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Improving ethanol production from switchgrass using a symbiotic bacteria/yeast consortium

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1. ABSTRACT

A cooperative microbial consortium of the cellulolytic *Clostridium phytofermentans* and a cellodextrin fermenting yeast *Saccharomyces cerevisiae* cdt-1 was developed for consolidated bioprocessing of lignocellulosic materials to ethanol. Symbiotic cooperation was induced through diffusion of oxygen into the culture which acts to inhibit the growth of one consortium partner. When provided a soluble carbon source via cellulose hydrolysis, the yeast partner metabolizes oxygen relieving the inhibition. The consortium outperformed mono-cultures of each organism by producing over two times more ethanol from purified cellulose and 50% more from switchgrass.

2. INTRODUCTION

Consolidated bioprocessing (CBP) of lignocellulose is simultaneous biological enzyme production, hydrolysis and fermentation. In contrast to traditional approaches like simultaneous saccharification and fermentation (SSF), the capital costs and synergies of CBP are thought to make it a more economically feasible approach to biofuel production. CBP, however, requires an organism that is capable of efficiently performing all three steps and, to date, no such "super-bug" exists. A consortium (mixture) of organisms in which each provides one or more function may be a viable alternative. The goal of this work was to develop a controlled consortium and apply it to consortia-mediated bioprocessing of lignocellulosic materials to ethanol.

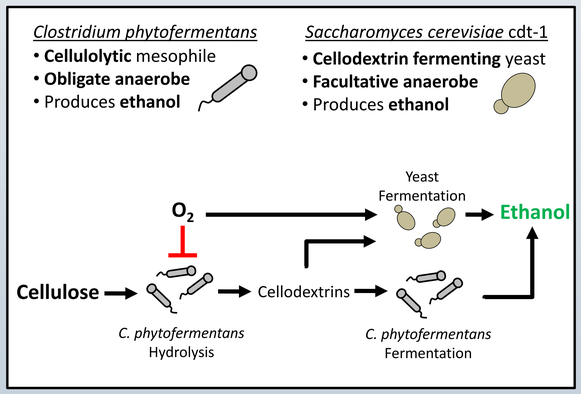


Figure 1. Top: Selected organism characteristics. Bottom: *C. phytofermentans*/yeast consortium design based on a mutualistic exchange of soluble cellodextrins and removal of a toxic inhibitor.

In the controlled consortium design ***C. phytofermentans* provides cellulolytic capability to convert cellulose to soluble sugars for conversion to ethanol by *S. cerevisiae* cdt-1**. Controlled diffusion of oxygen into the culture induces a symbiosis between the two organisms (Figure 1). *C. phytofermentans* relies on *S. cerevisiae* cdt-1 to remove oxygen in exchange for soluble carbohydrates liberated via cellulose hydrolysis.

3. MATERIALS & METHODS

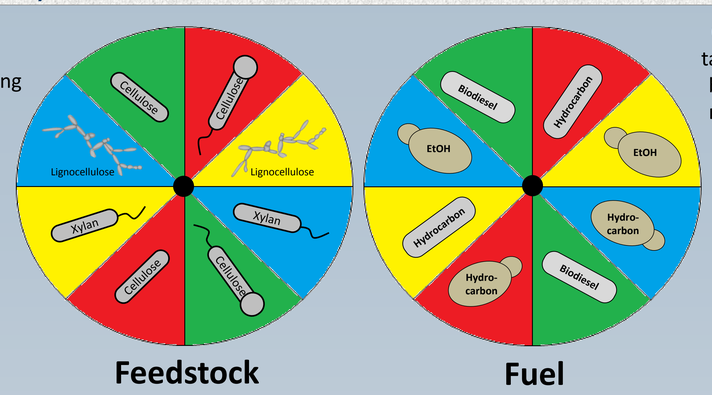
Controlled consortia were grown in 100 mL serum bottles with or without air flow through submerged neoprene (OD = 6.35 mm , ID = 2.9 mm, ~ 8 µmol/L hr) tubing (Figure 2). GS2 medium with ergosterol, tween 80 and glutathione was used for all consortium fermentation experiments. Reactors were incubated at 30˚C and 200 rpm. SSF of 100 g/L α-cellulose contained 400 mg/L (3.8 IU/mL) endoglucanase from *T. viride* and SSF of 100 g/L 2mm milled *P. virgatum* contained 1 mL of CTec 2 (Novozymes; approximately 2-5 FPU/mL).

Colony forming units (CFU) were determined by serial dilutions in PBS followed by selective plating on GS2 + Lactose under anaerobic conditions (*C. phytofermentans*) and ½ Sabouraud Agar under aerobic conditions (yeast). Ethanol and glucose concentrations were determined using an Agilent 1100 HPLC with an Aminex HPX-87H column and a Jasco RI detector.

Figure 2. Oxygen transfer reactor.

STEP RIGHT UP, SPIN THE WHEEL OF CONSORTIA-MEDIATED BIOPROCESSING

Spin the arrows, note which feedstock you will convert to which fuel using a dual-species microbial consortium.



Congratulations! You have just taken part in a new paradigm of biofuels production: **consortia-mediated bioprocessing** or the utilization of multiple defined microorganisms in an engineered interacting community.

Technologies to engineer and control microbial consortia could open up the possibility of feedstock and fuel choice based on consortium organism choice. Much as you have done here today, we envision a future in which we can "plug-and-play" with various microbes in a controlled consortium to convert lignocellulose to various fuels and chemicals.

4. RESULTS AND DISCUSSION

YEAST "PROTECTS" FROM OXYGEN INHIBITION

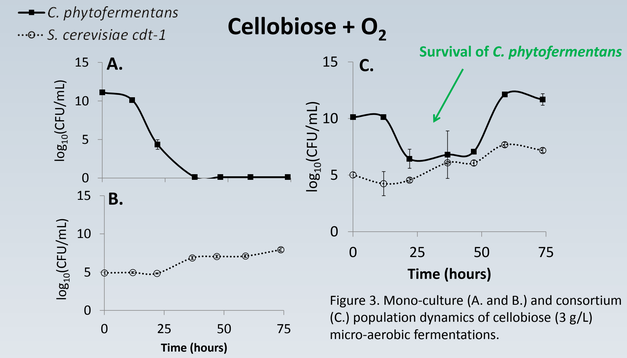


Figure 3. Mono-culture (A. and B.) and consortium (C.) population dynamics of cellobiose (3 g/L) micro-aerobic fermentations.

When grown on cellobiose with atmospheric oxygen *C. phytofermentans* does not survive (Figure 3 A) while yeast grows well (B). When co-cultured with yeast, *C. phytofermentans* recovers from the initial population decline and reaches a high final cell concentration (C). This demonstrates that **yeast provides respiratory protection to *C. phytofermentans* when a soluble carbon source is available**. Next it was necessary to demonstrate that the soluble substrate could be provide via cellulose hydrolysis.

CONSORTIUM HYDROLYZES CELLULOSE UNDER MICRO-AEROBIC CONDTIONS

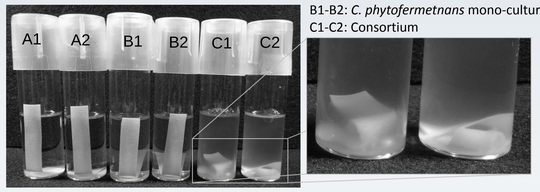


Figure 4. Semi-aerobic mono-cultures and co-culture in non-degassed GS2 medium with Whatman No. 1 filter paper. Photograph taken after 15 days of incubation at 30˚C with slight agitation. Zoom: Close up image of *C. phytofermentans*/ *S. cerevisiae* cdt-1 consortium cultures to show degraded filter paper.

Whatman No. 1 filter paper strips were placed, as the sole carbon source, in culture tubes that allow slow gas transfer. After 15 days only the consortium cultures degraded the paper strips (Figure 4). This demonstrates that **oxygen introduction establishes a symbiotic cooperation between the two organisms allowing cellulose hydrolysis under semi-aerobic conditions**. However, it appeared that cellodextrins *and* ethanol were being consumed by the yeast partner.

CONTROLLED OXYGEN DELIVERY PROMOTES SYMBIOSIS AND ETHANOL PRODUCTION

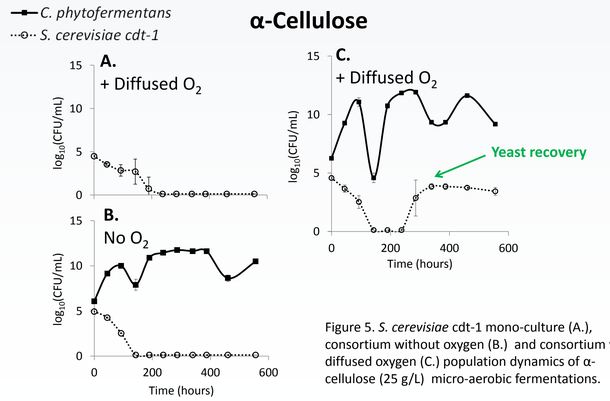


Figure 5. *S. cerevisiae* cdt-1 mono-culture (A.), consortium without oxygen (B.) and consortium with diffused oxygen (C.) population dynamics of α-cellulose (25 g/L) micro-aerobic fermentations.

To limit ethanol consumption while promoting the symbiosis, diffusive oxygen transfer was utilized. When grown both alone with oxygen (Figure 5 A) and in co-culture without oxygen (B) on α-cellulose, the yeast does not survive. However, when oxygen is diffused into the culture medium the yeast population recovers and the consortium reaches stable population levels (C). The oxygen transfer rates are not sufficiently high to allow ethanol consumption and thus the product is maintained. This demonstrates that ***C. phytofermentans* "feeds" yeast soluble substrate from cellulose** in return for "protection" from diffused oxygen and that **the symbiosis promotes conversion of cellulose to ethanol**.

4. RESULTS AND DISCUSSION CONT'D

CONSORTIUM FERMENTATIONS

The controlled consortium was applied to the conversion of 100 g/L α-cellulose and 100 g/L *P. virgatum* under simultaneous saccharification and fermentation conditions.

<u>α-Cellulose at 640 hours</u>

- **Consortium produced 30 g/L ethanol** compared to 12 g/L and 6 g/L for the yeast and *C. phytofermentans* mono-cutlures, respectively.

<u>Switchgrass at 300 hours</u>

- **Consortium produced almost 50% more ethanol** from un-treated switchgrass than either of the mono-cultures.

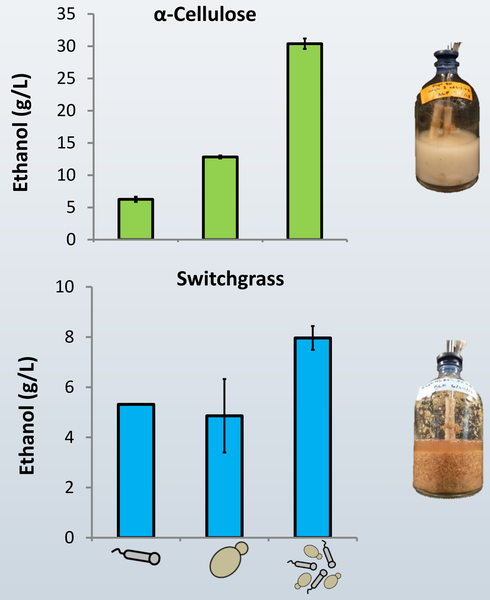


Figure 6. Top: 25 day fermentation of α-cellulose with endoglucanase (400 mg/L or 3.8 IU/mL). Bottom: 16 day fermentation of *P. virgatum* with Novozymes CTec2. *C. phytofermentans* mono-cultures were cultured anaerobically, *S. cerevisiae* cdt-1 mono-cultures and consortium cultures had oxygen transfer through 10 cm neoprene tubing.

5. CONCLUSIONS

1. Developed method for controllable, stable cultivation of a symbiotic consortium for conversion of cellulose to ethanol
2. Demonstrated benefit of consortium approach for switchgrass conversion to ethanol
3. Created a novel strategy for co-cultivation of microorganisms which could be applied to production of alternative fuel and chemical products

6. ACKNOWLEDGEMENTS

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