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Microbial consortia for saccharification of woody biomass and ethanol fermentation

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HIGHLIGHTS

- It reports newly isolated *Sistotrema brinkmannii* and *Agaricus arvensis* producing high levels of lignocellulase.
- The mixed fungal culture exhibits a higher lignocellulase activity than each monoculture.
- Optimization of saccharification by response surface methodology exhibits the highest saccharification of plant biomasses.
- The co-culture of *Saccharomyces cerevisiae* and *Pichia stipitis* produces more ethanol than that produced by each mono culture.

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ABSTRACT

Newly isolated *Sistotrema brinkmannii* and *Agaricus arvensis* were co-cultured using rice straw (20 g/L) as a carbon source and yeast extract (10 g/L) as a nitrogen source to produce high levels of cellobiohydrolase (12.6 U/mL), β-glucosidase (21 U/mL), and endoglucanase (16.4 U/mL). The filter paper activity (FPU) of the mixed fungal culture was enhanced (1.61 FPU/mL) compared to that of *S. brinkmannii* (0.3 FPU/mL) or *A. arvensis* (0.5 FPU/mL) monoculture. Enzyme loading, substrate concentration, pH, and temperature were optimized by response surface methodology (RSM) to improve the saccharification yield of alkali-pretreated plant biomasses. The highest enzymatic hydrolysis (76.7%) was obtained from *Pinus densiflora* under the following conditions: crude enzyme loading 22.5 FPU/g-substrate, substrate 3.75%, temperature 35 °C, and pH 5. The enzymatic hydrolyzate of pretreated *P. densiflora* was used for ethanol production using *Saccharomyces cerevisiae*, *Pichia stipitis*, and a co-culture of both the strains. The co-culture of *S. cerevisiae* and *P. stipitis* produced 23% more ethanol than that produced by *S. cerevisiae* alone and 38% more ethanol than that produced by *P. stipitis* alone. This study shows the potential of exploiting a microbial consortium for the cost-effective production of cellulases for bioethanol processes.

1. Introduction

Lignocellulosic biomass is an abundant, renewable source of carbohydrates for microbial conversion to chemicals and fuels. Due to the heterogeneity and complexity of lignocelluosic biomass, bioconversion requires multiple enzyme activities [1]. Cellulases are the key enzymes recruited for the bioconversion of lignocellulosic biomass to useful products. They consist of three major components: endoglucanase (EG; EC 3.2.1.4), exoglucanase (EC 3.2.1.91), and cellobiase or β-glucosidase (BGL; EC 3.2.1.21), which act synergistically to convert the complex carbohydrates present in a lignocellulosic biomass into glucose [2]. Due to a diverse range of applications, cellulases play a pivotal role in the commercialization of industrial enzymes [3]. However, the cost of cellulase enzymes remains a major concern for the commercialization of lignocellulosic biomass ethanol processes. Significant work to reduce the cost of cellulases has focused on improving the efficiency of known cellulases, identifying new, more active cellulases, creating multi-component cellulase systems optimized for selected pretreated substrates, and minimizing the costs of cellulase production [4,5]. In spite of these efforts, the bioconversion of lignocellulosic biomass continues to be challenged by its low conversion efficiency.

A variety of microorganisms, including fungi and bacteria, degrade cellulosic biomass to glucose monomers. Most bacteria, however, cannot degrade crystalline cellulose because their cellulase systems are incomplete [1]. However, fungi are well known cellulase producers that can utilize inexpensive and surplus lignocellulosic raw materials as the chief carbon source under various conditions [6], and thus are useful in the saccharification

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of renewable, pretreated lignocellulosic materials. Many fungal species, such as *Trichoderma*, *Penicillium*, and *Aspergillus*, are able to produce large amounts of extracellular cellulases [7]. *Trichoderma reesei* is the most widely employed fungus for the production of cellulolytic enzymes, and it has been extensively studied. However, research showed that a single component cellulase or a cellulase from pure cultures could not convert a highly ordered polymer into a monomer efficiently [8,9]. The improvement of microbial and enzymatic processes in lignocellulosic biomass conversion is thus an important area of research in sustainable "green" biotechnology. Microbial consortium, or the mixing of several types of enzymes that act cooperatively, has been proposed as an efficient approach for the production of antibiotics and several types of fermented food and for the bioconversion of apple distillery waste and domestic wastewater sludge [10]. Mixed fungal cultures have many advantages compared to monocultures, including improved productivity, adaptability, and substrate utilization [7]. In a previous study, co-culturing improved the fungal cellulolytic activity of *T. reesei* and *A. niger* [11]. The cellulases from *T. reesei* usually have high levels of endo- and exoglucanase components but lower BGL levels, whereas *A. niger* has high BGL activity but lower endoglucanase levels, and hence it has limited efficiency in cellulose hydrolysis. Therefore, it is appropriate to mix cellulolytic cocktails derived from different sources in order to obtain satisfactory yields of sugars after enzymatic hydrolysis.

Glucose and xylose are the two dominant sugars in lignocellulosic hydrolyzates. Both need to be fermented efficiently for economic ethanol production [12]. Current approaches are inefficient, since no native microorganism can convert all sugars into ethanol at a high yield [13]. Therefore, microorganisms that can efficiently convert both hexoses and pentoses to ethanol would be more useful. *Saccharomyces cerevisiae* is the most applied and traditional microorganism for ethanol production. It has a high ethanol tolerance, as well as high yields and rates of fermentation. However, its inability to ferment xylose, the second most abundant sugar in nature, limits its use in biofuel production. *Candida shehatae, Pachysolen tannophilus*, and *Pichia stipitis* are capable of fermenting xylose [12–14]. By co-culturing *S. cerevisiae* with one of these microorganisms in an appropriate ratio, it could be possible to obtain the desired yield of ethanol from lignocellulosic hydrolyzates. The objective of this study was to construct an efficient cellulolytic enzymes-producing consortium and to evaluate statistical methods with which to optimize the enzymatic hydrolysis of an alkali-pretreated *Pinus densiflora* biomass, following ethanol production by co-culture of yeast.

2. Materials and methods

2.1. Isolation of microorganisms

Soil samples collected from Sorak Mountain (South Korea) by the capillary tube method were diluted in sterile dilution solution (0.9% saline). Aliquots were spread on potato dextrose agar (PDA) plates, and the plates were incubated for 13 days. The initial screening of cellulase producing fungi was carried out in agar plates containing 0.5% carboxymethyl cellulose (CMC). Based on the zone of clearance observed by Congo red staining, strains were selected to analyze cellobiohydrolase (CBH), BGL, and EG activity. The different colonies were inoculated into 50 mL of growth medium containing 8 g/L peptone, 2 g/L yeast extract, 5 g/L KH_2PO_4, 5 g/L K_2HPO_4, 3 g/L $MgSO_4{\cdot}7H_2O$, 0.005 g/L thiamine·HCl, and 20 g/L microcrystalline cellulose (Sigma). The colonies were cultivated at 30 °C with agitation at 180 rpm for 10 days.

2.2. Identification of microorganisms

The fatty acid composition was analyzed by performing gas chromatography (Agilent 6890N, CA, USA), and the identity of the isolated strain was determined using the MIDI database. For sequence analysis, the ITS1-5.8 S-ITS2 rDNA region of the fungus was amplified by performing PCR using the primer set pITS1 (5′-TCCGTAGGTGAACCTGCCG-3′) and pITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [15]. The 604 bp and 566 bp amplicons thus obtained were cloned and sequenced. The sequences were submitted to GenBank with accession numbers HM004552 (*Agaricus arvensis*) and HQ717718 (*Sistotrema brinkmannii*). Pairwise evolutionary distances and a phylogenetic tree were constructed using the MEGA 4 software [16].

2.3. Growth conditions and production of enzyme

The fungal strains were routinely sub-cultured after 4 weeks and stored at 4 °C on PDA plates. A 500 mL flask containing 50 mL of potato dextrose broth (PDB) was used for seeding the culture. For enzyme production, *A. arvensis* and *S. brinkmannii* were inoculated at a ratio of 5:1 into above mentioned growth medium (20% v/v). The effects of the carbon or nitrogen sources on cellulase production and filter paper unit (FPU) activity were investigated after 7 days of cultivation in flasks (25 °C and 180 rpm) containing medium composed of 20 g/L of the carbon sources (cellulose, rice straw, avicel, cellobiose, CMC, maltose, sucrose, lactose, and glucose) and 10 g/L of various nitrogen sources (peptone yeast extract, corn steep powder, tryptone, ammonium sulfate, ammonium chloride, urea, and potassium nitrate).

The effects of temperature and pH on enzyme production were analyzed in a 7-L fermenter by varying the growth temperatures (20–40 °C) and pH values (3.5–7.0). For fermenter culture, the mycelia of *A. arvensis* and *S. brinkmannii* were inoculated into 100 mL of potato dextrose broth separately. Pre-cultures (20% v/v) were inoculated into 3 L of medium in a 7-L fermenter at a ratio of 5:1 (*A. arvensis*:*S. brinkmannii*) in final production medium.

2.4. Pretreatment of the biomass and hydrolysis conditions

The biomass samples were procured from Phygen Co. Ltd. (Daejeon, Korea). Five different selected lignocellulosic biomasses (*Catalpa ovata, Populus nigra, P. densiflora, Populus tomentiglandulos*, and *Pinus radiata*) were used as the substrates for hydrolysis. The wood chips were milled using a laboratory hammer mill. Milled material was further separated (size reduced to approximately 2–50 mm) using a portable sieve shaker. The wood chips were screened to remove all the particles greater than 35 mm and less than 6 mm in length to ensure smooth operation in disk milling. The thickness of the accepted chips ranged from 2 mm to 6 mm. The biomass was dried in an oven at 70 °C until the weight was constant. Then the biomass was stored in an airtight container at room temperature. Ten grams of biomass sample and 40 mL of NaOH (2.0 wt.%) solution were added to 200 mL Erlenmeyer flasks with a solid to liquid ratio of 1:4. Sodium hydroxide pretreatment was performed in an autoclave at 121 °C for 60 min. Pretreated biomass was recovered by filtration and washed with deionized water to remove excess alkali and dissolved byproducts that might inhibit enzymes in the subsequent hydrolysis. Then the pretreated biomass was dried in an oven at 70 °C until constant weight was observed. The pretreated biomass was either used immediately for hydrolysis experiments or stored in airtight containers at 4 °C until use. A typical hydrolysis mixture, which consisted of 0.2 g of substrate, 10 FPU of enzyme, and 10 mL of sodium acetate buffer (pH 5.0), was supplemented with the antibiotics tetracycline (40 μg/mL) and cycloheximide (30 μg/mL) to prevent any

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