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A versatile and robust aerotolerant microbial community capable of cellulosic ethanol production

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HIGHLIGHTS

- A cellulolytic aerotolerant microbial community was enriched from compost.
- Cellulolytic activity was observed in non-reduced as well as pre-reduced media.
- Ethanol and acetate were major fermentation products.
- Cellulolytic activity continued when sterile wastewater was provided as nutrient.
- The culture consisted of both facultative anaerobic and anaerobic members.

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ABSTRACT

The use of microbial communities in the conversion of cellulosic materials to bio-ethanol has the potential to improve the economic competitiveness of this biofuel and subsequently lessen our dependency on fossil fuel-based energy sources. Interactions between functionally different microbial groups within a community can expand habitat range, including the creation of anaerobic microenvironments. Currently, research focussing on exploring the nature of the interactions occurring during cellulose degradation and ethanol production within mixed microbial communities has been limited. The aim of this study was to enrich and characterize a cellulolytic bacterial community, and determine if ethanol is a major soluble end-product. Cellulolytic activity by the community was observed in both non-reduced and pre-reduced media, with ethanol and acetate being major fermentation products. Similar results were obtained when sterile wastewater extract was provided as nutrient. Several community members showed high similarity to *Clostridium* species with overlapping metabolic capabilities, suggesting clostridial functional redundancy.

1. Introduction

Fossil fuels are unsustainable, finite resources and their production and consumption has caused widespread environmental impacts and led to rapid climate change. With such a dramatic increase in the global energy demand over the last century, the complete depletion of fossil fuel reserves is predicted to occur within 50 years (Rodolfi et al., 2009). Consequently, there is an undeniable need for better renewable energy sources.

Bio-ethanol has garnered significant attention as a potential long-term replacement for fossil fuels. Currently, bio-ethanol is produced mainly from sugars derived from food crops such as corn and sugarcane. Because the use of edible crops for fuel production puts a burden on agricultural lands and contributes to rising food prices (Inderwildi and King, 2009), cellulose is an attractive alternative feedstock for bio-ethanol production, due to its abundance and renewability.

To date, much attention has been paid to utilizing pure cultures of anaerobic cellulose-degrading bacteria such as *Clostridium thermocellum* to overcome the challenges related to cellulose recalcitrance (Lynd et al., 2002; Xu et al., 2010). This organism is capable of simultaneously hydrolyzing cellulose and fermenting the resulting sugars to produce ethanol in a process known as consolidated bioprocessing (CBP) (Lynd et al., 2005). Pure culture systems, however, often persist within a narrow range of growth conditions (pH, temperature, oxygen content) and their activity

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may be altered by contamination from other microorganisms (Brenner et al., 2008).

Naturally-occurring microbial communities conversely, are adapted to exploit a wide variety of nutrient and energy sources, often as specialized consortia that utilize specific resources (Brenner et al., 2008). Cellulose utilization, for example, is commonly accomplished by microbial communities consisting of physiologically cooperative species. Consortia enriched from these natural communities can exhibit cellulolytic activity greater than the sum of their parts, and have been shown to perform more complex activities and persist within a wider range of environmental parameters than the separated members (Brenner et al., 2008; Kato et al., 2004).

Previous studies have aimed to characterize cellulose-degrading communities enriched from natural sources such as manure, paper mill waste, and various types of compost (Izquierdo et al., 2010; Okeke and Lu, 2011; O'Sullivan et al., 2005; Sizova et al., 2011; Zyabreva et al., 2001). Most often however, the communities described in such studies are cultivated under strict anaerobic conditions using pre-reduced media.

In order to improve the economic competitiveness of biofuels, efforts must focus on simplifying and streamlining their production, and reducing process-related costs. Anaerobic cellulose degraders such as *C. thermocellum* require strict anaerobic conditions, which necessitates the inclusion of reducing agents in the culture medium. On an industrial scale, this may represent a significant extraneous cost (Maddipati et al., 2011). The use of cooperative consortia enriched from natural cellulose-degrading communities that are aerotolerant and do not require pre-reduced media may help to overcome this limitation, as the diversity within them allows for efficient cellulolysis coupled with increased tolerance to environmental fluctuations (Kato et al., 2008; Okeke and Lu, 2011). Research focused on such aerotolerant communities has been somewhat limited with a relatively small number of studies describing cellulolytic consortia cultivated in non-reduced medium (Kato et al., 2004; Wang et al., 2011; Wongwilaiwalin et al., 2010).

Yeast extract (YE) is often a stimulatory constituent of media used to cultivate cellulose-degrading bacteria (Haruta et al., 2002; Kato et al., 2004; Miyazaki et al., 2008; Wongwilaiwalin et al., 2010), and is not a cost-effective option at industrial scale (Maddipati et al., 2011). The ability to replace YE with a low-cost and easily attainable waste-derived complex nutrient source, without compromising the cellulolytic activity of the culture, would help to circumvent such financial hurdle. It is also possible that cross-feeding of essential nutrients between community members may eliminate the requirement for such supplementation.

In addition to the applied relevance related to the search for improved cellulose hydrolysis, such research provides an opportunity to study microbial interactions when challenged with a recalcitrant substrate. The objectives of this study were therefore to: (i) enrich an aerotolerant cellulolytic consortium, (ii) obtain a time-resolved profile of selected soluble end-products in non-reduced (initially aerobic) and pre-reduced (anaerobic) media, as well as YE-free non-reduced media supplemented with either compost tea or wastewater from a municipal wastewater treatment plant, and (iii) obtain a time-resolved analysis of bacterial diversity within the consortium under these conditions.

2. Methods

2.1. Inoculum and enrichment conditions

Approximately 15 g of material from a household composter was added to 60 mL of non-reduced RM medium (Ozkan et al., 2001) containing urea (2 g/L), KH_2PO_4 (2 g/L), K_2HPO_4 (3 g/L), yeast extract (2 g/L), and the oxygen indicator resazurin (0.002 g/L). Whatman Grade 1 Qualitative Filter Paper (Whatman, Piscataway, NJ) was used as the cellulosic substrate and was added prior to autoclave sterilization. A filter-sterilized trace minerals solution containing $MgCL_2{\cdot}6H_2O$ (20 g/L), $CaCl_2{\cdot}2H_2O$ (5 g/L), and $FeSO_4{\cdot}7H_2O$ (0.25 g/L) was added after autoclave sterilization at a ratio of 1:100 (v/v). Incubation was done at 60 °C under static, initially aerobic conditions. Flasks were tightly capped to eliminate diffusion of oxygen into the culture. Over time, oxygen present in the headspace and medium at the beginning of the incubation period was depleted as a result of microbial activity, and was not replenished.

The resulting culture was sequentially transferred to fresh medium at a 1:5 ratio (v/v). Consistent and predictable cellulose-degrading activity (disappearance of filter paper) was observed through six enrichments. The sixth enrichment culture was used in subsequent bacterial diversity and end-product analyses.

2.2. Variation in growth medium composition and incubation conditions

Time-resolved analysis of selected end-products and microbial community structure was carried out in four different media. In addition to the non-reduced RM medium, experiments were also conducted with pre-reduced RM medium by the addition of L-cysteine hydrochloride monohydrate (Sigma–Aldrich, Oakville, ON) at a final concentration of 1 g/L, YE-free non-reduced compost tea RM medium, and YE-free non-reduced wastewater RM medium. Sealed 50 mL serum vials (Sigma–Aldrich, Oakville, ON) were used for anaerobic culturing, with the headspace in each evacuated by vacuum and flushed with grade 4.8 nitrogen (BOC Gasses, Mississauga, ON) for 30 s intervals until the resazurin had changed from pink to colourless.

YE-free non-reduced compost tea RM medium was used to assess the feasibility of replacing yeast extract with inexpensive sources of nutrients and growth factors. Compost tea was first prepared by combining compost and water at ~1:5 (w/v) and mixing for 30 min using a magnetic stirring-rod and stirrer at room temperature. After a 2.5 h settling period, the supernatant was gently pumped into a clean bottle using a Masterflex® Console Drive pump (Cole-Parmer, Montreal, QC). When preparing non-reduced compost tea RM medium, this compost tea was used as the solvent into which the various media components (excluding YE) were dissolved, prior to autoclave sterilization.

YE-free non-reduced wastewater RM medium was prepared in a similar fashion. Wastewater collected from the aeration tanks of the secondary treatment process were obtained from the Humber Wastewater Treatment Plant in Toronto, Canada. After mixing for 30 min at room temperature using a magnetic stirring-rod and stirrer, flocs and other solid particles were allowed to settle and the supernatant was gently pumped to a separate container and stored at 4 °C in the dark. When preparing non-reduced wastewater RM medium, the various RM medium components (excluding YE) were dissolved into this wastewater supernatant and autoclave-sterilized.

2.3. Experimental setup

Three millilitres of the enriched cellulolytic consortium grown in non-reduced RM medium was transferred to 27 mL aliquots of the respective media in 50 mL sealed serum vials and cultivated at 60 °C. Each 30 mL culture contained a small 80 mg (±3 mg) piece of Whatman Grade 1 Qualitative Filter Paper (Whatman, Piscataway, NJ), generating a cellulose concentration of 2.66 g/L. The consortium was also inoculated into cellulose-free controls of each