast, *Saccharomyces cerevisiae* TISTR 5339, was purchased from the yeast collection of Thailand Institute of Scientific and Technological Research (TISTR). Celluclast® 1.5 L containing cellulase activity from *Trichoderma reesei* ATCC 26921 and Liquozyme® SC DS containing α-amylase activity from *Bacillus licheniformis* were obtained from Novozymes A/S, Denmark. All chemicals used in this study were purchased from Sigma Chemical Co., USA.

2.2. Preparation of cellulosome and recombinant glucoamylase from C. thermocellum S14

Cellulosomes were prepared from cell-free broths using *C. thermocellum* S14 grown culture in BM7CO medium supplemented with 10 g/l microcrystalline cellulose powder (Sigmacell type-20; Sigma–Aldrich, St. Louis, USA) for 4 days at 60 °C and recovered by the affinity digestion method (Tachaapaikoon et al., 2012). Preparation of chromosomal and plasmid DNA, and transformation were carried out according to standard procedures or supplier protocols (Qiagen, Frederick, MD, USA). Recombinant β-glucosidases (rCglT) from *T. brockii* ATCC33075 was prepared in modified DSMZ 122 medium pH 7.0 (supplemented with 5 g/l cellobiose) according to Waeonukul et al. (2012). All BM7CO and DSMZ 122 media were degassed in boiling water and bubbled with high purity carbon dioxide gas. DNA sequence encoding glucoamylase (GA) was amplified by PCR using *C. thermocellum* S14 DNA as a template. The primers were designed to add a *Nde*I (Takara Biotechnology Co. Ltd.) site to the 5′ end and a *Bpu*1102 (Takara Biotechnology Co. Ltd.) site at the 3′ end. The forward primer C.thermof15F1(NdeI) 5′-GGAATTCCATATGGCGAACACATACTTT-3′ and the reverse primer C.thermof15R1(Bpu1102) 5′-ATTGCTCAGCT AAAACCCCCGCCTCTTT-3′ were used. The GA-encoding fragments were inserted between the *Nde*I and *Bpu*1102 sites of pET19b to generate pET19b-C.thermoGA. The C.thermoGA was purified through a two-step purification using a Bio-Scale™ Mini Profinity™ IMAC cartridge (5 mL, Bio-Rad, USA) and Bio-Scale™ Mini Bio-Gel® P-6 Desalting Cartridge (50 mL, Bio-Rad, USA). The two columns were respectively attached to the Profinia™ Protein Purification System (Bio-Rad, USA). The purification was performed according to the instruction of the Profinia™ native IMAC purification and buffer kit (Bio-Rad, USA).

Optimum pH and temperature of *C. thermocellum* S14 GA was determined using 0.5% (w/v) soluble starch as substrate. A 1.0 mL reaction mixture consisting 20 μL enzyme, 0.5% (w/v) soluble starch, 100 mM varied buffers was incubated for 30 min at varied temperatures. The amount of released glucose was measured by the Glucose Assay Kit (Mutarotase-GOD method; Glucose C2, Wako Pure Chemical Industries, Ltd., Japan). Determination of GA activity was based on measurement of the release of glucose from maltose. One unit of enzyme releases 1 μmol equivalent of glucose per min. *C. thermocellum* S14 GA was cloned and successfully expressed in *E. coli* BL21(*DE3*). Its optimum pH and temperature were shown to be at pH 6.0 and 60 °C, respectively.

2.3. Saccharification of dry cassava pulp using commercial amylase, GA, cellulosome, and rCglT

The saccharification was performed in 10 mL reactions containing 5% dry cassava pulp in 50 mM sodium acetate buffer pH 6.0. The enzymes mixture contained *T. brockii* rCglT (20 U β-glucosidase/g dry cassava pulp), *C. thermocellum* S14 GA (20 U glucoamylase/g dry cassava pulp) and Liquozyme® SC DS (55 U α-amylase/g dry cassava pulp) in combination with *C. thermocellum* S14 cellulosome (6 mg/g dry cassava pulp) or Celluclast® 1.5 L (6 mg/g dry cassava pulp). The reaction was incubated at 60 °C for 96 h with 120 rpm shaking. The reaction without enzymes loading was used as a control. Samples were collected at time intervals for analysis of released reducing sugar.

2.4. Ethanol fermentation of dry cassava pulp

After 48 h of enzymatic saccharification of 5% dry cassava pulp, the reactions were centrifuged at 10,000*g* to separate liquid and solid. The liquid, containing sugar solution, was further fermented with a working volume of 4 mL, containing 5% DM of *S. cerevisiae* TISTR5339 suspension. Glucose solution was prepared to serve as a fermentation control. The fermentation was incubated at room temperature (30 °C) with 120 rpm shaking for 48 h. The samples were periodically withdrawn for analysis of glucose by HPLC and ethanol by GC as described below.

2.5. Analysis

Starch content in cassava pulp was estimated using the total starch assay kit following manufacturer's instructions (Megazyme, Ireland). The contents of lignin, α-cellulose, holocellulose and pentosan were determined using the Technical Association Pulp and