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Synergistic effects of cell wall degrading enzymes on rheology of cassava root mash

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ABSTRACT

Control of rheological characteristics of substrates is important in ethanol fermentation, particularly in a very high gravity (VHG) process. In this study, the synergism of cell wall degrading enzymes on rheology of cassava root mash was studied under VHG condition. The root mash exhibited a shear-thinning behavior. Crude enzyme mixture from *Aspergillus aculeatus* showed a dose-dependent effect on reduction of complex viscosity and storage modulus of mash. The enzyme showed strong synergism with *Trichoderma reesei* cellulase and *Aspergillus* pectinase leading to marked reduction in mash viscosity compared with individual enzymes. The optimal mixture comprising the three enzymes at a 1:1:1 ratio at 0.05 mg protein/g dried solid led to a remarkable decrease of complex viscosity to 1.01 Pas at 15.79 rad/s compared with 832.40 Pas of the control after incubation at 45 °C for 2 h. Synergism could be related to complementary activities of glycosyl hydrolases from different microbial sources, according to proteomic analysis. Simultaneous saccharification and fermentation of the pretreated mash by *Saccharomyces cerevisiae* led to a final ethanol concentration of 15.47% (w/w) corresponding to 88.14% theoretical yield. The work showed for the first time the enzymatic modification of cassava root mash rheology related to degradation of cell wall polysaccharides.

1. Introduction

Very High Gravity (VHF) processes provide an effective strategy for production of fermentation products with higher batch productivity and improved process economics [1,2]. When applied to fuel ethanol production, increased ethanol concentrations can be achieved by the VHG method, which therefore leads to savings in water use and distillation cost with higher outputs at a given plant capacity. However, processing starchy substrates under VHG conditions containing a high total dissolved solid content (>300 g/L) needs good understanding of the rheological properties of the starting material characterized with high viscosity as a basis for reactor and piping design as well as for efficient control of the fermentation process.

Rheological studies are performed to elucidate the deformation and flow properties of materials in response to applied forces. These studies are particularly important for determining the property of high solid content substrates used for the fermentation conditions in VHG processes. In particular, a suitable range of substrate viscosity is required to ensure optimal performance of enzymatic and microbial processes related to mass and heat transfers. Substrates composed mainly of starch and lignocellulosic materials are viscous because of the swelling characteristic and high water holding capacity of plant cell wall polysaccharides [3,4]. A variety of cell wall degrading enzymes from various microbial sources have been used for reduction of substrate viscosity in VHG fermentation, particularly substrates from cereal grains [5–9]. A mixture of commercial cellulases, hemicellulases and β-glucanases from different

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fungal origins was reported to have a cooperative action on viscosity reduction of vinasse from wheat [10]. Root and tuber mashes are more viscous than grain mashes owing to their high pectin contents, which entrap water in the cell wall matrix. Different combinations of crude and recombinant enzymes degrading cellulose, xylan, mannan and pectin have been shown to reduce the viscosity of root mashes to varying degrees [4,11–13].

Cassava (*Manihot esculenta*) is one of the cost-effective substrates for biofuel production [14] owing to its high crop productivity and starch content, with potential for production of commodity chemicals and plastic monomers [15]. So far, most rheological studies on cassava have focused on native or modified starches for determining their gelling properties in food applications [16,17] while the study on rheological properties of cassava mash related to its processing under VHG conditions has not been reported. A complex enzyme mixture containing a variety of plant cell wall degrading enzyme has been demonstrated on their efficiency on non-thermal saccharification [18] and viscosity reduction [19] of cassava substrates. In this present study, the synergism of crude enzyme from *Aspergillus aculeatus* with cellulolytic and pectinolytic enzymes from different microbial sources on modification of cassava mash rheology was investigated. The basis on enzyme synergism was shown by complementation of hydrolytic activities and composite enzyme components revealed by proteomic analysis. The study aimed to provide a better understanding on the effects of enzymatic treatment on rheological behavior of cassava root mash and provides a more highly efficient enzyme candidate with marked improvement in viscosity reducing efficiency than the previously reported single enzyme for ethanol industry.

2. Materials and methods

2.1. Materials, microbial strains and enzymes

Fresh cassava roots were obtained from the Chonchareon Co. factory (Chonburi, Thailand). The roots were peeled manually, chopped, and homogenized in a blender. The chemical compositions of cassava roots including moisture, crude fiber, protein, ash, sand and starch content (by polarimetric method) were analyzed according to the Standard for Tapioca Starch [20]. The acidity was analyzed using the Association of Official Analytical Chemists (AOAC) method [21]. Neutral and acid detergent fibers (NDF and ADF) and lignin were analyzed using the standard AOAC method [22]. The amount of sugars (measured as water soluble non-starch carbohydrate) in cassava roots was estimated as the total carbohydrate content in 80% ethanol extractant by the phenol-sulfuric method [23], according to Hall et al. [24]. The galacturonic acid in cassava roots was quantified as the uronic acid content by colorimetric method using dimethylphenol, according to Theander 1995 [25]. *A. aculeatus* BCC17849 isolated from forest soil was obtained from the BIOTEC Culture Collection, Thailand (www.biotec.or.th/bcc) and maintained on potato dextrose agar (PDA). The crude cell wall degrading enzyme from *A. aculeatus* (A) was prepared by solid state fermentation in a production medium containing 3.5:1.5 (w/w) of dried cassava pulp: soybean meal, supplemented with 20 g/kg yeast extract, 20 g/kg peptone, and 250 mL/kg salt solution (40 g/L KH_2PO_4, 90 g/L Na_2HPO_4, and 6 g/L KCl), and 10 mL/kg trace element solution (14.3 g/L $ZnSO_4{\cdot}H_2O$, 0.5 g/L $NiCl_2{\cdot}6H_2O$, 2.5 g/L $CuSO_4{\cdot}5H_2O$, and 13.8 g/L $FeSO_4.H_2O$). The cultivation was performed in a tray fermenter system according to Poonsrisawat et al. [19]. *T. reesei* cellulase (Cellulase C (C)) and *Aspergillus* pectinase (Pectinase C (P)) were obtained from Siam Victory, Inc. (Bangkok, Thailand). The starch-degrading enzymes, α-amylase (EC 3.2.1.1; Liquozyme SC DS) and glucoamylase (EC 3.2.1.3; Spirizyme Fuel) were supplied by Novozymes A/S (Bagsvaerd, Denmark). Polysaccharides used as substrates in enzymatic activity analysis were obtained from Sigma-Aldrich. Active dry yeast *Saccharomyces cerevisiae* (Thermosacc® Dry; Lallemand Ethanol Technology, Duluth, GA) was used for ethanol production.

2.2. Enzyme activity assay

Polysaccharide degrading activities were analyzed based on the amount of liberated reducing sugars using the 3,5-dinitrosalisylic acid method [26]. One-hundred microliter reaction mixtures contained the appropriate dilution of enzyme in 50 mM sodium acetate buffer, pH 5.0 and 1% (w/v) of the corresponding substrate: carboxymethyl cellulose for CMCase activity, Avicel PH101 for avicelase activity, birchwood xylan for hemicellulase activity, pectin from citrus for pectinase activity as polygalacturonase (PG), β-glucan for β-glucanase activity, locust bean gum for mannnase activity, and soluble starch for amylase activity. The reaction was incubated at 45 °C for 30 min. Cellulase activity, expressed as filter paper unit (FPase), was analyzed in a 1 mL reaction using Whatman number 1 filter paper (size 1 × 6 cm) as the substrate and incubated at 45 °C for 60 min. The amount of reducing sugars was determined from the absorbance measurement at 540 nm and interpolated from a standard curve of the corresponding sugar. β-Glucosidase, β-xylosidase, β-galactosidase, α-arabinosidase, and α-arabinofuranosidase activities were determined using *p*-nitrophenyl-β-D-glucopyranoside (PNPG), *p*-nitrohenyl-β-D-xylopyranoside (PNPX), *p*-nitrophenyl β-D-galactopyranoside, *p*-nitrophenyl α-L-arabinopyranoside and *p*-nitrophenyl-α-L-arabinofuranoside (PNP-α-L-Ara*f*) as the substrates, respectively [27]. Reactions contained 0.1% w/v of the respective substrates in 50 mM sodium acetate buffer, pH 5.0 at 45 °C for 30 min before terminated by addition of 100 mM Na_2CO_3. The quantity of *p*-nitrophenolate was quantitated spectrophotometrically at 405 nm. One unit (U) was defined as the amount of enzyme which produced 1 μmole of reducing sugar or *p*-nitrophenolate in 1 min under the assay conditions. Pectin esterase activity was analyzed by determining carboxyl groups released by titration using 0.02 M NaOH according to the method modified from Kertez 1955 [28]. Total protein concentrations of the crude enzyme extracts were determined using Bradford's method with the BIO-RAD protein assay reagent (BioRad, Hercules, CA, USA) using bovine serum albumin as the standard. The experiments were performed in triplicate.

2.3. Enzymatic modification of cassava mash rheology

The enzymatic effects on viscosity reduction of cassava root mash were studied under a high solid condition for VHG fermentation. The solid content of cassava root mash was adjusted to 32% (w/w) on dried weight basis with tap water. The substrate were adjusted to pH 5.0 and treated with 0.02–0.30 mg protein of the individual enzymes or enzyme mixtures per g dry matter at 45 °C with 160 rpm mixing. Samples were collected after 2 h of enzyme treatment for viscosity measurements.

Rheological measurements were performed using a controlled stress rheometer (Bohlin CVO, Malvern Instruments Ltd., Worcestershire, UK), fitted with a parallel plate system (25 mm diameter) at a gap size of 2 mm. Small amplitude oscillatory shear rheology was carried out using frequency sweep test over the frequency range of 0.1–20.0 rad/s at 25 °C. Data Analysis software built into the instrument was used to obtain the experimental data and to calculate the complex viscosity (η^*, a measure of overall resistance to flow) and storage modulus (G′, a measure of elastic response). The apparent viscosity of the substrate was also analyzed using a Rapid Visco Analyzer (RVA 4, Newport Scientific, Macquarie Park, NSW, Australia). The mixture was incubated at 45 °C for 2 h with agitation

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