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Phanerochaete chrysosporium pretreatment of biomass to enhance solvent production in subsequent bacterial solid-substrate cultivation

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ABSTRACT

This study investigated pretreatment of corn stover using solid substrate cultivation (SSC) of *Phanerochaete chrysosporium* (*P. chrysosporium*) to improve subsequent accessibility to cellulose. Thereafter, *Clostridium thermocellum* (*C. thermocellum*) was directly inoculated onto the pretreated biomass to accomplish hydrolysis, followed by solventogenesis by introducing *Clostridium beijerinckii* (*C. beijerinckii*).

An enzyme suite containing laccase, lignin peroxidase and manganese peroxidase activity was detected during the cultivation of *P. chrysosporium* on corn stover within 288 h at an initial moisture content $w_{H_2O} = 80\%$ (mass of water/total mass). Incubation factors, such as substrate moisture content and cultivation temperature affected the percent of lignin removal which ranged from 14.4% to 36.4% of the original lignin. Lignin removal increased as the cultivation of *P. chrysosporium* continued but was accompanied by increased cellulose loss. The 7-day fungal cultivation sufficiently delignified the corn stover for the subsequent processing. Approximately 25% of the original lignin was removed; however 18% of the initial cellulose was also removed with the lignin. The investigations of the effect of fungal pretreatment were extended to miscanthus, wheat straw and switch grass. The yield of reducing sugar produced by *C. thermocellum* on pretreated biomass was doubled compared with non-pretreated biomass, demonstrating that pretreatment resulted in a more accessible carbon source for the solvent-producing bacterium. The final comprehensive comparison between the pretreated biomass and non-pretreated biomass on the three-stage SSC for butanol production showed pretreatment by *P. chrysosporium* improved microbial utilization of lignocellulosic materials for solvent production by approximate 4–7 folds.

1. Introduction

The global energy crisis has spurred interest in producing alternative biofuels from clean, renewable feedstocks via biological processes. Because of several desirable physical and chemical properties, butanol is currently a favored alternative to ethanol [1]. Butanol can be produced by an acetone, butanol and ethanol (ABE) fermentation via the anaerobic conversion of carbohydrates by *Clostridium* strains [2]. ABE fermentation was popular in the early 20th century. However, the ABE process eventually suffered from the high cost of

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conventional starch (maize, wheat, millet, etc.) or sugar (molasses) substrates, and was abandoned in favor of chemical synthesis of these chemicals. Current interest in biofuel production has stimulated research into the use of less expensive substrates [3,4] for the ABE fermentation. Being the most abundant renewable resource, lignocellulose is recognized as a promising feedstock for use in biofuel fermentation, provided that the cellulose components can be deconstructed and utilized efficiently [5]. Since the ability of butanol-producing bacteria to catalyze the complex lignocellulosic feedstocks is limited, lignin removal or modification followed by cellulose hydrolysis is required to convert the lignocellulose into simpler sugars prior to the butanol fermentation. Chemical pretreatment combined with enzymatic saccharification of agricultural residues has been adopted as the conventional method to generate reducing sugars prior to solvent production [6–8]. However, inhibitors present after pretreatment and the high cost of hydrolytic enzymes have hindered process industrialization [9,10]. These concerns motivated this study to develop a mild pretreatment with cost-effective hydrolysis for the ABE fermentation process.

Among the methods of pretreatment available, biological pretreatment with lignolytic enzymes is said to be superior to the current chemical and thermochemical methods in terms of energy intensity, environmental impact, and reduced production of chemicals toxic to fermentation microorganisms [11]. The enzymes commonly found in a lignolytic enzyme complex consist mainly of enzymes with lignin-degrading peroxidase activity (LiP; E.C.1.11.1.14), manganese peroxidase activity (MnP; E.C.1.11.1.13) and the lignin-degrading enzyme laccase activity (E.C.1.10.3.2). Some or all of these enzymes and their isozymes are produced by a number of wood-rotting fungi [12,13]. The white-rot basidiomycete, *Phanerochaete chrysosporium* (*P. chrysosporium*) is reported to have high lignolytic activity. This strain is considered to be a model strain for the development and understanding of the lignolytic enzyme production system because it can produce a more complete lignolytic enzyme complex than most other strains [13,14].

Following pretreatment, the cellulose hydrolysis step could be accomplished using commercial enzymes (predominately fungal enzymes), however an alternative approach was used in this study. The alternative approach involved cultivating the solvent-producing *Clostridia* with a microorganism that can enzymatically convert cellulose into reducing sugars. There have been several reports of co-cultivations [15–17] using a cellulolytic strain like *Clostridium thermocellum* (*C. thermocellum*), *Clostridium cellulolyticum* or *Bacillus thermoamylovorans* to produce cellulase for saccharification of lignocellulose or cellulose (rice straw with swine dung, cellulose or solka floc), followed by butanol production achieved by adding a solventogenic species such as *Clostridium acetobutylicum* or *Clostridium beijerinckii*. These studies report that sequential co-culture increased the total fermentation products formed from cellulosic substrate by 1.7–2.6-fold compared to *C. beijerinckii* monoculture.

The hypothesis for the current study was that a biological pretreatment followed by a sequential co-culture could efficiently utilize lignocellulosic substrate to produce solvents. To our knowledge, there are no previous studies investigating the effect of biological pretreatment on increasing substrate utilization and butanol yield, particularly for this co-culture process. Moreover, although sequential co-cultures have been investigated as a way of increasing the production of butanol using ideal feedstocks (pretreatment unnecessary), the studies were only performed in liquid fermentation. Solid substrate co-culture cultivation, which has been shown to have advantages over liquid cultivation [15], has not been previously conducted using sequential co-cultures for ABE production.

To address this research gap, the objective of this study was to develop an appropriate biological pretreatment method which would increase the fermentability of lignocellulosic feedstocks intended for subsequent co-culture; specifically to increase cellulose accessibility and solvent accumulation in solid substrate cultivation. The fungal pretreatment of corn stover using SSC of *P. chrysosporium* was investigated as a method to improve accessibility to cellulose in the pretreatment stage by preferentially degrading the lignin. Thereafter, *C. thermocellum* was directly inoculated onto the pretreated biomass to accomplish hydrolysis, followed by solvent production initiated by introducing *C. beijerinckii*. The study investigated the effect of cultivation conditions (moisture content of initial substrate and culture temperature) on the lignin removal by *P. chrysosporium* and then investigated the effects of lignin degradation on the subsequent fermentability by a bacterial co-culture, quantified by availability of carbohydrates and solvent production. A comprehensive analysis between fungal pretreated and non-pretreated biomass (corn stover, miscanthus, switch grass and wheat straw) on metabolite accumulation was performed to evaluate the effect of pretreatment on the solid substrate co-culture fermentation.

2. Materials and methods

2.1. Strain cultivation

The white-rot basidiomycete, *P. chrysosporium* strain (ATCC MYA-4764) was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained as a frozen culture (−80 °C) in 30% glycerol. Propagation of the organism for SSC was performed as described by Shi et al. [16]. Spore suspensions were prepared by washing the slant with 10 cm^3 of sterilized sodium acetate buffer (50 mmol dm^{-3}, pH 4.5). The final spore inoculum concentration was 5×10^6 spores cm^{-3}, determined using a hemocytometer.

C. thermocellum ATCC 27405 was obtained from ATCC and grown in basal medium that contained (per L) [17]: 1530 mg Na_2HPO_4, 1500 mg KH_2PO_4, 500 mg NH_4Cl, 500 mg $(NH_4)_2SO_4$, 90 mg $MgCl_2 \cdot 6H_2O$, 30 mg $CaCl_2$, 4000 mg yeast extract, 10 cm^3 standard vitamins [18], 5 cm^3 modified metals [18], 500 mg cysteine hydrochloride, 1 cm^3 resazurin, and 4000 mg sodium carbonate. The medium pH was adjusted to 6.7 with NaOH and maintained under a 100% carbon dioxide atmosphere by sparging with CO_2 followed by sealing the container. *C. beijerinckii* ATCC 51743 purchased from ATCC was used in this study. For seed culture preparation, stock cultures were heat-shocked at 80 °C for 10 min, and transferred anaerobically into