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Soybean waste (okara) as a valorization biomass for the bioethanol production

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ABSTRACT

Okara, an agricultural waste product that is generated in abundance by the soy industry, has tremendous potential for use as renewable biomass rather than being disposed off in landfills or incinerated. We investigated the feasibility of using okara as a raw material for the production of bioethanol using hydrolytic enzymes produced in-house and analyzed its content in fermentable sugars such as glucose and galactose, which can be fermented by *Saccharomyces cerevisiae*. We also performed a comparative study of the activities of hydrolytic enzymes produced in-house from various fungal species on okara biomass. The in-house enzymes were produced from fungi using okara as a carbon source and tested on okara biomass for their hydrolytic activity. The okara biomass was used raw or pretreated in an autoclave (moist heating) for 20 min at 121 °C. The chemical compositions content of raw and autoclaved biomass exhibited little difference; however, the enzymatic conversion rate increased significantly from 21.9% for the raw okara to 82.9% for the pretreated okara. The ethanol conversion yield (based on sugar content) from enzymatic hydrolysis after *S. cerevisiae* fermentation was 96.2%.

1. Introduction

The increasing demand for alternative energy resources such as biofuel has led to increasing interest in biomass as an important renewable source as an alternative to conventional fossil fuel resources. The demand for biofuel production from biomass and the use of food crops as biomass caused increased cost of raw materials leading to higher food prices. An opportunity, therefore, exists to shift the use of agricultural waste streams to renewable resources [1–3].

Approximately 200 billion tons of agricultural waste are produced annually by food processing industries, creating significant environmental concerns [4]. Much of the agricultural waste that is produced has no current use other than disposal in landfill or first generation recycling such as composting or use as animal feed. This waste contains high levels of nutrients [5], which can cause serious environmental problems associated to odors and leachates in particular. Often these waste materials are simply disposed off by dumping in landfills or in the ocean. Waste disposal in landfills is becoming increasingly expensive and, in many parts of the world as the available land area is limited. Improved agricultural waste utilization would have a positive impact on environmental and economic problems, produce new growth, and help to achieve a zero waste society [6,7].

Okara is a major agricultural waste that is generated from the processing of soymilk, tofu, and their derivatives. The production of 1000 L of soy beverage can result in 250 kg of okara. Based on soy beverage consumption, approximately 14 million tons of okara are estimated to be generated annually worldwide, with associated environmental problems [8]. In addition, okara spoils and putrefies naturally when not refrigerated because it has high water and protein contents [9]. Many studies have examined ways to use okara, some of which have involved developing okara as a processed food for humans. Several studies on okara have focused on its fermentation [10,11], its use for the extraction and purification of protein or oil [12,13], and its enzymatic digestion for animal feed [14] or mushroom medium.

Okara contains 50% carbohydrate, consisting of cellulose, hemicellulose, and pectin, which make it a biomass that is attractive for bioethanol production. The carbohydrates in okara include

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fermentable sugars such as glucose, galactose, and mannose. Okara also contains pectin which is a complex polymer consisting of galacturonic acid bound by many different monosaccharides, such as arabinose and rhamnose. Despite the high carbohydrate content relative to other biomass, information on the use of okara in ethanol production is limited. Enzymatic degradation of okara and soybean has been reported previously [15], but successful enzymatic hydrolysis and fermentation of okara have been briefly reported [16,17].

In this study, we investigated bioethanol production from okara, including an autoclave pretreatment, and identified enzymes that enable an efficient hydrolysis process. We also determined the effect of autoclave pretreatment on the chemical composition of okara and the efficiency of hydrolytic enzymes produced in-house.

2. Materials and methods

2.1. Raw material and pretreatment

Okara (moisture content 79%) was kindly supplied from local market (Gwang-ju, Korea). Okara was lyophilized at −20 °C and stored until further use. Okara was treated using the autoclave equipment for 20 min at 121 °C. The effect of pretreatment was evaluated the amount of sugar release during enzymatic hydrolysis.

2.2. Chemical composition analysis

The chemical composition (protein, fat/oil, ash) of okara on a dry weight basis was analyzed using the A.O.A.C method [18]. Monosaccharide composition rate of raw and pretreated okara were treated using a modified alditol acetate method, and the neutral sugar contents were measured by gas chromatography. Thirty milligrams of each raw and pretreated sample was treated with 0.25 mL of 72% H_2SO_4 for 45 min at room temperature and diluted with distilled water to 4% H_2SO_4, followed by autoclaving for 1 h at 121 °C. The solution contained *myo*-inositol as an internal standard and was neutralized with ammonia solution until reaching pH 7.0. Sodium borohydride solution (1 mL, 0.5 M in dimethyl sulfoxide) was added. Glacial acetic acid (0.1 mL, 18 M) was added to degrade the sodium tetrahydroborate. To ensure that the acetylating process was complete, 0.2 mL of methyl immidazol and 2 mL of anhydrous acetic acid (catalyst) was added. Finally, 5 mL of deionized water and 2 mL of dichloromethane were added. Gas chromatography analysis conditions were as follows: gas chromatography (GC-2010, Shimadzu, Japan) using a DB-225 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness, J&W) operated with He, injector temperature of 220 °C, FID (flame ionization detector) at 250 °C, and oven temperature programming at 100 °C for 1.5 min, 5 °C/min to 220 °C [19].

2.3. Enzyme preparation

Commercial cellulase (C8546, Sigma) and pectinase (Pecinex Ultra SPL, Novozyme) were prepared to determine okara hydrolysis. *Aspergillus flavus* (KCTC 6405), *Penicillium verruculosum* (KCTC 6116), and *Trichoderma longibrachiatum* (KCTC 6507) were purchased from the KCTC (Korean Collection for Type Cultures), and *Penicillium funiculosum* (KACC 41346) was obtained from the KACC (Korean Agricultural Culture Collection). The lyophilized fungi were revitalized on potato dextrose broth with 1.2% (w/v) agar PDA (potato dextrose agar) and incubated for spore production for 10 days at 30 °C. One hundred mL of PDB (potato dextrose broth) was sterilized in 500 mL Erlenmeyer flasks. The medium contained 10 g/L okara as carbon source. The other components was (in g/L); 40, peptone; 24, KH_2PO_4; 5, $(NH_4)_2SO_4$; 4.7, $K_2C_4H_4O_6 \cdot 4H_2O$; 2, urea; 1.2, $MgSO_4 \cdot 7H_2O$ and (in mg/L) 10; $ZnSO_4 \cdot 7H_2O$, 9.28; $MnSO_4 \cdot 7H_2O$, 8.74; $CuSO_4 \cdot 7H_2O$ with 1 mL Tween 80. The pH was adjusted to 4.0 using hydrochloric acid. The medium was sterilized at 121 °C for 15 min. Cultures were conducted in a 5 L fermenter (FMT-05, Fermentec, Korea) equipped with a 3 L working volume at 180 rpm for 10 days at 30 °C (pH 4.0). The produced culture broth was centrifuged, and the supernatant was stored at 4 °C.

2.4. Enzyme activity and hydrolysis

Two commercial enzymes and four enzymes, produced in-house from *A. flavus*, *P. funiculosum*, *P. verruculosum*, and *T. longibrachiatum*, were evaluated. The protein concentration was measured by the Lowry method using bovine serum albumin as standard [20]. Enzyme activities were assayed with specific substrate solution, 50 mM sodium citrate buffer pH 4.8 (at 37 °C for 30 min). Endoglucanase (CMCase) and exoglucanase (Avicelase) activities were determined with 1% carboxymethylcellulose and microcrystalline cellulose (Avicel) as substrate, respectively. Xylanase activity was determined by the same procedure described for endo- and exo-cellulase, but using beechwood xylan (Sigma) as substrate. Polygalacturonase acitivity was measured with a 0.5% polygalacturonic acid (Sigma) in 50 mM sodium citrate buffer (pH 4.8) at 37 °C for 5 min. Reducing sugars were quantified with DNS (dinitrosalicylic acid) and the absorbance was measured at 540 nm by modified Miller [21] using Thermo Scientific Multiskan EX (Thermo Fisher Scientific, Finland). One unit of activity was defined as the amount of enzyme required to release one μmol of glucose, xylose or galacturonic acid per min. Specific activities were expressed as enzyme units per milligram of protein.

Commercial and extracellular enzymes were added to okara at concentration of 40–140 ug protein/g okara. Enzymatic hydrolysis was performed on 1% (w/v) dry matter with 50 mM sodium acetate buffer (pH 4.8) at 180 rpm for 72 h at 37 °C. Optimization of enzyme loading volume and time course change okara during enzymatic hydrolysis were measured using colorimetric method (DNS) for determination of sugars. Based on a small scale study, the enzymatic hydrolysis process was scaled up to a 5 L bioreactor (1% dry matter).

2.5. Vacuum evaporation and fermentation

To increase the fermentation efficiency, low concentrated fermentable sugar solutions from hydrolysate were vacuum evaporated. The vacuum evaporation process was undertaken in a vertical stainless reactor equipped with an electronic heater on the bottom [22]. Samples were evaporated at 60 °C under a pressure of 0.04 Mpa for 4 h, after which the water samples were removed. As a consequence of evaporation, a low sugar concentration solution was separated into a high-level sugar solution in the reactor.

After obtaining concentrated hydrolysate, yeast fermentation process was conducted. *Saccharomyces cerevisiae* KCTC 7906 was obtained from the KCTC (Korean Collection for Type Cultures) and activated in YPD (yeast peptone dextrose) media which contained (w/v) 0.5% yeast extract, 0.5% peptone, 2% dextrose. Okara fermentation experiment was performed in a fermenter, FMT-05 (Fermentec, Korea) with prepared fermentation culture medium (pH 5.0) excluding glucose [5 g/L yeast extract, 5 g/L peptone, 3.3 g/L KH_2PO_4, 0.2 g/L $(NH_4)_2SO_4$, and 0.4 g/L $MgSO_4$]. The fermenter containing medium was autoclaved at 121 °C for 15 min. The yeast cell seed culture 10% (v/v, 6.9×10^7 yeast cell/mL) was transferred to fermentation medium (pH 5.0) and incubated at 300 rpm for 36 h at 30 °C. Samples were withdrawn aseptically from the fermenter periodically from 0 to 36 h to analyze ethanol production. The sample supernatants were analyzed for soluble sugar and