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PNNL-33008

Enhancing organic matter removal, product recovery, and hydrogen generation from fermentation wastewater

July 2022

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U.S. DEPARTMENT OF ENERGY

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Abstract

Existing ethanol biorefineries produce billions of gallons of wastewater (stillage) that must be treated extensively prior to discharge or concentrated in an energy intense evaporation step to produce animal feed as a value-added bioproduct. Yeasts and filamentous fungi were screened for growth and reduction of soluble organic compounds, carbohydrates, and protein from stillage to identify species capable of rapidly concentrating organic compounds into readily separable biomass in an attempt to concentrate the product using a much more efficient centrifugation step. Yeasts amenable to production of biomass derived products including bio-oils (*Yarrowia lipolytica* and *Rhodotorula pallida*), chitosan (*Aspergillus niger*) and animal feed (*Cyberlindnera jadinii*) were identified as well as microbes capable of efficient production of small organic acids with applications as bioderived polymers including 3-hydroxypropionic acid (engineered *A. niger*) and lactic acid using a co-culture of *Debaryomyces udenii* and *Lactobacillus pentosus*. The filamentous fungus *A. niger* eliminated all specifically measured soluble organics (glycerol, acetate, lactate, ethanol, and citrate) in under 48 hours and reduced total soluble protein content by 46% and total carbohydrate content by 83% within 96 hours, the greatest reduction in organic content observed. During this process an *A. niger* strain engineered to produce 3-hydroxypropionic acid achieved a yield of 0.35 C-mol 3HP / C-mol non-protein organics consumed at an overall rate of 0.09 g/Lh in unmodified stillage without pH control. Stillage treated by the fodder yeast *C. jadinii* as well as stillage containing acetic or lactic acid was found to be most suitable for growth by electrogenic bacteria with applications in production of electricity or hydrogen from wastewater. This work establishes fungal and bacterial strains appropriate for biological treatment of stillage to produce biomass derived products, soluble commodity chemicals, energy, and treated water to reduce the energy intensity and improve the economics of ethanol biorefineries.

Acknowledgments

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1.0 Introduction

Existing corn to ethanol biorefineries produce around 60 billion gallons per year of wastewater known as thin stillage after recovery of the ethanol by distillation. Thin stillage is typically acidic (pH 3-4), has a high percentage of undissolved solids (> 2%) and dissolved organic matter (5-10% COD), and must be treated extensively before it is safe to discharge using aerobic and anaerobic processes to reduce COD. Existing full-scale wastewater treatment of effluents from biorefineries typically includes a combination of anaerobic digestion and a two-stage extended aeration process [1]. Alternative processes used by the U.S. corn ethanol industry derive value from thin stillage through centrifugation and energy intense evaporation to produce water and concentrated syrup as a component of animal feed [2]. The shortfalls of existing technologies include fuel consumption for evaporation and incineration, power consumption for air, diffusion for aerobic oxidation of organic matter, dilution of effluent to meet the disposal standards, land requirement for lagoon disposal and potential pollution of groundwater and surrounding lands by leaching of pollutants from the aerated lagoon. Although, many technologies have been developed to treat fermenter wastewater, none have shown convincing results to achieve safe and economically feasible disposal.

Reduction of soluble COD and reclamation of nutrients are the primary goals of stillage treatment. Stillage is a particularly nutrient-rich “waste stream” that has value as animal feed [3] if it can be suitably concentrated for transport and can be processed to produce a variety of value added products including oils, phytate, glycerol, or used as a feedstock for bioconversion processes [2]. The pH, chemical composition, and undissolved solids present in stillage are well suited for biological processing, particularly with fungi capable of rapid growth on mixed carbon sources at acidic pH. Biological treatment of biorefinery wastewater has the potential to reduce the dissolved and suspended organic matter in stillage from existing upstream fermentation processes and produce a value-added biosolids stream separable by low-energy methods such as sedimentation or light centrifugation. Fungi like *Aspergillus awamori*, [4] and other fungi capable of secreting enzymes that enable break down and utilization of the carbon and nitrogen from the solids found in biorefinery effluents like thin stillage [5-8] will enable production of biosolids rich in chitin, proteins, vitamins, or lipids as an additive to existing animal feed streams or for production of bio-oil.

Direct production of electricity using a microbial electrochemical cell (MEC) is another promising technology to derive value in the form of clean energy from aqueous carbon streams of heterogeneous composition such as stillage. Electrogenic microorganisms are capable of producing H_2 from short chain fatty acids (the major component of many bioprocess waste streams) at 80% efficiency, which is favorable to the 60-70% efficiency achieved with water electrolysis. However, functionality of the MEC is sensitive to dissolved and undissolved solids present in many bioprocess waste streams. The use of an upstream fungal system in conjunction with a MEC in a two-stage integrated process will allow the reduction of COD and concentration of nutrients into readily removeable value-added biomass followed by the production of electricity or hydrogen [9], and the efficient purification of water that can be bolted on to existing facilities today as a module and integrated into future biorefinery designs. This approach improves upon the existing biorefinery economics and energy efficiency by reducing energy intense evaporation steps associated with nutrient concentration and mitigates the cost of wastewater cleanup.

2.0 Experimental Methods

2.1 Cultivation conditions

Stillage was a generous gift from Solar Spirits (Richland, WA). Yeasts and filamentous fungi were cultivated on stillage or post-centrifuge supernatant (thin-stillage) in 24-well plates or 125 mL shake-flasks at 30C, 200 rpm that were either sealed with a foil film (plates) or a stopper (flasks) to produce oxygen limited conditions or a permeable membrane or stopper to produce aerobic conditions. Growth was assessed by direct measurement of cell dry weight after washing and lyophilization or at OD600 on a spectrophotometer for plate assays. Growth of electrogenic bacteria was assessed after adjustment of treated stillage to pH 6.8-7.0 at 30C with gentle shaking in a Biotek neo2 plate reader either in aerobic conditions or in a glove box to produce anaerobic conditions. For bioreactor analysis strains were pregrown in stillage in shake-flasks over-night at 30C and 200 rpm and inoculated to OD600 of 0.2 in 0.5 L Sixfors bioreactors (Infors HT, Basel, Switzerland) equipped with temperature, dissolved oxygen, and stir-rate control.

2.2 Analytical methods

Absolute quantification of specific metabolites was performed by HPLC. Samples were filtered with a 0.2 micron syringe filter and analyzed using an Aminex HPX-87H ion exclusion column with a 1 mM H_2SO_4 flow of 0.6 ml/ml. The temperature of the column was 60C. The refractive index at 45C and the UV absorption at 210 nm were measured. Total protein was measured using a ThermoFisher BCA protein assay (Waltham, MA) according to the manufacturer's instructions. Total carbohydrate was measured using a Sigma Total carbohydrate assay kit (St. Louis, MO) according to the manufacturer's instructions.

3.0 Results and Discussion

3.1 Characterization of stillage

Whole stillage from a batch distillation process to produce single malt whiskey was obtained from Solar Spirits (Richland, WA) immediately after two distillation runs on 1/20/21 and frozen at -20C for further processing and analysis. Whole stillage was centrifuged for 5' at 4000g to remove solids and produce a soluble fraction (Figure 1). The soluble fraction, which is most representative of "thin stillage" was characterized chemically by high-pressure liquid chromatography and colorimetric assays (Table 1). Fifty-seven chemicals analyzed for were not present above the limit of detection including sorbitol, arabinose, xylitol, levoglucosan, levulinic acid, glycolate, furfural, acetate, acetaldehyde, acetoin, butanoic acid, butanol, butanediols, propanoic acid, propanol, propanediols, methyl acetate, and ethyl acetate.

Figure 1. Stillage samples from Solar Spirits (Richland, WA). (A) Stillage samples from distillation 1 and 2 after centrifugation. (B) Compositional makeup of the soluble fraction from a mixture of distillation 1 and 2 used for experimental work.

Table 1. Compositional analysis of stillage soluble fractions. All values are expressed in g/L and are the average of three technical replicates. Error indicates standard deviation. A mixture of stillage soluble fractions 1 and 2 was used for the work presented.

Sample	Carbohydrate	Protein	Ethanol	Glycerol	Methanol	Xylose	Trehalose	Citric acid	Succinic acid	Acetic acid	Formic acid	pH
Distillation 1	na	5.1±0.3	3.4±0.0	2.8±0.0	1.8±0.0	1.4±0.0	1.1±0.0	0.7±0.0	0.5±0.0	0.3±0.0	0.3±0.0	4.1
Distillation 2	na	5.1±0.2	4.0±0.0	2.7±0.0	1.7±0.0	1.2±0.0	1.0±0.0	0.6±0.0	0.4±0.0	0.3±0.0	0.2±0.0	4.1
Mixed 1/2	14.1±0.2	5.1±0.1	3.7±0.0	2.8±0.0	1.8±0.0	1.3±0.0	1.0±0.0	0.6±0.0	0.5±0.0	0.3±0.0	0.2±0.0	4.1

3.2 Identification of biocatalysts for stillage conversion

The soluble fraction from a mixture of distillation 1 and 2 was sterile filtered and used as a substrate to screen for robust and metabolically diverse yeast, bacteria, and filamentous fungi capable of growth on stillage without any processing or amendments to its composition (e.g. addition of nutrients or modification of pH). Microorganisms were inoculated into 24-well plates

and incubated with 2 mL of stillage at room temperature for four days to identify species capable of growth in either aerobic or oxygen limited (microaerobic) conditions. Thirty-four yeasts, two bacteria, and two filamentous fungi from a variety of genera were identified that are capable of growth on unmodified stillage at moderate temperature in either aerobic or microaerobic conditions and include species from the genus *Aspergillus*, *Brettanomyces*, *Candida*, *Debaryomyces*, *Dipodascopsis*, *Lactobacillus*, *Lipomyces*, *Pichia*, *Rhodotorula*, *Yarrowia*, *Zygoascus*, and *Zygosaccharomyces*.

End-point analysis of the stillage growth assays was used to score species based on their ability to remove undesired organics, particularly protein, and produce desirable products including biomass and short chain fatty acids (lactic acid and acetic acid) amenable to conversion by electrogenic bacteria in a microbial electrochemical cell. Overall, the filamentous fungus *Aspergillus niger*, yeasts from the genera *Debaryomyces* and *Rhodotorula*, as well as the bacterium *Lactobacillus* performed well in terms of protein and other organic compound removal. Most of the fungi screened, with the exception of some *Debaryomyces* species, consumed any short-chain fatty acids present.

Promising species that performed well at 2 mL scale were down-selected and tested at 30 mL scale in 125 mL shake-flasks to verify the results (Figure 2A). In aerobic conditions most of the fungi completely consumed the specifically monitored organics (ethanol, citric acid, glycerol, xylose, citric acid, lactic acid, and acetic acid) after four days while removing up to 95% of the soluble carbohydrates (*Aspergillus niger*; ATCC 11414) or 92% of the soluble protein (*Rhodotorula pallida*; Phaff 83-461). In oxygen limited conditions, the *Debaryomyces* species consumed up to 62% of the carbohydrates while the *Brettanomyces* species consumed up to 40%. Protein consumption was poor in oxygen limited conditions. The greatest reproducible reduction in soluble protein content was 28% using *Pichia kudriavzevii*; NRRL Y-7551. Consumption of the specifically monitored organics was modest. *Lactobacillus pentosus*; ATCC8041 removed 100% of the citric acid and 80% of the glycerol, but many of the species produced ethanol, lactic acid, and acetic acid.

Since short chain fatty acids and potentially small alcohols are desirable as a feedstock for electrogenic bacteria we examined their production further using *Brettanomyces naardinensis*; NRRL Y-17526, *Debaryomyces udenii*; NRRL Y-17354, *Pichia kudriavzevii*; NRRL Y-5396, *L. pentosus*; ATCC8041, and co-cultures of the yeast and bacteria species in aerobic and O_2 limited conditions (Figure 2B). *L. pentosus* is notable for producing lactic and acetic acid in both culture conditions while the yeasts were selected based on production or very limited consumption of ethanol, lactic and acetic acid. Time-course cultivation (Figure 3) revealed that *L. pentosus* in isolation initially produces a mixture of lactic acid and acetic acid to day 4 but that lactic acid diminishes in favor of acetic acid. Addition of yeasts that tend to produce, or at least not consume, the short chain fatty acids enhanced production of lactic acid at the expense of acetic acid during co-cultivation with *L. pentosus* even though the yeasts in isolation did not produce lactic acid (Figures 2B & 3). The yeasts in question (*B. naardinensis*, *D. udenii*, and *P. kudriavzeviil*) are all notable for their niche lifestyles as secondary fermenters of pentoses and oligosaccharides in conditions that are characterized by a variety of stresses including limited oxygen, acidic pH, and high concentrations of alcohols or other osmolytes. These yeast species performed best in terms of carbohydrate conversion and *L. pentosus*, which itself is capable of converting pentose sugars, may be benefiting from extracellular release of sugar monomers from more complex oligosaccharides catalyzed by secreted fungal enzymes. The fungal species are also effective scavengers of oxygen and may be accelerating transition from the initial O_2 limited state to a fully anaerobic environment.

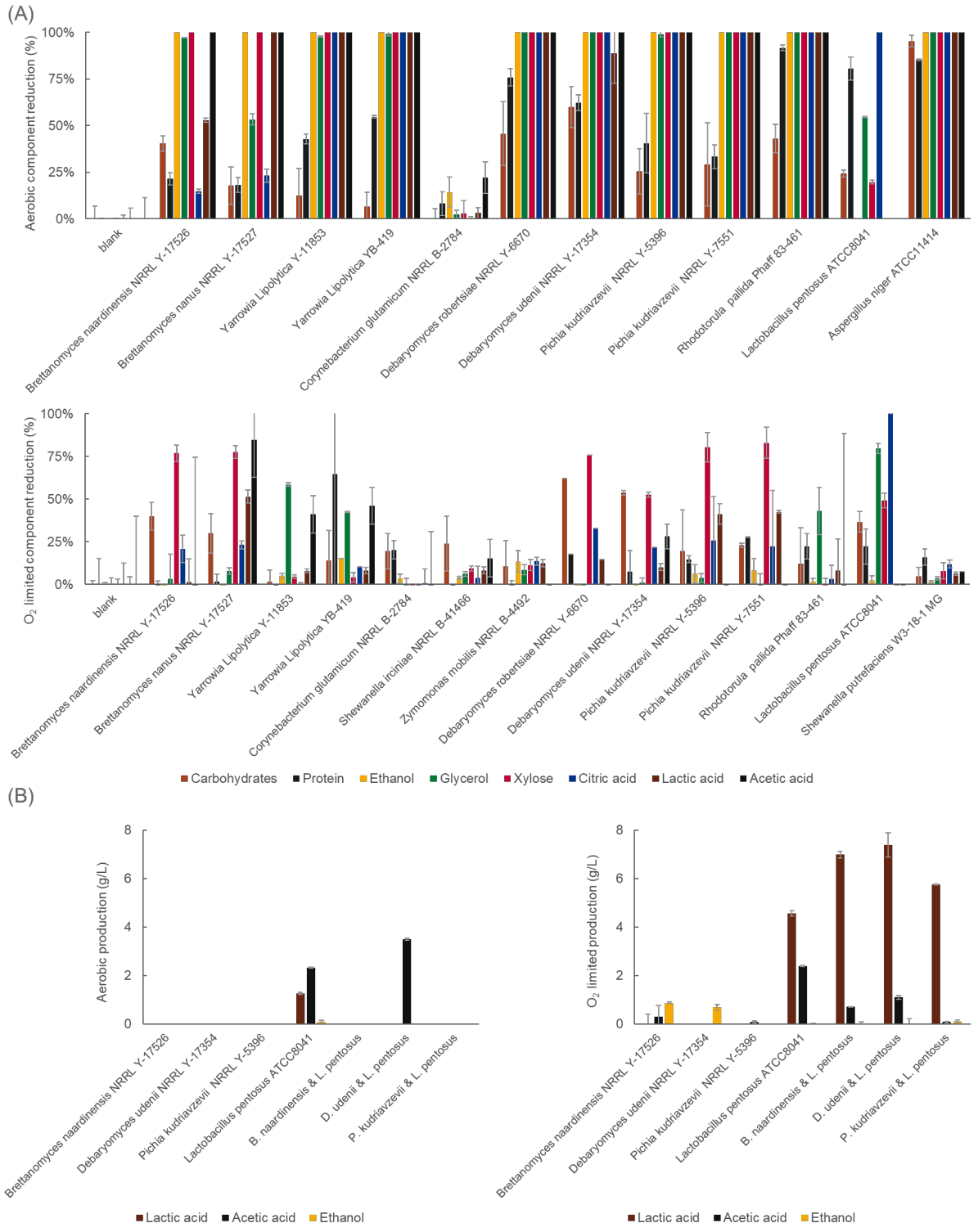

Figure 2. Conversion of soluble organic components of stillage. Strains were inoculated to OD_{600} = 0.2 in sterile filtered soluble fraction of stillage and incubated for 4 days at 200 RPM and 30C in shake-flasks with an air permeable cap (aerobic) or a sealed cap (O_2 limited). (A) Reduction of soluble organic components of stillage. (B) Production of short chain fatty acids and ethanol using pure strains and co-cultures.

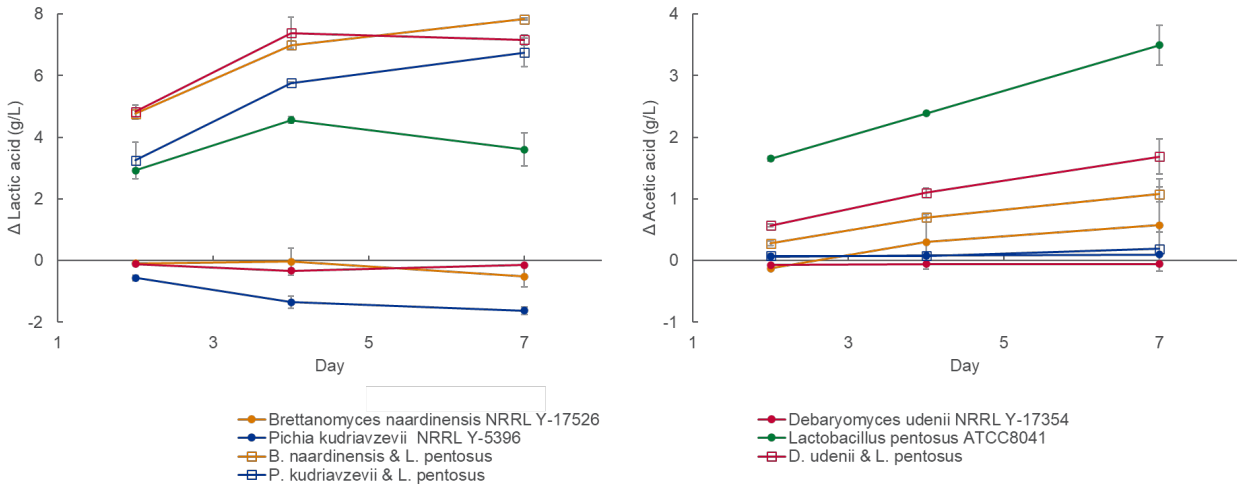

Figure 3. O_2 limited production of short chain fatty acids in selected species and co-cultures. Strains were inoculated to OD_{600} = 0.2 (or 0.1 for each species in co-cultures) in sterile filtered soluble fraction of stillage and incubated for 7 days at 200 RPM and 30C in sealed shake-flasks.

3.3 Bioreactor scale stillage conversion

To validate the screening results obtained at microtiter plate and shake-flask scale we examined the most promising conversion organisms in 0.5 L bioreactors. The co-culture of *D. udenii* and *L. pentosus* was chosen for further study because of its ability to maximize output of short chain fatty acids as a feedstock for bacteria growing in a MEC. The yeasts *Y. lipolytica*, *R. pallida*, and *Cyberlindnera jadinii* were chosen for their ability to concentrate soluble organics into readily separable biomass with value as animal feed or oil rich biomass. The filamentous fungus *A. niger* was chosen for its ability to rapidly reduce the concentration of soluble organic material in stillage. For scale-up, an *A. niger* strain engineered to convert carbohydrates to 3-hydroxypropionic acid (3HP) via the β-alanine pathway [10] was tested. 3HP is a β-hydroxy acid similar in structure to the α-hydroxy acid lactic acid with commercial potential as a bioderived commodity chemical [11] as well as the possibility of being utilized as a carbon source by bacteria growing in a MEC.

The co-culture of *D. udenii* and *L. pentosus* was tested for conversion in anaerobic, microaerobic and aerobic conditions with equivalent inoculum to examine the impact of oxygen level on reduction of organics and production of short-chain fatty acids (Figure 4A). In aerobic conditions all specifically monitored organics were eliminated from the stillage by 72 hours. In microaerobic or anaerobic conditions, organics were consumed more slowly and the total protein and carbohydrate content was reduced to a lesser degree (Figure 4D). Microaerobic conditions favored production of acetic acid while the fully anaerobic fermentation favored lactic acid production. In all cases, reduction of soluble protein content by this co-culture was minimal reaching only 11% reduction in the aerobic condition.

Conversion of stillage using yeasts with the potential to produce nutrient or oil rich biomass was examined. The yeast *C. jadinii*, which is used commercially for the production of animal feed, eliminated all the specifically monitored soluble organics in under 20 hours while yielding 6.3 g/L of biomass. Conversion by the yeasts with oil rich biomass as a target (*Y. lipolytica* and *R. pallida*) was slower and resulted in lower utilization of the soluble carbon. Conversion of stillage using *A. niger* was originally examined because of its ability to reduce soluble protein and

carbohydrate content. Bioreactor cultivation confirmed that *A. niger* eliminates all the specifically measured organics in under 48 hours (Figure 4B) and reduced total protein content 46% and total carbohydrate content 83% by 96 hours post-inoculation resulting in the highest total reduction of organics by any species studied (Figure 4C). We used an *A. niger* strain that had been engineered previously to produce the platform chemical 3HP. From the mixture of organic compounds present in stillage, 8.2 g/L of 3HP was produced at hour 88 representing a yield of 0.35 C-mol 3HP / C-mol non-protein organics consumed at an overall rate of 0.09 g/Lh in unmodified stillage without pH control.

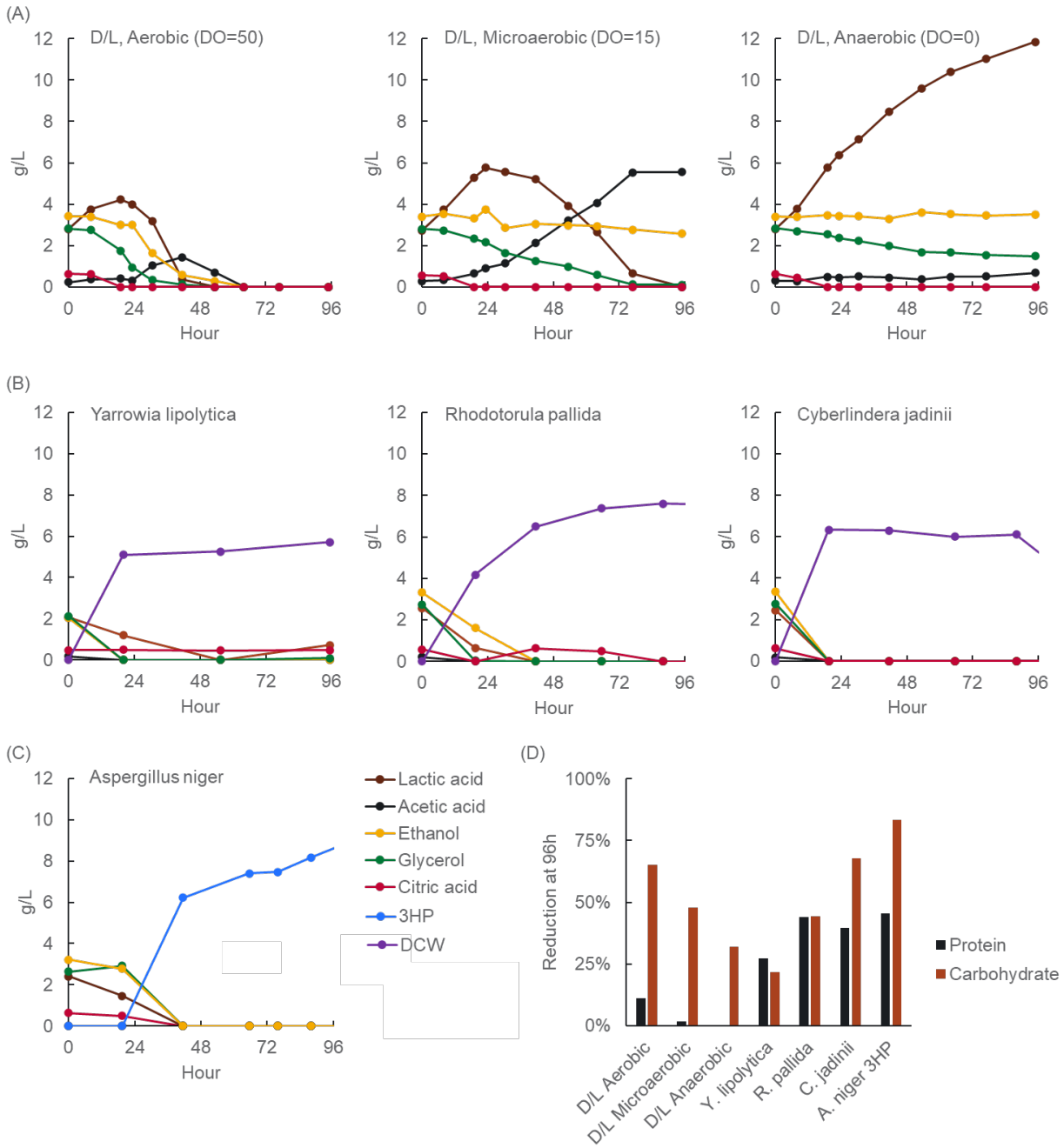

Figure 4. Bioreactor scale conversion of stillage. (A) Impact of oxygen availability on conversion using co-cultures of *D. udenii* and *L. pentosus*. (B) Aerobic production of 3-

hydroxypropionic acid (3HP) from stillage by engineered *A. niger*. (C) Reduction of soluble total protein and total carbohydrate content after 96 hours of fermentation.

3.4 Growth of electrogenic bacteria on stillage after fungal treatment

As a secondary step for processing of stillage we examined conversion of organics by electrogenic as a method to produce electricity or hydrogen while treating the fermentation wastewater. In all cases, the stillage still contained organic compounds after fungal treatment and in the case of the *D. udenii* and *L. pentosus* co-cultures, short-chain fatty acids were specifically produced as carbon sources for the electrogenic bacteria in a microbial electrochemical cell (MEC). The suitability of treated stillage produced by the fungal or co-culture processes for conversion in a MEC was assessed using two bacterial species, *Geobacter* SD1 and *Shewanella* W3-18-1. After fungal or co-culture treatment, solids were removed from the stillage and the pH was adjusted to between 6.8-7.0. Growth by *Geobacter* SD1 was assessed in anaerobic conditions from a 10% mid-log phase preculture with 10 mM ferric citrate as an electron acceptor, while *Shewanella* W3-18-1 was assessed in aerobic conditions from a 1% mid-log phase preculture. Growth experiments were carried out in a Biotek neo2 plate reader at 30 C while slowly shaking for 125 hours. Both species grew well on stillage treated by wild-type but not 3HP producing *A. niger*. Overall, *Geobacter* SD1 grew on a wider range of treated stillage samples including treated stillage containing short-chain fatty acids from the co-cultures as well as stillage treated by the fodder yeast *C. jadinii* and the lipid accumulating yeast *Y. lipolytica* (Figure 5A). Interestingly, *Shewanella* W3-18-1 did not grow well on the short-chain fatty acid containing stillage samples but grew rapidly on the *C. jadinii* treated stillage (Figure 5B).

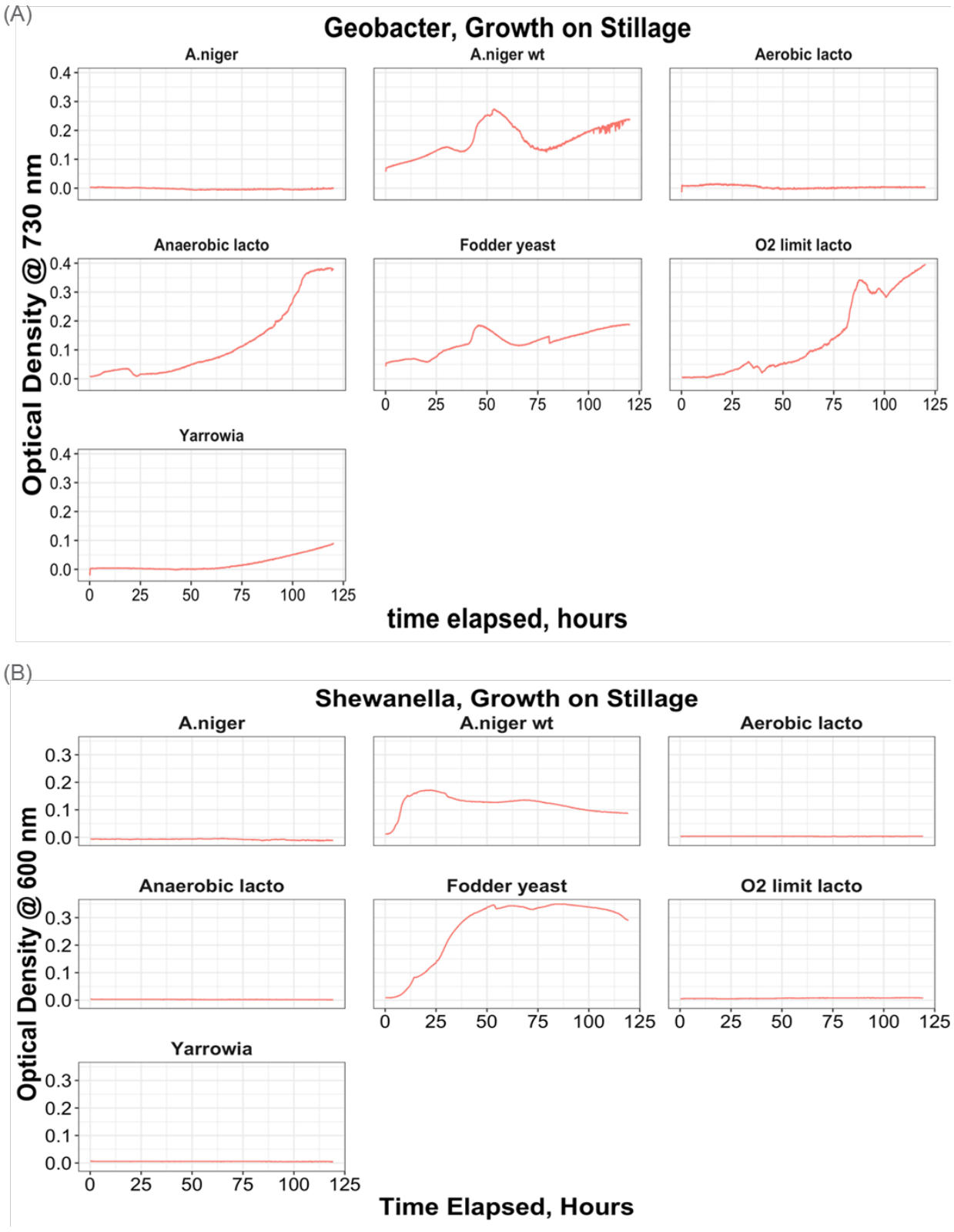

Figure 5. Growth of (A) *Geobacter* SD1 and (B) *Shewanella* W3-18-1 on stillage after treatment by yeast, filamentous fungi, or bacteria/yeast co-cultures.

To confirm the growth experiments in stillage treated by wild-type and 3HP producing strains of *A. niger*, which reduced the organic content to the greatest degree, growth was assessed in 30 mL aliquots and the ash free cellular biomass was determined. The results confirmed that both *Geobacter* SD1 and *Shewanella* W3-18-1 produced significantly more biomass on stillage treated by the wild-type *A. niger* strain and that *Shewanella* W3-18-1 produced more biomass on both *A. niger* treated stillages samples, reaching 0.39 g/L ash-free cellular biomass after 10 days growth on the wild-type sample. The reason for the difference in growth on stillage treated by wild-type *A. niger* and the 3HP producing *A. niger* was not clear. To assess this, stillage treated by 3HP producing *A. niger* was supplemented with various macronutrients and iron to identify nutrients that may be limiting growth. Addition of 10 mM pyruvate most dramatically increased growth of *Shewanella* W3-18-1 (Figure 6) suggesting an appropriate carbon source may be limiting in the stillage treated by the 3HP producing *A. niger* strain.

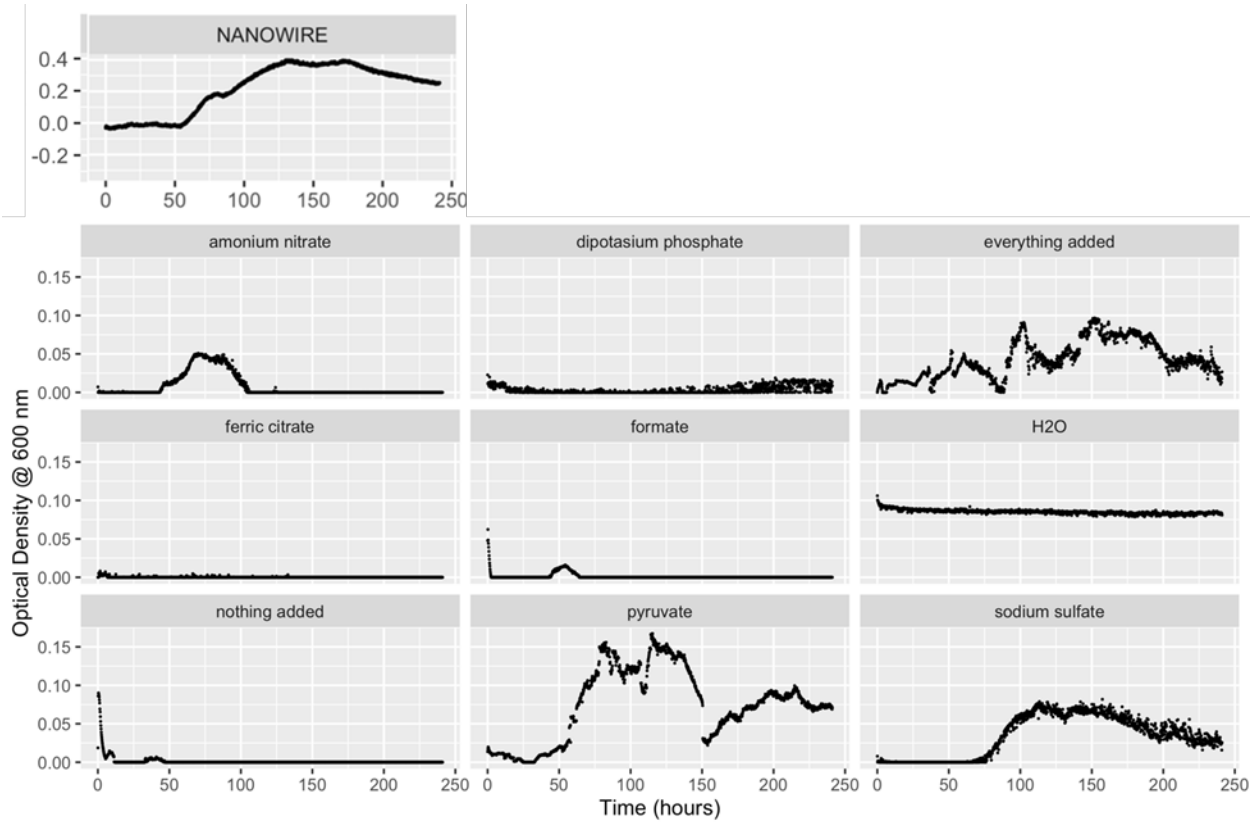

Figure 6. Growth of *Shewanella W3-18-1* on stillage after conversion by 3HP producing *A. niger*, adjustment of pH to 6.8-7.0, and addition of selected nutrients. Nanowire media is a positive control for growth.

4.0 Conclusions and future direction

First-generation corn ethanol production has been increasing in the US, and second-generation cellulosic ethanol production from corn stover has been briefly attempted by both Dupont and POET-DSM at biorefinery scale [12] before ultimately shutting down due to challenges in biomass collection, processing, and pretreatment as well as interdependency of unit operations and novel operations [13]. From these attempts at commercialization, it became clear that handling and processing a novel feedstock (corn stover) is a major challenge to be solved and that reduction of process complexity and improvements in energy efficiency will aid the next generation of ethanol biorefineries. The thin profit margin faced for fuel ethanol production is a challenge that requires production of high value co-products. In the case of first-generation corn ethanol plants this has been met primarily by production of animal feed products derived from stillage which is produced at higher volume than the primary product ethanol [2].

Production of co-products from next-generation ethanol biorefineries is expected to be important for financial viability, however, stillage from lignocellulosic feedstocks will be complicated by being compositionally biased toward difficult to convert pentose monomers and any unhydrolyzed cellulose or hemicellulose. To address this, fungi have been proposed as natural hosts for the bioconversion of stillage to a variety of co-products from cellulosic biorefineries [14]. Here we screened for and identified yeast and filamentous fungal hosts capable of growth and reduction of soluble organic compounds on unmodified stillage. Hosts amenable to production of biomass-based products including animal feed, lipids, carotenoids, and chitosan were identified and evaluated at 0.5 L scale. Conversion of the mixed soluble carbon source present in stillage to specific products valuable to the biobased polymer industry (lactic acid and 3-hydroxypropionic acid) was also demonstrated, expanding the repertoire of organic acids capable of being produced from stillage by fungi beyond citric acid [15] and malic acid [16]. Finally, stillage treated by fungi or co-cultures of fungi and bacteria was tested for utilization by electrogenic bacteria capable of enabling on-site electricity production in a microbial electrochemical cell. Future work will focus on testing the efficiency of electricity and hydrogen production from fungal treated samples that enabled growth of electrogenic bacteria.

The results presented here identified a variety of pathways to concentrate organics in stillage within fungal biomass, thereby reducing the energy intensity of separating biomass for animal feed or other biomass derived products. Further exploration of the data produced within a techno-economic model of first and second-generation biorefineries will guide optimization of specific processes. Treatment and valorization of heterogeneous aqueous waste is a problem common to processing of organic material. The two-part approach described herein is broadly enabling across many dilute aqueous streams with organic contaminants, and in many cases may provide greater recovery of benefits from waste-streams than methane for heat, steam and power.

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