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First proof of concept of sustainable metabolite production from high solids fermentation of lignocellulosic biomass using a bacterial co-culture and cycling flush system

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HIGHLIGHTS

- First proof of cycling flush system applied in co-cultural SSC.
- Five different flushing strategies were investigated.
- Cycling via cellulolytic phase significantly improved glucose availability.
- Cycling via cellulolytic phase sustainably convert lignocellulose into products.
- Cycling process was simplified with no re-inoculation of *C. thermocellum*.

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ABSTRACT

To improve the lignocellulose conversion for ABE in high solids fermentation, this study explored the feasibility of cycling the process through the cellulolytic or/and solventogenic phases via intermittent flushing of the fermentation media. Five different flushing strategies (varying medium ingredients, inoculum supplement and cycling through phases) were investigated. Flushing regularly throughout the cellulolytic phase is necessary because re-incubation at 65 °C significantly improved glucose availability by at least 6-fold. The solvents accumulation was increased by 4-fold using corn stover (3-fold using miscanthus) over that produced by flushing only through the solventogenic phase. In addition, cycling process was simplified by re-incubating the flushed cellulolytic phase with no re-inoculation because the initial inoculum of *Clostridium thermocellum* remained viable throughout sequential co-culture. This study served as the first proof of the cycling flush system applied in co-cultural SSC and the knowledge gained can be used to design a farm-scale flushing system.

1. Introduction

The global energy crisis has spurred interest in producing alternative biofuels from clean, renewable feedstocks via biological processes. Lignocellulose is recognized as a promising feedstock for use in biofuel fermentation due to its abundance and renewability, provided that the lignin recalcitrance can be destructed and cellulose components can be utilized efficiently (Kumar et al., 2009). Butanol, used as a biofuel, is currently a favored alternative to ethanol (Nigam and Singh, 2011; Qureshi and Blaschek, 2001). Butanol can be produced via acetone–butanol–ethanol (ABE) fermentation by anaerobically cultivating *Clostridium* strains (Clark et al., 2012). Since butanol-producing bacteria do not have the ability to catabolize complex lignocellulosic feedstocks, lignin removal or modification followed by cellulose hydrolysis is required prior to butanol fermentation. Our previous study explored a biological pretreatment followed by a sequential co-culture of *Clostridium thermocellum* (*C. thermocellum*) ATCC 27405 and *Clostridium beijerinckii* (*C. beijerinckii*) ATCC 51743 to ferment ABE solvents from lignocellulosic substrate in batch solid substrate cultivation (SSC) (Yao and Nokes, 2014). Fungal pretreatment of corn stover using SSC of *Phanerochaete chrysosporium* (*P. chrysosporium*) ATCC MYA-4764 was investigated as a method to preferentially degrade the lignin. Thereafter, *C. thermocellum* was directly inoculated onto the pretreated biomass to enzymatically convert cellulose into reducing sugars, followed by solvent production initiated by introducing *C. beijerinckii*. *C. thermocellum* in SSC on fungal pretreated biomass degraded the substrate into a significantly higher yield of glucose (19–20 mg/g biomass) than cultures grown on

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non-pretreated biomass and consequently significantly higher yields of butanol (3 mg/g biomass) were generated by fermenting fungal pretreated biomass compared to the non-pretreated biomass (0.5 mg/g biomass). However, the batch SSC–ABE process still suffered from low yield of acids and solvents even using pretreated biomass. The batch SSC–ABE experienced high initial production rates for all fermentation products, but could not sustain these rates. This decrease in rate, and consequently yield, is undesirable as it offsets the advantages of working at high solids levels.

Several mechanisms behind the decreasing conversion in SSC or high solids substrate (high SS), such as osmotic stress, mass transfer limitations and product inhibition, have been proposed and investigated. Bacteria grown in SSC experience a reduced liquid phase and in addition the substrate is exceptionally hypotonic. A shell of hydration forms around the solid particles and the bacteria grow in this water layer. This shell of hydration may experience high concentrations of metabolites, which likely desiccate the bacterial cells by osmosis thus resulting in a decreased final yield and a slowing rate of conversion as the fermentation progresses (Dharmagadda et al., 2010). In addition, whatever extracellular enzymes that are available experience decreased enzyme activity due to the low availability of water (Gervais and Molin, 2003; Todd, 1972).

When *C. thermocellum* bacteria are grown in substrate-excess conditions, growth can be inhibited by the accumulation of fermentation products, either through enzymatic feedback inhibition or metabolic inhibition from high osmotic stress. Termination of growth due to enzymatic feedback inhibition, has been reported; Johnson et al. (1982) saw a 35% inhibition of *C. thermocellum* cellulase activity at 60 g/L of glucose (Johnson et al., 1982). However, product inhibition by glucose and cellobiose as enzymatic feedback inhibition at high solids loading has not fully accounted for the decreasing conversion. In previously established batch SSC–ABE processes, the level of glucose generated (4–5 g/L) by *C. thermocellum* is far below the reported critical levels when the inhibition occurred (Yao and Nokes, 2014).

Another type of inhibition is caused by hydrolysis sugars released from cellulose and hemicellulose present in SSC. The presence of water soluble sugars (glucose, xylose, etc.) acts to draw water away from insoluble substrate surfaces and could significantly reduce water activity near insoluble substrate surfaces (Selig et al., 2012). The lower water activity may in turn act to reduce the effectiveness of some enzyme systems, specifically enzyme adsorption at these substrate surfaces. Kristensen et al. (2009) found that the amount of adsorbed cellulases dramatically dropped at increasing solids substrate concentrations, with only approximately 17% of the added enzyme adsorbed at 30% solids filter paper as compared at 70% adsorbed at low solids. The drop in cellulose adsorption correlated to a decrease in cellulose conversion from 63% to 38%. Thus the inhibition of cellulase adsorption may be responsible for at least a portion of the decrease in yield in SSC.

In addition to sugars, acids generated by *C. thermocellum* and C. *beijerinckii* will also exacerbate the already unfavorable osmotic conditions of SSC by prohibiting the cells to maintain isotonic intracellular conditions. This combination (fermentation acids and low water content) has been applied throughout history to prevent microbial growth in food (*e.g.* cheese, fermented sausage) (Dharmagadda et al., 2010).

One advantage of using a SSC is that the products are produced at a higher concentration than in liquid fermentation. However, these inhibitory issues mentioned above may occur in the SSC process, particularly in the batch mode and do not allow for continuous product accumulation. To address this challenge, this study aims to modify the batch SSC–ABE fermentation developed in our previous study into a periodically flushed process during the cellulolytic or/and solventogenic phases. Our hypothesis is that intermittent flushing with fresh media will provide essential nutrients to the microorganisms while decreasing the osmotic stress and removing the fermentation acids, promoting cell metabolism and growth and thus achieving a higher extent of lignocellulosic biomass conversion. The study investigated five different flushing strategies by varying medium ingredients, re-inoculations after flushing, and the timing of the flushing relative to the fermentation phases. These processing variables were evaluated by quantifying the fermentability of lignocellulose by a bacterial co-culture of *C. thermocellum* and C. *beijerinckii*. Fermentability was quantified by the total amount of carbohydrates released, and acids and solvents produced.

2. Methods

2.1. Strain cultivation and inoculum preparation

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. (2009). Spore suspensions were prepared by washing the slant with 10 cm^3 of sterilized sodium acetate buffer (50 mM, pH 4.5). The final spore inoculum concentration was 5×10^6 spores/mL, determined using a hemocytometer.

C. thermocellum ATCC 27405 was obtained from ATCC and grown in basal medium that contained (per L) (Dharmagadda et al., 2010): 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 mL standard vitamins (Cotta and Russell, 1982), 5 mL modified metals (Cotta and Russell, 1982), 500 mg cysteine hydrochloride, 1 mL 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. For seed culture preparation, a bacterial culture from a −80 °C stock was grown for 24 h in Balch tubes at 60 °C containing 10 mL of basal medium supplemented with 4 g per liter cellobiose. This initial culture was then used to inoculate 80 mL of cellobiose-containing medium (4 g/L). After 15 h of growth, this secondary culture was diluted with fresh basal media (without cellobiose) to prepare the standard inoculum stock (final optical density of 0.143 OD_{600}; ~0.07 g dry cells/l) for use in flushed solid-substrate cultivation (FSSC).

C. beijerinckii ATCC 824 purchased from ATCC was also used in this study. Laboratory stocks of *C. beijerinckii* ATCC 824 were routinely maintained as spore suspensions in sterile double-distilled water at 4 °C. For seed culture preparation, stock cultures were heat-shocked at 80 °C for 10 min, and transferred anaerobically into Reinforced Clostridial medium (RCM, Difco Laboratories, Detroit, Mich.) at 35 °C for 24 h.

2.2. Biomass composition

The corn stover and miscanthus used in this study were harvested using a rotary disc mower at 10 cm above the ground from the University of Kentucky North Farm in 2011, windrowed and baled. Biomass samples consisted of the whole plant above ground (including stems and leaves), however grain had been previously removed from the corn stover. The biomass was stored in small square bales with $w_{H_2O} < 15\%$ for 9 months. The bales were ground to 5 mm, thoroughly mixing the plant tissue. Samples were air-dried and stored at room temperature.