CHARACTERIZATION OF EXTREMOPHILIC BACTERIA FOR POTENTIAL IN THE BIOFUEL AND BIOPROCESS INDUSTRIES

A Dissertation

by

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ABSTRACT

Industrial bioprocesses are constrained by the availability of microbes that are optimized for harsh bioprocess conditions. Over 500 soil and sediment samples collected from 77 saline and/or thermal sites in the continental United States, Hawaii, and Puerto Rico were used to inoculate fermentations in an effort to optimize the microbial communities for a biofuel process developed at Texas A&M (MixAlco™).

A diverse bacterial isolate library (N= 1,866 isolates) was constructed by employing a variety of culturing techniques across thirty-four of the fermentation communities selected based on superior performance in the biofuel platform. Phylogenetic analysis of partial 16S rDNA sequences placed 1,200 of these isolates in the genus *Bacillus*. Other genera include, but are not limited to: *Ureibacillus*, *Bacillus*, *Geobacillus*, and *Caldalkalibacillus*.

The central hypothesis of this dissertation was that selection due to the original site conditions of the natural inocula, and also, survival during fermentation likely favored isolates in the library that possess biofuel/bioenergy relevant traits (e.g. hydrolysis of lignocellulosic biomass, utilization of hydrolysis products, and tolerance to inhibitory compounds released during hydrolysis).

The phylogeny for this library was used to identify and prioritize a diverse subsample of the library (n=207) for high-throughput screens of extracellular cellulase activity, *n*-butanol tolerance, vanillin utilization, and lignin degradation, as indicated by decolorization of the surrogate Congo red. Many isolates exhibited the capacities

screened, including several isolates that were positive for more than one of these traits. Subsequently, a subset of the 207 screened isolates were studied further for tolerance and/or utilization of bioprocess byproducts (e.g. lignocellulosic hydrolysate, bio-oil) that are known to harbor both compounds with inhibitory effects on growth and pentose sugars released after hydrolysis. Polyhydroxyalkanoates (PHA) are bio-plastics produced by some bacteria. There is interest in industry in the identification of bacteria that can utilize wastes from some bioprocesses while creating bio-plastics, thus, a subset of the library (n=43 isolates) was screened for PHA accumulation associated with growth with the bio-diesel byproduct glycerol as the carbon source. Several isolates from the library are PHA producers with glycerol based on a fluorescence screen conducted. Microfluidic microbial fuel cells (MFCs) take advantage of microbial metabolism to convert organic matter to electricity. Microbial communities collected directly from soils were screened in a MFC array developed at Texas A&M. The screening of natural microbial communities identified electricigens with enhanced power generation abilities.

The variation identified in these industrially relevant traits across isolates provides a proof of concept for both the existence of this variation in nature and the efficacy of employing fermentation and culturing approaches to enrich for these phenotypes. Also, it became clear this library could serve as a resource for bioprocess isolates for industry, either directly or as a starting point to advance the bioprocess optimization prior to some form of genetic engineering targeting a particular function.

DEDICATION

I would like to dedicate this dissertation to my mom, Jacqueline, my sisters Jackie, April and Angel, and all of my nieces and nephews. I would like to especially dedicate this work to Christopher D. Magee without his unyielding support, encouragement and love I would not have been able to come this far.

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CHAPTER I

INTRODUCTION

Energy security is vital to economic prosperity and environmental safety and is a global concern [8]. In the past five years there have been a variety of high profile energy commotions: the Deepwater Horizon oil spill of 2010, the Fukushima nuclear reactor accident of 2011, protests over the Keystone Pipeline 2011 – present, along with oil-price instability and unpredictability of the petroleum product supply (e.g. plastics). These disturbances along with the effect of petroleum consumption on the environment (e.g. CO_2 emissions increasing) and fossil fuel depletion are the reason for the development of alternative energy sources.

Oil is the world's primary energy source [9]. Developed and developing countries are dependent on petroleum and petroleum derivatives. With world population growth and the transition within developing countries toward more energy intensive standards of living, the energy demand is steadily increasing, estimated to increase six times by 2100 [10]. There is a growing demand to develop and use renewable energy sources. Nonetheless, even if this demand is partially met over the next few decades, the world will still depend greatly on fossil fuels.

Wastes from agriculture (e.g. plant and animal) and industry (e.g. pulp and paper) can be converted to energy. Furthermore, it seems reasonable to expect solid and liquid waste streams will increase with world population growth. In addition to waste to energy approaches, renewable energy can be harnessed from geothermal and solar

energy, cellulosic, algal and microbial biofuels. These renewable forms of energy do not compete with agricultural crops and benefit the environment. Energy security will not be sustained until petro-based fuel alternatives are produced from renewable sources (e.g. microbes).

Biofuels are fuels made from living organisms. First-generation biofuels are produced from edible crops (e.g. corn), while second-generation biofuels are made from non-edible, recalcitrant, raw materials (e.g. lignocellulosic biomass). Third-generation biofuels are predominantly produced from algae. The most common biofuels are biodiesel and bioethanol.

Electricity is the world's fastest growing energy sector, with electricity generation rising on an average of 2.3% per year [8]. Thus, this is also an important area of need for new energy sources, specifically renewable energy. Microbial fuels cells (MFCs) are devices that convert energy stored in chemical bonds in organic compounds to electricity, and are considered to become an important part of bioenergy [11-13]. The generation of electricity in a MFC is green, recyclable and efficient. MFC research is focused on electrochemically active species and device design (e.g. stability requirements)[14]. Microbes have the ability to utilize waste products and MFCs can also be used to convert waste (e.g. wastewater or lignocellulosic biomass) to electricity.

The increasingly limited supply of petro-based chemicals have led to interest in developing microbial systems for the production of fuels and other chemicals such as plastics [15].

Bioprospecting microbes can help confront the challenges of dwindling energy supplies and a polluted environment. Bioprospecting involves finding communities, single organisms or genes of interest in nature to provide for a desired phenotype. Bioprospecting is important in bioprocesses; many traits and phenotypes desired in industrial bioprocesses are found in environmental isolates, including extremophiles. These include but are not limited to traits in biofuel and bioprocess industries: degradation of complex compounds (e.g. lignocellulose), tolerance to inhibitory products (e.g. ethanol, acetic acid) and substrate derived compounds that act as inhibitors to processes (e.g. furans and phenolics)[16].

Using bioprospected isolates can be difficult in some cases owing to differences in the natural environment from which these microbes were isolated and the selective pressures/conditions in the bioprocess they are to be utilized in, along with limitation in the cellular regulations of multiple industrially advantageous traits (e.g. substrate utilization, compound tolerance, and production). Though industrial bioprocessing is progressing in availability of molecular tools for environmental isolates, it is still difficult to transfer genes to these isolates [17] . The ultimate microorganism that encompasses all sought-after bioprocess traits has yet to be found. Industrial bioprocesses require microbes and catalysts optimized for unfavorable reaction conditions (e.g. high temperatures, high product concentrations). Interest in utilizing extremophiles (e.g. halophiles and thermophiles) such as *Bacillus* and related genera for industrial processes is increasing [18-22]. Extremophiles are robust organisms that produce thermo stable enzymes. As a result of extremophiles having fluid membranes,

isolates can readily tolerate changes in environmental conditions (e.g. pH and temperature). Thermophiles can ferment both pentose and hexose sugars and complex carbohydrates. Thermophilic industrial bioprocesses are less likely to be contaminated by mesophilic microbes. These processes do not require cooling steps in turn generating lower energy inputs [23].

Bacillus and related species (e.g. *Geobacillus, Ureibacillus)* are economically important in bioprocesses, specifically in the fields of medicine, biodefense, bio-pesticides and biofuels [24] . *Bacillus* species have high growth rates leading to shorter fermentation times, many have low nutrient requirements, salt and organic solvent tolerance, and these microbes produce a number of extracellular proteins. *Bacillus* and *Geobacillus* species produce novel enzymes adapted for extreme environments that would serve as catalysts in various industrial bioprocesses. Because of these characteristics, *Bacillus* species are becoming regarded as industrial organisms that have well-developed fermentation machinery [25].

The ideal microbe utilized in biofuel production should be capable of degrading the biomass substrate, efficiently fermenting the hydrolysis products (e.g. hexose and pentose), and tolerating high concentrations of end products, high temperatures and pH, and inhibitory compounds released during degradation (Figure 1). Microbial biodiversity is vast and an ideal host for biofuel production may be naturally occurring in nature, but microbial biofuel research is currently focused on combining desirable traits into an engineered host such as *E. coli* [1]. Microbes have great potential to help resolve the world's diminishing energy supply.

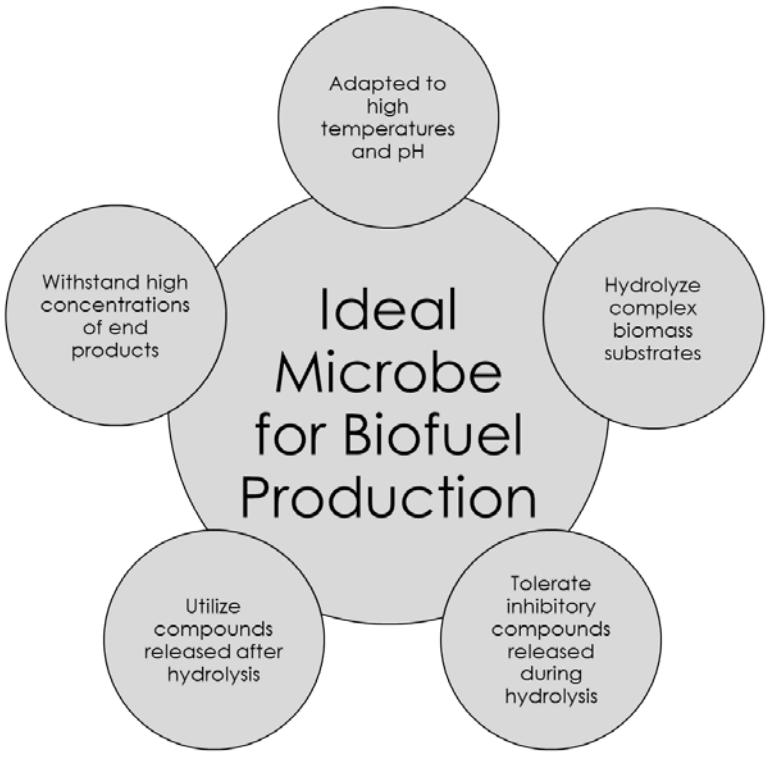


Figure 1. Model for the ideal biofuel host. This model was adapted from CR Fischer, D Klein-Marcuschamer and G Stephanopoulos [1]. The ideal microbe for the production of biofuels should be able to withstand high temperatures and pH, hydrolyze complex biomass substrates and compounds released during hydrolysis and tolerate inhibitory compounds produced during biomass hydrolysis and high concentrations of end products.

In this study microbes from saline and/or thermal environments were screened for industrially relevant traits to identify candidate isolates that encompass one or multiple traits associated with biofuel and bioenergy production. A central hypothesis for this work was that selection imposed by the sediment environments, subsequent fermentation conditions, and culture conditions from which these isolates originated

would favor isolates with the industrially relevant phenotypes of interest. Furthermore, phylogenetic comparisons of these bioprospected isolates to published isolates might provide insights in to the novelty of any traits identified, thus possibly revealing traits not previously detected in isolates from less extreme environments. The results of these screens of bioprospected isolates will serve to help prioritize which isolates will be sequenced and further characterized.

In total, this dissertation provides insight into the potential for a library of isolates from extreme environments in nature. Chapter II describes the construction of the isolate library, phylogenetic analysis of the library, and screening some representative groups of isolates for traits related to biofuel production, such as cellulose degradation, organic solvent tolerance, vanillin utilization, Congo red decolorization and the ability to grow at both 37ºC and 55ºC. Lignocellulosic substrates are used as energy feedstock to produce materials and essential compounds. Cellulose sources converted to volatile fuel by microbes can be separated through distillation and other techniques and used for energy. Lignin is a complex aromatic polymer consisting of phenylpropanoid units. Unlike cellulose, lignin contains few hydrolyzable bonds and cannot be depolymerized to soluble fragments [26]. Microbes are known to secrete a variety of enzymes during delignification (e.g. laccase, cellulase, amylase, xylanase). Congo red is an azo dye that is structurally similar to lignin [2]. Microbial enzymes involved in lignin degradation are some of the same enzymes involved in decolorization of azo dyes [27]. Thus, it has been asserted that microbes capable of decolorizing Congo red have potential in lignin degradation in biofuel production, resulting in straightforward

laboratory screens for delignification potential [2] . Vanillin is a methoxylated, phenolic aldehyde that is found in lignin [28].Vanillin is a by-product of bioconversion, specifically the chemical oxidation of lignin [29] . Vanillin has toxic effects on most microbes used in industrial fermentation resulting in its recognition as an extreme inhibitor [30]. Bioconversion processes that produce solvents benefit from tolerant microbes and their stable enzymes. Butanol is regarded as a potential alternative to liquid transportation fuel, compared to other alternatives (e.g. ethanol), butanol is more hydrophobic, has a lower volatility and a higher energy density [31]. Generally, butanol production is characterized by low yields, presumably due to microbial intolerance to product accumulation [32] . Chapter III describes screens targeting a few isolates, based on the results of the phylogenetic analysis, for the ability to tolerate and/or utilize complex byproducts associated with biofuel and bioenergy processes, lignocellulosic hydrolysate and bio-oil and the ability to utilize five-carbon fermentable sugars associated with lignocellulosic hydrolysis. Furthermore, as a test of concept, isolates capable of growth on these complex substrates were characterized for production of industrially valuable compounds (e.g. organic acids). Chapter IV presents the results of screening a subset of isolates for polyhydroxyalkanoates (PHA) accumulation during growth with glycerol as the substrate. PHA is used in the production of bioplastics and glycerol is a byproduct of biodiesel production. Chapter V presents an analysis of the electro-chemical potential of microbial communities from the original sediments in a microbial fuel cell array [6]. Finally Chapter VI provides a summary of this dissertation and a discussion of some future research directions.

CHAPTER II

ASSESSING THE GENETIC AND PHENOTYPIC DIVERSITY WITHIN A LIBRARY OF BACTERIA ISOLATED FROM CARBOXYLATE PLATFORM FERMENTATIONS INOCULATED WITH SEDIMENTS FROM EXTREME ENVIRONMENTS

II.1 Introduction

Industrial bioprocesses require microbes and catalysts optimized for reaction conditions (e.g. high temperatures, high product concentrations.) Metagenomic sequence analysis approaches allow for cataloging of species and metabolic potential present in natural microbial communities. An advantage of this approach is that it is independent of the ability to culture the microbes. However, to advance fundamental understanding necessary to utilize microbes in industrial bioprocesses it remains essential to characterize pure cultures (e.g. collecting phenotypic and genetic data). Assembly of bacterial isolate libraries is a valuable approach for acquiring, characterizing, and cataloging extremophiles. Bacterial cultures provide physiological insight into whole organisms. Cultures can provide resources allowing for testable hypotheses that come out of genomic data [33] ; homologous genes may have different functional roles than their database-predicted role [34]. Cultured microbes also allow for the study of whole genomes. Thus, in order to capture the potential of microbes from extreme environments for use in industrial processes, it is essential to endeavor with efforts to culture, maintain,

and characterize these organisms that often are quite fastidious. Screening microbes for industrial applications targets traits that influence bioprocess efficiency, specificity and/or stability. Bioprocesses utilize living cells or living cell products (e.g. enzymes) to produce favored physical or chemical changes. Cell metabolism can be optimized to drive a bioprocess. Bacteria allow for the synthesis of numerous products for food, pharmaceutical sectors, while some are used in waste treatment and pollution control [35]. In a large-scale effort to screen the carboxylate platform fermentation potential of diverse microbial communities from extreme environments [4, 36]collected more than 500 sediments from 77 saline or thermal sites. This chapter describes the concomitant effort to assemble and begin characterization of a library of bacterial isolates from 34 of the top performing communities identified by [5] and two other communities, one from a microbial fuel cell array discussed in Chapter V and the other from [7]. The library assembly involved employment of a variety of culturing strategies in order to maximize the diversity of isolates collected. Further, to characterize the diversity captured in the library, all 1,866 isolates were subjected to phylogenetic analysis based on sequences of the partial 16S rDNA.

Frequently microbial libraries are screened for phenotypic diversity based on standard approaches to catalog nutritional requirements [37]. Herein, based on a specific interest in biofuel and bioprocess industrially relevant traits, an alternative approach was taken. Given the original selection in biofuel platform fermentations, it seemed reasonable to expect the individual isolates might possess variation in traits associated with advanced generation biofuel processes. Thus, to further evaluate and establish the

phenotypic diversity present in the library a variety of screens for biofuel related traits were employed across a subset of the library, selected to represent diversity of both geographic origin and phylogeny. Specifically the screens included temperature tolerance (growth at both 37 and 55 ºC), cellulose degradation, lignin-like dye (Congo red) decolorization, vanillin utilization, and butanol tolerance.

MixAlco™ Process

The MixAlco™ process, a carboxylate biofuel platform developed at Texas A&M University, uses a mixed-acid fermentation to produce alcohols such as 2-butanol and other higher alcohols [38]. To increase digestibility biomass is treated with lime then fed into a fermentor, which contains a mixed culture of carboxylic acid-forming microorganisms and a methanogenesis inhibitor to prevent methane production. Calcium carbonate is added to the mixture as a buffer. The calcium carbonate reacts with the carboxylic acids to form carboxylate salts, which are then dewatered and dried. The dried salts are thermally converted to ketones, which can then be hydrogenated to alcohols. Carboxylic acids have a variety carbon-chain lengths and structures [39] . Carboxylic acids are produced biochemically from carbohydrates through microbial fermentation. With increasing concerns about oil supplies, prices and environmental pollution caused by petrochemical processes [26], more attention has been focused on using bioprocesses to produce carboxylic acids [40, 41].

Optimizing Microbial Communities in Carboxylate Biofuel Platform Fermentations

A recent collaboration among the Wilkinson, Gentry, and Holtzapple laboratories at Texas A&M University involved efforts to identify extreme environmental conditions

that select for microbial communities pre-adapted to superior performances in carboxylate platform fermentations [4, 5, 36] . Briefly, natural microbial communities were passed through a 30-day batch fermentation performance screen at 55°C utilizing a cellulosic substrate (e.g. office paper). Thirty-four of the natural soil communities collected from extreme environments and screened in the platform were selected for further study because they were top performers based on biomass conversion and/or acid production (e.g. total acids and total high molecular weight acids), and they were geographically diverse. Community sequencing determined that initial inoculum community diversity improved fermentation performance and the acid spectrum diversity [4, 36].

Phylogenetic Characterization of Bacterial Libraries

Using DNA sequences to approximate phylogenetic diversity is rapid and efficient with the increasing accessibility of 16S rDNA, which encodes for the 16S rRNA gene. The Ribosomal Database Project maintained by the Center for Microbial Ecology (CME) at Michigan State University has over 2 million 16S rRNA sequences. The 16S rRNA gene is present in almost all bacteria, the gene is highly conserved and the gene is large enough for sequencing (~1.4 Kbp) [42] . The 16S rDNA sequences of multiple bacterial isolates can be phylogenetically grouped in Operational Taxonomic Units (OTUs). The taxonomic level chosen by researchers defines an OTU. Approximately 97% sequence identity of the 16S rDNA defines an OTU at the species level.

Developing High-throughput Screens for Bacterial Libraries

High-throughput bacterial screens for industrial traits allow us to investigate biodiversity. High-throughput refers to the capacity to completely assay for a large number of isolates (e.g. isolate library) within a short period of time. Screens for industrial applications depend on traits that allow for bioprocesses to take place more efficiently. Bioprocesses utilize living cells or living cell products (e.g. enzymes) to produce favored physical or chemical changes. The living cell used in a bioprocess is vital to the desired outcome. Cell metabolism can be utilized to drive a bioprocess. Bacteria allow for the synthesis of numerous products for food and pharmaceutical sectors, while some are used in waste treatment and pollution control [35] .

In high-throughput screening, each isolate in a library must be individually screened for various traits and activities (e.g. cellulose and lignin degradation) and isolates positive for that trait must distinctly display the phenotype. Most libraries typically consist of environmental isolates while other libraries consist of mutant isolates that were optimized for specific traits through directed evolution [43]. Assays for a particular trait can be manipulated by changing parameter pressures, such as temperature and oxygen conditions.

Libraries can be screened using assays that test growing microbial colonies or cultures for activity, cell survival, and/or the presence of a trait. These assays are potentially high-throughput, but are complicated to develop and execute [44] . All industrial bioprocesses utilize microbes (e.g. enzyme production, fermentation).

Most culturable bacteria can be grown on agar plates. A single agar plate can culture thousands of different bacterial colonies. Colonies with superior performance of a particular trait can be identified through substrate stains or cell survival on the agar plate. The agar plate method is an older microbiological technique, but an effective one. For example, agar plates containing lipids stained with Victoria Blue can be used to screen multiple bacterial colonies for lipase production. Those colonies that produce lipases will grow with clear halos around the colony and those isolates that do not produce lipase will grow without a halo [45] . In this example, lipids are the substrate of interest and used as the sole carbon source to distinguish microorganisms that may produce an enzyme capable of degrading this substrate. An example of a cell survival assay on agar plates is shown during a butanol tolerance screen where bacterial colonies were grown on agar plates containing butanol, those that survived on the plate in the presence of a certain concentration of butanol were positive for the butanol tolerance phenotype [46]. The positive isolates are candidates for bioprocess conditions that contain high concentrations of butanol.

High-throughput screening is most often carried out using analogous experiments in small volume (5-200µL) samples dispersed in microtiter plate wells [47]. Microtiter plates can make use of a number of sample types, including: individual growing cultures, microbial communities and isolated enzymes. Microtiter plates are compatible for spectroscopic reading using ultraviolet, visible light or fluorescence plate readers. High-throughput screening was conducted on subsets of the library to examine the potential

for five traits relevant to various biofuel bioprocesses: carboxymethyl cellulose degradation, Congo red decolorization, vanillin utilization, and butanol tolerance.

Carboxymethyl Cellulose Degradation

Cellulose is the primary constituent of plant cell walls making it the most abundant organic compound in nature [48, 49]. Cellulose is a linear polymer comprised of beta-D-glucose molecules linked by beta-1, 4 glycosidic bonds [50] . Cellulose has a crystalline arrangement with varying amorphous regions depending on the cellulosic substrate [51].

Large amounts of cellulose are required for a wide variety of industrial processes. Cellulosic substrates are used as energy feedstock to produce materials and essential compounds (e.g. glucose). Cellulose sources converted to volatile fuel by microbes can be separated through distillation and other techniques and used for energy. Cellulosic materials such as wood pulp are used in paper manufacturing, while others materials are used in textiles and agriculture. Microbes capable of degrading cellulosic substrates can secrete extracellular enzymes that solubilize the substrate to metabolizable products.

Microbial cellulases are utilized in textile, paper, biofuels and agricultural industries. Novel bioprocesses utilize microbial conversion of cellulosic biomass. Optimization of cellulase production and cellulase efficiency are important for industrial advancement. Cellulases in the paper industry are used to refine fibers and improve drainage. In the textile industry, cellulases are used in bio-polishing and the finishing of textile fabrics [52]. Biofuel production requires enzymatic conversion of cellulosic biomass to energy-rich chemical products. Agricultural animal feed can be

pre-treated with cellulases, which improves digestibility and increases nutritional value of the feedstock [53] .

Microbial extracellular cellulases are either bound to the cell wall of the microbe or secreted. There are three different types of enzymes simultaneously involved in cellulose degradation: endoglucanases, exoglucanases and beta-glucosidases. Endoglucanases initiate the hydrolysis of cellulose by randomly attacking the intramolecular beta-1, 4 glucosidic bonds of cellulose chains, generating new chain ends. Exo-glucanases cleave the chain ends releasing cellobiose or glucose. Beta-glucosidases hydrolyzes short chain cellulo-oligosaccharides and soluble cellobiose to glucose.

Fibrous cellulose and microcrystalline cellulose are insoluble forms of cellulose that are used in laboratory screens to detect endoglucanase, exoglucanase, and total cellulase activity. Fibrous cellulose is ground Whatman no. 1 filter paper that is made from long cotton fiber pulp. Microcrystalline cellulose is wood pulp that has been partially hydrolyzed by acid. Carboxymethyl cellulose (CMC) is a soluble derivative of cellulose used in identifying endoglucanase activity. CMC is generally generated through a reaction between cellulose and chloroacetate.

Cellulase activity can be determined quantitatively using three factors: 1) product accumulation 2) reduction in substrate quantity and 3) the physical property change of cellulosic substrates [54]. Measuring the reducing sugars after hydrolysis is a common product accumulation determination method. After the cellulosic substrate is hydrolyzed, reducing sugars are released, and activity is measured based on sugar concentration [55]. Reductions in substrate quantity can be measured gravimetrically, monitoring dry weight

before and after hydrolysis. The viscometric method is used to quantify physical property change in cellulosic substrates by calculating the change in viscosity over time. With a large number of isolates, as in this study, plate-clearing assays that stain un-degraded substrate are utilized to qualitatively observe cellulose degradation [56].

Congo Red Decolorization

Plant cell walls are formed with lignin and cellulose, this arrangement is one of earth's most biologically and chemically recalcitrant materials. In this arrangement, cellulolytic microbes are unable to degrade the cellulose without the capacity to partially degrade lignin. Some microbial enzymes degrade lignin. Organisms capable of degrading lignin and cellulose wood prove useful in bioconversion, paper and agricultural industries.

Lignin is a complex aromatic polymer consisting of phenylpropanoid units. Unlike cellulose, lignin contains few hydrolyzable bonds and cannot be depolymerized to soluble fragments [26] . Microbes are known to secrete a variety of enzymes during delignification (e.g. laccase, cellulase, amylase, xylanase).

Dyes and pigments used in industrial processes (e.g. textiles) are released in the environment as hazardous effluents. Synthetic dyes used in these industries are carcinogens and mutagens [57] . Biodegradation is used as a remediation method for reducing and eliminating the toxic compounds. Congo red is a toxic azo dye used in the textile industry that is structurally similar to lignin and lignin related compounds [2] . Microbial enzymes involved in lignin degradation are some of the same enzymes involved in decolorization of azo dyes [27]. Microbes capable of decolorizing Congo red

have potential in bioremediation of industrial waste and lignin degradation in biofuel production and the pulp industry.

Vanillin Utilization

Vanillin is a methoxylated, phenolic aldehyde that is found in lignin [28] and an aromatic compound used as flavoring in foods and perfumes. Vanillin can be produced through bioconversion, specifically the chemical oxidation of lignin [29]. Lignin is a potential substrate for biofuel production. Lignin is recalcitrant and is oxidized before fermentation. Most pretreatments involve acid hydrolysis or steam explosion, which results in the release of inhibitory compounds [58]. During this oxidation of lignin by-products such as vanillin are released. Vanillin is an extreme fermentation inhibitor due to its toxic effects on microbes used in fermentation. Clark et al. 1984 found a complete inhibition of biofuel producing microorganisms by vanillin during fermentation. Concentrations of vanillin differ in fermentations based on solids in the reactor, raw material used and pre-treatment conditions [58]. Inhibitors are detoxified in the pretreatment through evaporation, activated charcoal adsorption and solvent extraction [59]. Some of these methods are expensive and generate waste products. To increase biofuel yield and to attain maximum productivity during fermentation, microbial detoxification of inhibitory compounds is under investigation.

Microbial detoxification involves utilizing microbes and microbe produced enzymes that change the structure or utilize inhibitory compounds (e.g. vanillin) present in lignin hydrolysates without utilizing fermentable sugars. Microbial detoxification allows for detoxification concurrent with fermentation, mild reaction conditions (e.g.

mesophilic temperature), fewer toxic products and a lowered energy demand [58] . Identifying microbes that are capable of detoxification can also create possibilities for genetically engineering biofuel-producing microbes. Okuda et al (2008) shows that *Ureibacillus thermosphaericus*, a thermophilic, fast-growing bacterium, can detoxify waste house wood. *Ureibacillus thermosphaericus* detoxifies various inhibitory compounds (e.g. phenols, furfurals), which increases ethanol yields by the ethanol-producing microbes, while utilizing less than 5% of the fermentable sugars.

Bare et. al. (1992) examines the ability of a soil-isolated *Pseudomonas* (BTP9) isolate to convert vanillin to vanillic acid during the oxidation of lignin. BTP9 is grown on a minimal media with varying concentrations of vanillin as the sole carbon source. Its growth is monitored in the same media in liquid phase. Isolates capable of growth on vanillin as the sole carbon source are considered capable of utilizing vanillin as the sole carbon source and are then cultured in a vanillin culture media to produce cells for a vanillin bioconversion assays.

Butanol Tolerance

Industrial bioprocesses utilize organic solvents in various reaction media due to lower microbial contamination and the quality of products generated (e.g. higher molecular weight products). Bioconversion processes that produce solvents benefit from tolerant microbes and their stable enzymes. Organic solvent tolerant bacteria have a role in bioremediation and wastewater treatment. Most polyaromatic hydrocarbon contaminated sites contain high levels of organic solvents.

Organic solvents damage bacterial cell membranes disrupting normal cell function [60] . Solvent tolerant bacteria have adapted methods (e.g. solvent efflux pumps, lower cell membrane permeability, and rapid membrane repair) to avoid these toxic effects. The solvent concentration accumulating in the cell membrane is most fundamental in establishing toxicity, and isolate tolerance is affected by environmental factors and genetics [61]. Organisms capable of tolerating high concentrations of these compounds are useful in industrial bioprocesses. Butanol is regarded as a potential alternative to liquid transportation fuel, compared to other alternatives, butanol is more hydrophobic, has a lower volatility and a higher energy density [31]. The fermentation of butanol is restricted by low productivity and yield from sugars involved in Acetone-Butanol production. Improving isolate tolerance will help improve limitations in the fermentation process. Butanol inhibits the ability of the cell to maintain a stable internal pH and carbon uptake hindering cell growth and multiplication [62]. The average bacterial cells cannot tolerate 0.3% (v/v) butanol while even some tolerant isolates cannot exceed 2.0% (v/v). To improve butanol tolerance for industrial bioprocess two strategies are implemented: engineering through variation and selection and creating butanol synthetic pathways in butanol-tolerant microbes [63]. Butanol tolerant microbes exist in nature signifying potential host isolates for synthetic pathway manipulation.

Li et al (2010) describes two methods for screening environmental isolates for butanol tolerance: agar plate method using screening solid medium and liquid culture media with varying butanol concentrations. Environmental sand and soil samples are washed with distilled water and the supernatant is streaked onto screening growth media

containing 2.0% (v/v) butanol. Colonies that grow are then plated onto higher concentrations of butanol until the isolates that exhibit high butanol tolerance are identified. Those isolates are then run through a butanol tolerance assay where growth rates of liquid cultures grown in the presence of varying butanol concentrations and in the absence of butanol are compared.

II.2 Methods

Assembling the Bacterial Culture Library

Cope et al (2014) screened diverse sediments as inocula for carboxylate platform fermentations in order to identify the sediment environments that favor microbial communities with optimal traits. The sediments were collected from a geographically and ecologically diverse set of 501 samples from sites across 11 states in continental United States, Puerto Rico, and Hawaii (Figure 2). The site selection for the original study was based on interest in screening extremophilic microbial communities native to saline and/or thermal environments. Cope et al (2014) describes the sediment collection, screening for fermentation performance, and a multivariate analysis of the relationships between sediment characteristics and process performance.

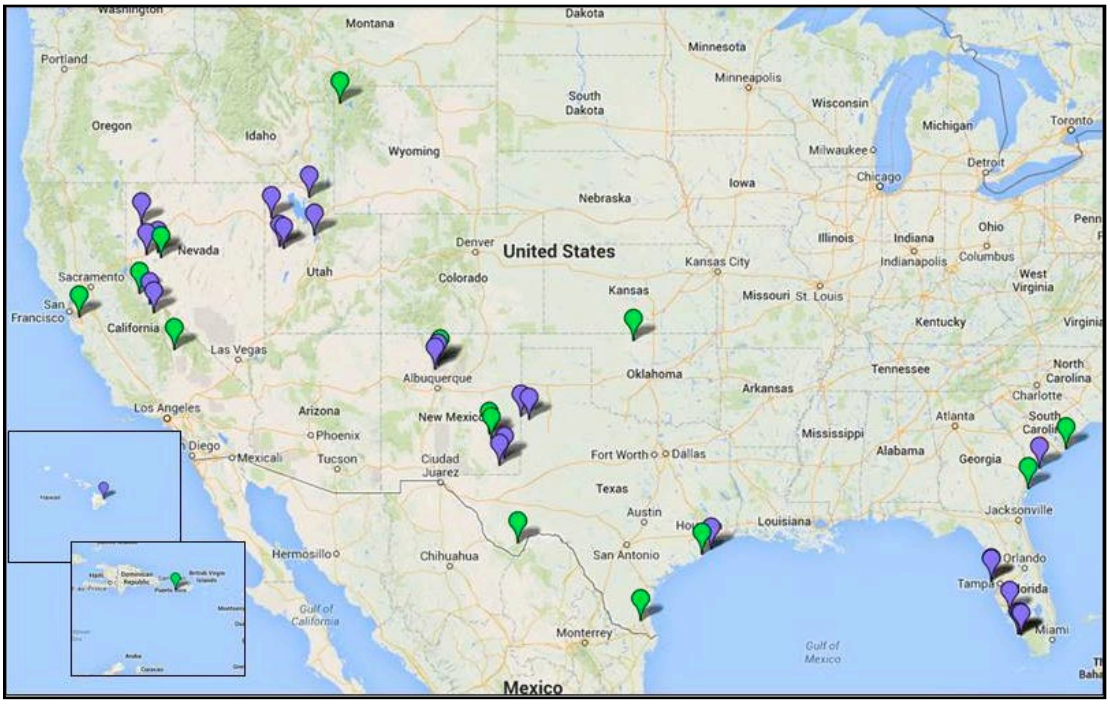


Figure 2. Locations in the United States and Puerto Rico where thermal and/or saline soils were sampled (Google Maps®)(accessed 13 December 2013). A green marker indicates locations where the inoculum for the 34 fermentation with top performing communities were collected. Isolates from these communities are those that are included in the library and some are used in the industrial trait screens. Isolates were first chosen for the industrial screens based on ecologically and geographically diversity of the soils from which the isolate originated.

The selection of communities for inclusion in the isolate library was based on the communities with superior conversion performance (g volatile solids digested/ g volatile solids fed), selected from the top ~5% of communities in the fermentation screen. Also included were communities from sites that maximized geographic diversity. In total, 34 fermentation screens were cultured. To further maximize diversity the selection strategy included using different media and oxygen conditions, and efforts were made to isolate similar numbers from each sample on each media.

Briefly, all fermentations were harvested at day 30 and the materials not used for

chemical analysis (liquids and solids associated with a particular microbial community) were collected and divided up for storage at 4°C and -20 °C [4]. In most cases to isolate bacteria for the library the 4°C stored materials were used, however, in instances of contamination or low viability due to extended period of storage the -20 °C material was used. The stored material was used to prepare 8-fold serial dilutions onto three types defined media: Drake's Thermophilic Acetogen Media [64](DTAM), modified by aerobic handling (Non-Hungate), and SL9 trace elements [65]; Cellulolytic Agar for Thermophiles [66] (CAT), modified by aerobic handling, and minimizing agar to 20g/L; and Modified Growth Medium [67] (MGM) a halophile medium. One hundred microliters of each dilution was streaked onto three agar plates of each medium type and grown at 55°C. For 6 of 34 fermentation communities two different approaches to limit oxygen were employed during culturing: growth in a nitrogen triple flushed and sealed food grade plastic bag (Sunbeam Products, Inc., Boca Raton, FL, USA, FoodSaverTM Model V2220) or an anaerobic chamber (Oxoid Limited, Hampshire, England, 2.5L AnaeroJar Assembly) with chemical reduction of oxygen (Oxoid Limited, Hampshire, England, AneroGen 2.5L Cat. #AN0025A), both performed after transferring within a biosafety cabinet with exposure to ambient oxygen levels. Based on the color of the oxygen indicator (Resazurin) in the CAT plates the oxygen appeared more limited in the chambers than in the sealed plastic bags. For the other 28 of the 34 samples only the nitrogen triple flushed and sealed food grade plastic bag method was employed.

Cultures were isolated to single colonies on solid medium and re-streaked 2 to 4 times to ensure a pure culture. At the final stage, most isolates were grown in the liquid

broth version of the given defined media under the same conditions as the primary culture; however, isolates that were more fastidious were cultured in LB broth [66] . Liquid cultures grew until turbid or until high colony numbers were observed after transfer back onto solid media. Stock cultures, established from these original liquid cultures, containing glycerol (20% of total volume) were stored at both -20°C and -80°C.

Phylogenetic Analysis of Bacterial Culture Library

Collecting DNA from an isolate involved transfer of a single colony into a 250µL polymerase chain reaction (PCR) tube containing 20µL of Alkaline Lysis Buffer (0.25% SDS, 0.05 N NaOH), then incubation at 95°C for 30 min. in a stable heat block with water, freezing at -20°C for 30 minutes, and combination of 80µL of room temperature Promega nuclease free water (Madison, WI, USA) with the lysed cell suspension. PCR amplification of the 16S subunit of the ribosome gene (16S rDNA gene) included 5µL of this DNA preparation. In instances when this process failed to provide a sufficient quality or yield of genomic DNA a Wizard® SV Genomic DNA Purification System (Promega Corp., Madison, WI, USA) extraction kit was employed. All lysis products were frozen at -20ºC until enough samples for a 96 well PCR amplification accumulated, or for a maximum of one week. A ~1450bp region of the 16S rDNA spanning variable region V1 to variable region V8 was amplified**.** Primers for PCR amplification were forward primer 27f: 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 1492r: 5'-CGGTTACCTTGTTACGACTT-3' (Lane 1991), supplied by Integrated DNA Technologies, Inc. (Coralville, IA, USA). PCR reaction constituents for a 50µL reaction included: 5µL 10x buffer (500mM KCl, 300mM TRIS pH 8.3, 15mM $MgCl_2$), 5µL bovine serum

albumin (BSA) (1.0mg/ml), 2µL $MgCl_2$ (25 µM), 4µL GeneAmp dNTPs (0.1mM, Life Technologies, Carlsbad, CA, USA, formerly Applied Biosystems), 1µL 27f, 1µL 1492r, 0.5µL *Taq* DNA Polymerase (2.5 Units New England Biolabs; Ipswich, MA, USA), 26.5µL H2O, and 5µL template DNA. Bovine serum albumin was used in the PCR reaction to alleviate inhibition of replication [68] . Amplification consisted of initial denaturation for 1 min. at 95°C followed by 35 cycles of 1 min. at 95°C (denaturation), 1 min. at 55°C (annealing), 1:30 min. at 72°C (extension); followed by one cycle of final extension for ten min. at 72°C; and then held at 4°C until separation by gel electrophoresis. Each group of PCR reactions included a positive and negative control (*E. coli* DNA and PCR grade water used as template for each respectively). All isolates were amplified by PCR with the nearly complete 16S rDNA primers listed even when smaller portions of the rDNA gene were the sequencing target, as the PCR product may be used for further sequencing should the need arise. Large 50µL PCR reactions were required for this project, as all samples were normalized to 15-25ng/µL for submission to Sanger sequencing. Products of PCR amplifications were visualized via agarose gel electrophoresis (SeaKem® LE Agarose, Lonza, Walkersville, MD, USA). PCR products quantification involved using an AlphaImager 2000 v5.5 with spot density software (Alpha Innotech Corp., San Leandro, CA, USA) to estimate intensity of each PCR product band to the intensity of a known concentration of DNA in the ladder bands. PCR products were diluted as necessary. Beckman Coulter Genomics, formerly Agencourt Bioscience Corporation (Danvers, MA,USA) sequenced the 16S rDNA gene of all isolates using the Sanger single pass method [69]. For the majority of samples sequenced

for this study the following primers were used: forward 27f: 5'-AGAGTTTGATCCTGGCTCAG - 3'[70] and reverse 519R-Gray: 5'-GTNTTACNGCGGCKGCTG - 3' [71].

Post Sequence Processing

Reads for each sample were assembled into contigs using Sequencher 4.10.1 (GeneCodes Corporation, Ann Arbor, MI, USA). Ribosomal Database Project (RDP) Classifier function (accessed 11-11-11) [72] was used to reference against the contigs for putative identities of the isolates. The RDP pyrosequencing pipeline aligner function (accessed 11-11-11) was used to align all sequences. The alignment was trimmed to a constant length using Bioedit v7.0.9 [73] . To assess phylogenetic diversity, all samples that formed a contig with a 97% identity cut off for assignment of OTU using the cluster command (average neighbor default) of mothur v1.22 [74] were analyzed and were deposited into the National Center for Biotechnology Information (NCBI) GenBank accession numbers KC847997 - KC848049 and KC849718 - KC851724. A representative library of different isolates as determined by OTU assignment and the get.oturep function in mothur v1.22 in the RDP Tree Builder program (accessed 11-11-11) with the inclusion of the highest similarity sequence in the RDP Hierarchy Browser (accessed 11-11-11) feature as nearest neighbors for classification were obtained for comparison from the SeqMatch feature of the RDP website (accessed 11-11- 11) were obtained. For comparison all nearest neighbor sequences were aligned using the RDP aligner function (accessed 11-11- 11) with the corresponding database of representative sequences and the sequences were trimmed to the same length as the project isolate

sequences using Bioedit. The RDP Tree Builder using Jukes-Cantor corrected distance matrix modeling, and Weighbor, a weighted neighbor-joining tree algorithm [75] were used for tree estimation with bootstrapping at 100 iterations.

Phenotypic Characterization

While a total of 1866 isolates exist in the library a subset of 207 isolates was selected for subsequent phenotypic screens. The inclusion in the subset was based on phylogenetic classification (OTU representation), to include OTUs that were closely related to known isolates possessing the traits of interest (Table 1), geographic diversity of the sites from which the isolates originated, and maximizing the variety of media types used to isolate the isolates. It is worth noting, all the isolates in the 207 subjected to screening were isolated under the less restrictive triple nitrogen flushing method for exclusion of oxygen.

Growth Temperature Assay

The original carboxylate platform fermentations and subsequent isolate collected occurred at 55°C, however, not all industrial processes occur at such high temperatures. Therefore, to determine whether any isolates possessed the capacity to grow at lower temperatures, all 207 isolates were grown both 37°C and 55°C.

Table 1. Taxonomic identification of representative isolates for each operational taxonomic unit and identified phenotypes. BLAST IDs were from the National Center for Biotechnological Information's BLAST 16s ribosomal RNA sequences (Bacteria and Archaea) alignment tool, which was accessed 24-04-13. The BLAST search did not include uncultured organisms, environmental samples or metagenomes. The representative sequence for each OTU was used in the search.

OTU	NCBI BLAST ID[a]	Industrially Relevant Phenotypes
1	*Geobacillus pallidus*	Thermophilic emulsifier-producer capable of hydrocarbon degradation [76]
2	*Geobacillus vulcani*	Produces acids from glycerol, lactose and ribose [77]
4	*Bacillus thermoamylovorans*	Thermophilic, facultative anaerobe capable of fermenting starch and cellobiose and producing ethanol [78]
5	*Bacillus asahii*	Hydrocarbon utilizing bacterium [79]
6	*Geobacillus thermoglucosidsus*	Thermophilic fermentative bacterium that produces mixed acids and is tolerant to ethanol [80, 81]
7	*Actinomadura rubrobrunea*	Anaerobic to facultative anaerobe that produces mixed acids during glucose fermentation [82]
8	*Bacillus smithii*	Arsenic tolerant, lipase producer [83]
9	*Geobacillus debilis*	Gram-negative, aerobic, thermophile capable of utilizing cellulose and starch. [84]
11	*Bacillus licheniformis*	Cellulose degrader used in industry for the production of proteases, amylases and antibiotics/optimal growth and enzyme production temperature is 37°C [85]
12	*Geobacillus pallidus*	Thermophilic, emulsifier-producer capable of hydrocarbon degradation[76]
14	*Ureibacillus thermosphaericus*	Thermophilic bacterium that degrades phenolics and other toxic compounds [86]
15	*Brevibacillus thermoruber*	Thermophilic bacterium that produces sulfates and exopolysaccharides [87]
19	*Geobacillus stearothermophilus*	Thermophilic bacterium that produces thermostable amylase and xylanases/biological indicator used in industry/isolate isolated from a hot spring Yellowstone National Park is used for thermophilic analyses, [88]
20	*Geobacillus stearothermophilus*	Thermophilic bacterium that produces thermostable amylase and xylanases/biological indicator used in industry/isolate isolated from a hot spring Yellowstone National Park is used for thermophilic analyses, [88]
21	*Bacillus thermoamylovorans*	Thermophilic, facultative anaerobe capable of fermenting starch and cellobiose and producing ethanol [78]
22	*Caldalkalibacillus uzonensis*	Thermophilic, aerobic alkali-tolerant gram-positive bacterium shown to tolerate high levels of CO and up to 6%w/v NaCl [89]
28	*Ureibacillus terrenus*	Soil inhabitant thermophile able to grow in pH 9.0 and in 5%NaCl and able to produce mesophilic and thermophilic compost related enzymes (e.g. xylanase, cellulase, amylase) [90]
32	*Bacillus aeolius*	Halophilic, thermophile capable of producing surfactants and utilizing hydrocarbons [91]
36	*Bacillus psychrodurans*	Gram-positive, psychrophile capable of growth at -2°C and in up to 5% NaCl [92]
37	*Luteimonas aestuarii*	Gram-negative, mesophile capable of growth in up to 3% NaCl isolated from tidal flat sediment [93]
40	*Geobacillus pallidus*	Thermophilic emulsifier producer capable of hydrocarbon degradation [76]
41	*Bacillus psychrodurans*	Gram-positive, psychrophile capable of growth at -2°C and in up to 5% NaCl [92]
44	*Geobacillus thermoleovorans*	Thermophile that can grow on a wide range of carbon substrates (e.g. cellobiose and hydrocarbons) [94]
45	*Pseudomonas gessardii*	Gram-negative, mesophilic fluorescent bacterium that produces lipase [94, 95]

This entailed streaking the isolates from -20°C glycerol stocks onto LB agar, incubation at both temperatures in the dark in vacuum-sealed bags triple flushed with nitrogen. Agar plates were monitored daily for growth. Furthermore, in this study two of the trait screens, n –butanol tolerance and vanillin utilization, are only effective at 37°C, thus only isolates with the capacity to grow at 37°C were included in screens for those traits.

Extracellular Cellulase Production

Isolates were transferred from glycerol stocks to LB media containing 1.5% bacto-agar (LB agar). Individual colonies were used to streak another LB agar plate and this plate was grown overnight in vacuum-sealed, nitrogen gassed bags in the dark at 55°C. One colony from the overnight agar plate was streaked onto one carboxymethyl cellulose (CMC) agar plate. The CMC agar plates were streaked in replicates of three with two isolates per plate, vacuum-sealed, nitrogen gassed and incubated at 55°C, in the dark for 7 days. After incubation, CMC agar plates were flooded with Gram's iodine for 5 minutes as previously described by [56] . The Gram's iodine was removed from the plate to identify zones of cellulose clearing. Isolate LMG 21723 *Cellulomonas xylanilytica* from Ghent University BCCM/LMG Bacteria Collection was used as a positive control.

Congo Red Decolorization

Liquid cultures of isolates were made using a single overnight colony grown on LB agar. Congo red was added to LB broth at 25 mg/L and 330 μL of this solution was added to each well of a 96-well plate. Twenty microliters of overnight liquid LB broth culture were randomly inoculated onto the 96-well plate in replicates of four. The plate

was incubated at 55°C in the dark for 24 hours, after incubation degradation of Congo red was visually observed for each replicate by the absence of color (Figure 3). The plate was centrifuged for 20 minutes at 2,250x *g*. The supernatant was transferred to a new 96-well plate and the absorbance was read by the TECAN spectrofluorometer (Tecan Group LtD., Switzerland) at 450 nm. Decolorization was calculated as a percent of the initial absorbance of un-inoculated Congo red media at 450 nm as previously described by [2].

Growth Curve Analysis for n-Butanol Tolerance and Vanillin Utilization

Assays for both n-butanol tolerance and vanillin utilization involved spectrophotometric data generated using an automated growth-curved generator, the Bioscreen C system (Lab systems, Helsinki, Finland) (Figure 4).

All isolates were grown overnight liquid culture in LB broth. Within each treatment isolates were grown in randomly assigned locations on a 100-well honeycomb plate represented by 4 replicates. The plates were loaded with 330 µL of media and 20 µL of an overnight culture. LB containing either 0% or 2% butanol was used for the butanol screening while MSM containing 0.2g/L of vanillin or no carbon source was used in the vanillin utilization screens. The optical density measurements were taken using the wide band filter at 450-580nm at 55°C for 48 hours for butanol and 37°C for over 72 hours for the vanillin screening. The plate was shaken for 10 seconds at medium speed before each four-hour measurement interval. The average absorbance at each time point was used to generate the growth curve.

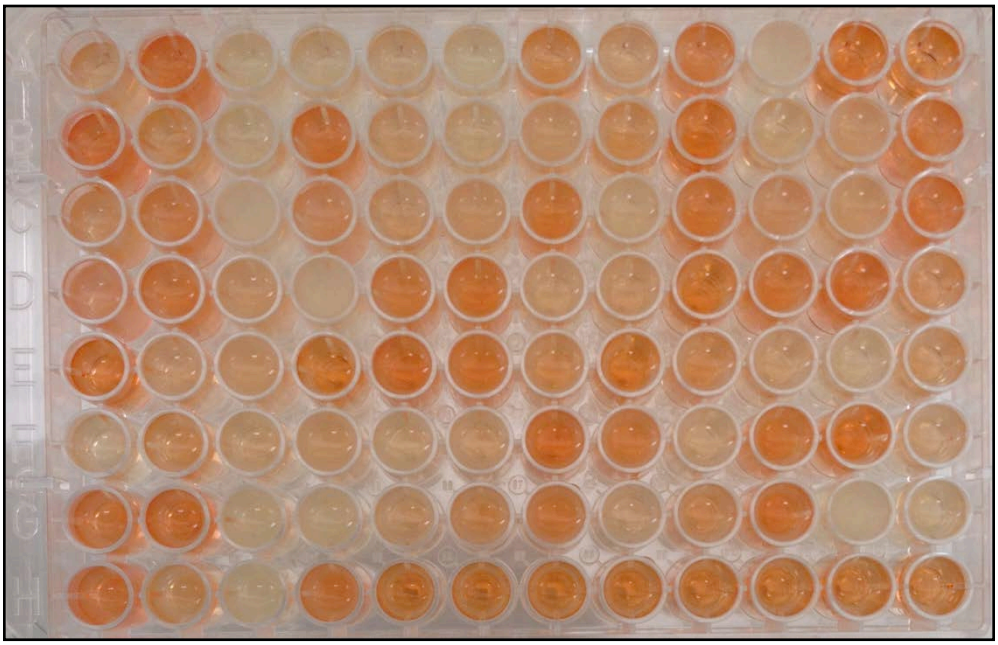


Figure 3. Congo red assay employs a 96-well plate. Pictured is an assay with 4 replicates of each isolate wherein isolates and replicates randomly distributed across the plate. These results are visible 48 hours after inoculation with library isolates. Decolorization was calculated as a percent of the initial absorbance of un-inoculated Congo red media at 450 nm as previously described by [2]. Those isolates that exhibited Congo red decolorization of more than 50% were considered positive based on the highest levels of decolorization seen in Bandounas et al 2011.

a. b.

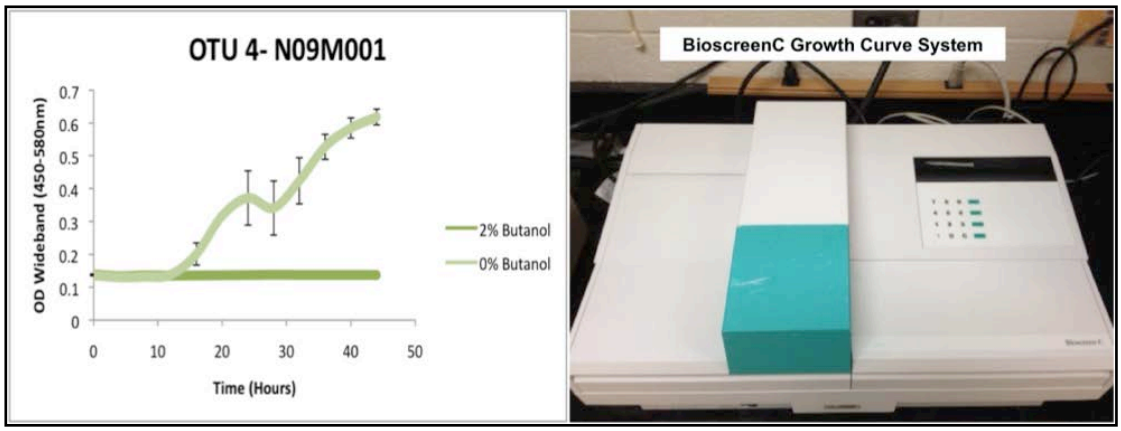


Figure 4. The BioscreenC growth curve system and growth curve data generated from the instrument. a. Growth curve of isolate in 2% butanol. b. BioscreenC. Two hundred and seven isolates were screened for butanol tolerance. Of these 207 isolates screened, zero isolates were tolerant to 2% butanol. Error bars indicate the standard error of four replicates. b. The BioscreenC used to generate growth curves. The BioscreenC is an automated growth curve generator used in tolerance and utilization screens.

Growth inhibition in the vanillin screening was calculated as a percent of the specific growth rate in the presence of vanillin.

Isolates were considered positive for vanillin utilization if the specific growth rate in the absence of vanillin was decreased by 85% or more in comparison to the specific growth rate in the presence of vanillin as a carbon source.

II.3 Results

Phylogenetic Analysis of the Library

Partial 16S rDNA sequences for a total of 1,866 isolates from 34 fermentation communities were generated (NCBI Genbank accessions: KC847997-KC848049,

KC849718-KC851724) (Table 2). Out of a total of 1,866 sequences analyzed there were a total of 230 unique, non-identical at the DNA sequence level, sequencing reads. The sequences were binned at 97% similarity, commonly associated at the species level with a total of 46 Operational Taxonomic Units (OTU). OTU 31 is an *Escherichia coli* control group sequenced as a positive indicator of quality during extraction, PCR, and sequencing reactions. Of the 1,866 isolates: 1,260 isolates collapsed into OTU 1, 251 into OTU 2, 110 into OTU 8, 102 into OTU 4, 57 into OTU 6, 73 into OTU 24, which mostly contains isolates that were isolated under stricter anaerobic conditions, and 44 into OTU 5. All other OTUs had 35 isolates or fewer within each (Table 3).

Figure 5 shows a phylogeny of the distinct bacterial lineages (OTUs) in the context of the type sequences identified within the Ribosomal Database Project (RDP). Of the 230 unique sequences, 223 or 97% were in the phylum *Firmicutes*. Of the *Firmicutes* class *Bacilli* was 85% (196/230) of unique isolates. Class *Clostridia* made up the remainder of those sequences within *Firmicutes* with 11.7% (27/230) of unique isolate sequences. Additionally phylum "Actinobacteria" class *Actinobacterida* occurred at 0.9% (2/230) unique level sequences, and phylum *Proteobacteria* class *Gammaproteobacteria* with 1.3% (3/230) of the library at the unique sequence level.

Extracellular Cellulase Production

The 207 isolates screened for cellulase production on CMC agar plates represented 24 OTUs. Slightly more than half of the 207 were showed zones of clearing (Figure 6) (positive) in the assay (Table 4), 107 from 15 OTUs. Out of twenty-one total isolates screened from OTU 1, only 3 were cellulase producers.

Table 2. Summary of isolate and sequence counts across fermentation experiments and culture conditions imposed. This table represents the sequence data included in the phylogenetic analysis. This library of isolates originated from fermentation experiments inoculated with soils from different sites selected as putative extreme environments [5]. Focus for isolate library construction efforts was mainly fermentations with superior conversion performances; other fermentations included were based on maximizing geographic diversity. Isolation targets with fermentation identification number, corresponding soil identification number, location of soil sample, site name of soil sample, conversion percentage of fermentation, isolate (Iso.), and sequence (Seq.) numbers of isolates, across media and oxygenation regime, and totals within sites of isolates and sequenced isolates. Table adapted from [4] with permission.

Fermentation ID	Soil Sample Name	State	Site Name	Facultative Aerobic Acetogenic		Facultative Aerobic Halophilic		Facultative Aerobic Cellulose		Anaerobic Acetogenic		Anaerobic Cellulose		Total Isolates Per Site	Total Seq. Per Site
				Iso.	Seq.	Iso.	Seq.	Iso.	Seq.	Iso.	Seq.	Iso.	Seq.		
S48	STYS3	WY	Yellowstone	30	30	20	19	30	27	0	0	0	0	80	76
S44	FHYS5	WY	Yellowstone	34	34	21	21	30	24	13	13	12	12	110	104
F02	Bra2	TX	Brazoria	32	30	26	26	27	27	0	0	13	13	98	96
G23	LL2	NM	Lazy Lagoon (Roswell)	44	39	20	20	24	27	0	0	12	12	100	98
H01	SFB1	CA	San Francisco Bay	28	23	27	25	12	11	0	0	0	0	67	59
G13	BL13	NM	Bitter Lake (Roswell)	45	43	20	20	30	24	0	0	0	0	95	87
J04	Big4	TX	Big Bend	32	30	15	13	25	27	0	0	23	23	95	93
G08	BL8	NM	Bitter Lake (Roswell)	26	23	22	21	25	26	0	0	0	0	73	70
F05	Bra5	TX	Brazoria	30	30	21	16	0	0	0	0	0	0	51	46
U22	OLCA1	CA	Owens Lake	30	30	27	27	30	28	9	9	7	7	103	101
J19	Big19	TX	Big Bend	30	28	19	19	30	20	0	0	0	0	79	67
H20	SFB20	CA	San Fran Bay	8	8	12	12	25	23	0	0	0	0	45	43
G19	BL19	NM	Bitter Lake (Roswell)	30	27	19	19	25	26	0	0	0	0	74	72
J18	Big18	TX	Big Bend	30	29	0	0	27	25	0	0	0	0	57	54
G24	LL3	NM	Lazy Lagoon (Roswell)	30	24	26	26	30	23	0	0	0	0	86	73
K49	BHS5	UT	Baker Hot Spring	30	16	23	23	30	26	0	0	0	0	83	65
P01	BWR1	PR	Puerto Rico	1	1	18	18	24	18	0	0	0	0	43	37
E08	GSP8	OK	Great Salt Plains	31	30	23	22	29	31	0	0	13	13	96	96

Table 2. Continued.

Fermentation ID	Soil Sample Name	State	Site Name	Facultative Aerobic Acetogenic		Facultative Aerobic Halophilic		Facultative Aerobic Cellulose		Anaerobic Acetogenic		Anaerobic Cellulose		Total Isolates Per Site	Total Seq. Per Site
				Iso.	Seq.	Iso.	Seq.	Iso.	Seq.	Iso.	Seq.	Iso.	Seq.		
M24	CR22	SC	Cape Romain	30	27	25	25	30	22	0	0	0	0	85	74
N09	SI9	GA	Sapelo Island	30	24	22	21	30	23	0	0	0	0	82	68
R08	NSS3	NM	Vellas Caldera	31	23	16	16	30	21	0	0	0	0	77	60
T02	SWRN2	NV	Still Water	30	20	19	19	30	25	0	0	0	0	79	64
A07	LSDR T1-325	TX	La Sal Del Ray	0	0	24	24	0	0	0	0	0	0	24	24
J11	Big11	TX	Big Bend	2	2	13	11	0	0	0	0	0	0	15	13
J20	Big20	TX	Big Bend	0	0	19	17	0	0	0	0	0	0	19	17
G09	BL9	NM	Bitter Lake	30	26	0	0	0	0	0	0	0	0	30	26
G21	BL21	NM	Bitter Lake	0	0	0	0	0	0	0	0	0	0	0	0
F09	Bra91	TX	Brazoria	30	30	9	9	0	0	0	0	0	0	39	39
F06	Bra61	TX	Brazoria	0	0	23	21	0	0	0	0	0	0	23	21
F22	Bra12	TX	Brazoria	0	0	7	7	0	0	0	0	0	0	7	7
F08	Bra81	TX	Brazoria	30	28	0	0	0	0	0	0	0	0	30	28
F01	Bra11	TX	Brazoria	0	0	17	17	0	0	0	0	0	0	17	17
E07	GSP7	OK	Great Salt Plains	0	0	14	13	16	16	0	0	0	0	30	29
G47	BLM21	NM	BLM; Laguna Gatuna	0	0	5	5	0	0	0	0	0	0	5	5
N/A	JGI	N/A	Day15 - EBH Experiment	0	0	0	0	0	0	0	0	30	30	N/A	30
N/A	EB7	N/A	Electric Bacteria	0	0	0	0	0	0	0	0	0	0	N/A	7
				734	655	572	552	589	520	22	22	110	110	1997	1866

Table 3. Distribution of operational taxonomic units by original fermentation experiment. Each fermentation experiment corresponded to a single sediment sample as inoculum [5] Rows correspond to each Operational Taxonomic Unit (OTU) numbers based on a phylogenetic analysis. Columns correspond to the fermentation community or other source for the isolates (Elec. indicates electrical fuel cell project [96]; JGI indicates Joint Genome Institute project isolates [7]; and E. coli indicates internal control isolates). The final column and final row correspond to the sum of all isolates within each OTU and each fermentation sample respectively. Coloring indicates red (no or few samples) to green (many samples) and is calculated by category. Table adapted from [4] with permission.

OTU	A07	E07	E08	F01	F02	F05	F06	F08	F09	F22	G08	G09	G13	G19	G21	G23	G24	G47	H01	H20	J04	J11	J18	J19	J20	K49	M24	N09	P01	R08	S44	S48	T02	U22	Elec.	JGI	E. coli	Sum
1	4	22	51	14	35	40	20	28	33	5	62	27	86	51	89	53	0	5	47	7	39	5	29	65	7	27	58	61	23	41	73	46	37	70	0	0	0	1260
2	0	17	8	0	41	1	0	1	2	0	4	0	11	21	0	10	13	0	4	11	18	0	2	12	3	0	6	6	7	16	18	10	0	9	0	0	0	251
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
4	20	0	2	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	6	12	4	0	0	0	0	0	0	4	4	2	22	0	24	0	0	0	0	102
5	0	0	1	4	23	6	0	0	4	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	3	0	0	0	44
6	0	0	2	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	1	0	0	0	2	26	0	22	0	0	0	57
7	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
8	0	0	17	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	2	40	0	16	1	0	23	0	0	0	0	1	0	0	6	0	0	0	110
9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	2	0	0	6	0	0	0	3	0	0	0	1	0	0	19
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1
11	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	5	0	0	5	4	0	0	0	0	0	0	0	0	0	0	0	17
12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	4	0	3	3	0	0	0	3	2	0	0	0	16
13	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6
14	0	0	0	0	2	0	0	1	0	0	0	0	0	11	0	0	11	0	0	0	0	0	0	0	0	4	0	0	0	0	0	6	0	0	0	0	0	35
15	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
16	0	0	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1
19	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2

Table 3. Continued.

OTU	A07	E07	E08	F01	F02	F05	F06	F08	F09	F22	G08	G09	G13	G19	G21	G23	G24	G47	H01	H20	J04	J11	J18	J19	J20	K49	M24	N09	P01	R08	S44	S48	T02	U22	Elec.	JGI	E. coli	Sum
20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
22	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
24	0	0	5	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	22	0	0	0	0	0	7	0	0	0	9	0	0	0	5	15	0	73
25	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	13	0	21
26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
27	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	0	0	9	0	0	0	0	0	0	0	0	0	0	0	16
28	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
29	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
30	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	1	3	0	7
31	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2
32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
33	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
34	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
36	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
37	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
38	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	2
39	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
41	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
42	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
43	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
44	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
45	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	2
46	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1

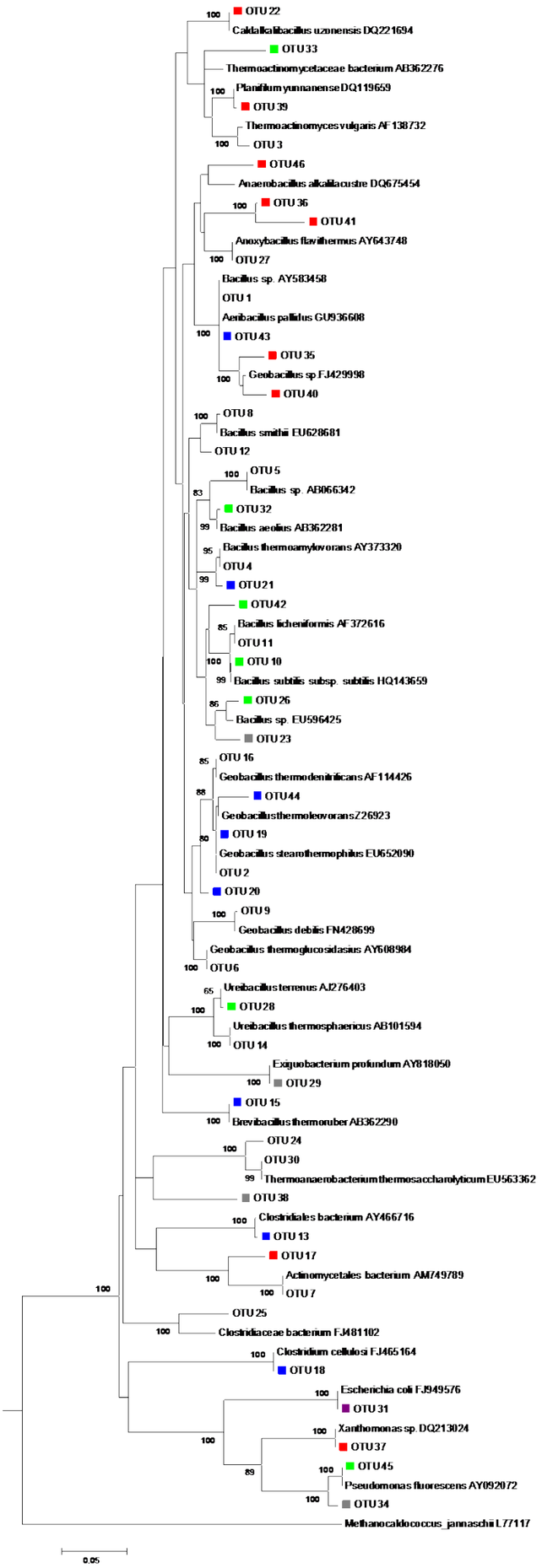


Figure 5. Neighbor joining phylogenetic tree of all representative operational taxonomic units (OTUs) and closest sequences within Ribosomal Database Project (RDP) SeqMatch (accessed 11/11/11) adapted from [4]. Isolates with a 97% partial 16s rRNA sequence similarity were collapsed into the same Operational Taxonomic Unit (OTU). Tree constructed using Jukes-Cantor corrected distance matrix modeling and weighted neighbor-joining with bootstrapping at 100 iterations. Colored squares indicate those branches resulting from a single media/oxygen incubation type: cellulose/ anaerobic chamber (grey), cellulose (blue), acetogen (red), halophile [3], other (purple).

Given that this OTU was the most abundant in the library, additional screening for this trait in that clade is certainly possible. Placing this phenotype in the context of the original media type from which the isolates were recovered, of the 94 isolates screened originating from CAT media 62 were positive, 38 were positive out of 76 originating on MGM5 media, and 7 out of 37 isolated on DTAM. Twenty-one of the isolates positive for this screen originated at one site, S48 (Yellowstone, WY), 26 isolates from this site were screened. Twenty of the S48 isolates were originally isolated using CAT media and fell into OTU 6. Twenty-two out of the 30 tested from sites in Brazoria, TX were positive for cellulose degradation. Sixteen out of the 29 tested from Big Bend, National Park were positive for cellulose degradation.

Congo Red Decolorization

Isolates positive for Congo red decolorization exhibited 50% or greater decolorization. Forty-two out of 207 isolates were positive for Congo red decolorization. One isolate each from OTUs 15, 20, 21, 44 was positive for this screen. Eighteen positive isolates were from OTU 6 (14 isolates originated in S48 sediment) while 9 were from OTU 4, 7 from OTU 2 and 2 from OTUs 1 and 9. Thirty of the 94 tested originally isolated with CAT media were positive for Congo red decolorization.

Growth at 37°C

As expected all 207 isolates grew at 55°C, the temperature used for the carboxylate platform fermentations and the original isolation for the isolate library. Forty-three isolates out of 207 isolates also grew at 37°C. Eight out of the 24 OTUs screened included isolates with this capacity.

Figure 6. Carboxymethyl cellulose (CMC) agar plates. Plates are inoculated with isolates from OTU 2: S44C007 and OTU 11: J04M004, J11M005 and J11M011. After seven days, the CMC agar plates were flooded with Gram's iodine for 5 minutes. Zones of clearing can be seen around isolates S44C007, J04M004 and J11M005 indicating carboxymethyl cellulose degradation.

Table 4. Screening results by OTU**.** 207 isolates were screened for the ability to degrade cellulose, decolorize Congo red, grown under mild temperature conditions. Isolates were chosen based on OTU, media type and original site, to maximize diversity. National Center for Biotechnological Information (NCBI) BLAST search was conducted on the representative sequence of each OTU using the 16s ribosomal RNA sequences (Bacteria and Archaea) alignment tool which was accessed 24-04-13. The BLAST search did not include uncultured organisms, environmental samples or metagenomes and differed from the OTU assignments generated on the phylogenetic tree. The unique column is the number of unique isolate sequences identified in that OTU out of the total sequences in that OTU.

OTU	NCBI Blast ID	Unique/Total	Total Screened	Growth at 37°C		Cellulose Degradation		Congo Red Decolorization	
				(+)	(-)	(+)	(-)	(+)	(-)
1	*Geobacillus pallidus*	102/1260	21	1	20	3	18	2	19
2	*Geobacillus vulcani*	26/252	19	1	18	15	4	7	12
4	*Bacillus thermoamylovorans*	7/102	32	18	14	19	13	9	23
5	*Bacillus asahii*	4/44	22	9	13	19	3	0	22
6	*Geobacillus thermoglucosidasius*	3/57	32	6	26	29	3	18	14
7	*Actinomadura rubrobrunea*	1/1	1	0	1	0	1	0	1
8	*Bacillus smithii*	6/110	16	0	16	5	11	0	16
9	*Geobacillus debilis*	8/19	9	0	9	3	6	2	7
11	*Bacillus licheniformis*	6/17	9	7	2	6	3	0	9
12	*Geobacillus pallidus*	3/16	13	0	13	2	11	0	13
14	*Ureibacillus thermosphaericus*	2/35	16	0	16	1	15	0	16

Table 4. Continued.

OTU	NCBI Blast ID	Unique/Total	Total Screened	Growth at 37°C		Cellulose Degradation		Congo Red Decolorization	
				(+)	(-)	(+)	(-)	(+)	(-)
15	*Brevibacillus thermoruber*	2/3	3	1	2	0	3	1	2
19	*Geobacillus stearothermophilus*	2/2	1	0	1	1	0	0	1
20	*Geobacillus stearothermophilus*	1/1	1	0	1	1	0	1	0
21	*Bacillus thermoamylovorans*	1/1	1	0	1	1	0	1	0
22	*Caldalkalibacillus uzonensis*	1/1	1	0	1	0	1	0	1
28	*Ureibacillus terrenus*	1/1	1	0	1	1	0	0	1
32	*Bacillus aeolius*	1/1	1	0	1	0	1	0	1
36	*Bacillus psychrodurans*	2/2	2	0	2	0	2	0	2
37	*Luteimonas aestuarii*	1/1	1	0	1	0	1	0	1
40	*Geobacillus pallidus*	1/1	1	0	1	0	1	0	1
41	*Bacillus psychrodurans*	1/1	1	0	1	0	1	0	1
44	*Geobacillus thermoleovorans*	1/1	1	0	1	1	0	1	0
45	*Pseudomonas gessardii*	1/2	2	0	2	0	2	0	2
			207	43	164	107	100	42	165

OTUs 1, 2, 8 and 15 contained one positive isolate per OTU. OTU 4 contained 18 positive isolates while 9 positive isolates were in OTU 5.

OTUs 6 and 11 contained 6 positive isolates each. Considering media associated with the original isolation, 29 (14 of which were from OTU 4 and 6 from OTU 11) of the 43 were from MGM5 media while 1 was from DTAM and 13 (6 of which were from OTU 6) were from CAT media. Nine isolates out of 12, which were from MGM5 media and OTU 4, were from A07 (La Sal del Ray, TX) and positive for growth at 37°C.

Tolerance to the Organic Solvent n-Butanol

The entire screening subset of the library (n=207) was screened for tolerance to 2% *n*-butanol and no isolate from any OTU was tolerant (data not shown).

Vanillin Utilization

Once again, since screens for microbial detoxification of compounds found in lignocellulosic hydrolysates require mild reaction conditions [58], the vanillin utilization screen was conducted at 37°C. Only the 43 isolates capable of growth at that temperature were included in the screen. Of the 43 isolates screened, 13 utilized vanillin. Nine isolates from OTU 4 were positive, 3 were originally isolated on CAT media and from H20 (San Francisco Bay, CA) and 6 were originally isolated on MGM5 media (5 isolates from A07, LOCATION). Three were positive from OTU 5, and they were all isolated originally on MGM5 media. One isolate was positive from OTU 2 and it was originally isolated on CAT media and the H20 site.

II.4 Discussion

Collection, cataloging, and characterization of microbial resources from extreme environments ought to provide for efforts to improve industrial bioprocesses. This isolate library was assembled from a geographically diverse group of 34 microbial communities collected from the best performing carboxylate platform batch fermentations originally inoculated with sediments from saline and/or thermal environments [5]. Furthermore, through the use of a variety of media and oxygenation conditions, the intent was to create a diverse microbial resource. These efforts were successful, a diverse array of distinct bacterial lineages (OTUs) were assembled in this library. Not surprisingly, the majority of the OTUs isolated from fermentations successful at biomass conversion and/or carboxylic acid production were from the phylum Firmicutes, which harbors facultative and obligate anaerobes including lactic acid bacteria and food spoilage agents. In the very well represented OTUs there tended to be overlap across the media types (Table 3) (Figure 7). Conversely, the more rare and unique OTUs were more likely to occur on fewer media. This might well be reasonable to expect since isolates rare in the original fermentation communities ought to have a low probability of being captured regardless of media type, simply because of the relative low abundance or frequency across sites. However, it is also to be expected that some isolates are only favored under particular culture conditions. There is some evidence of both possibilities within the distribution patterns of the rare OTUs. Specifically, 4% were captured on all three media, 6% on both CAT and MGM5, 9% on

CAT and DTAM, 4% on both DTAM and MGM5, and 63% of the OTUs resulted from a single media or a single media and oxygenation condition. Perhaps a most noteworthy evidence for the role of culture conditions was that only by employing the anaerobic chamber in combination with the cellulose agar for thermophiles (CAT) 4 OTUs (OTUs 23, 29, 34, and 38) were captured (Figure 5). Thus, this approach of relatively deep sampling of a reasonably large number of fermentation communities, using multiple media types and oxygenation conditions successfully resulted in a diversity of OTUs represented in the library.

To evaluate diversity in traits of interest for a variety of biofuel bioprocess applications a diverse subset of the library (n=207) was subjected to screens for cellulose degradation, growth temperature, and lignin-like dye decolorization (Table 4).

OTU1 corresponds to *Geobacillus pallidus.* This species has been described as a thermophile, which is consistent with the findings in this study; most of the isolates from OTU1 were unable to grow at 37ºC. While *Geobacillus pallidus* is known to produce cellulase on CMC agar [97], of the 21 OTU1 isolates screened from this library only three (14%) were positive for cellulose degradation (Table 4). This seems noteworthy since cellulose degradation was the most likely trait for isolates to possess across the 207 isolates within the screened library subset (52% positive for this trait; Table 4) and OTU1 was the most represented OTU in this culture library (68% of the 1,866 isolates fell in this OTU).

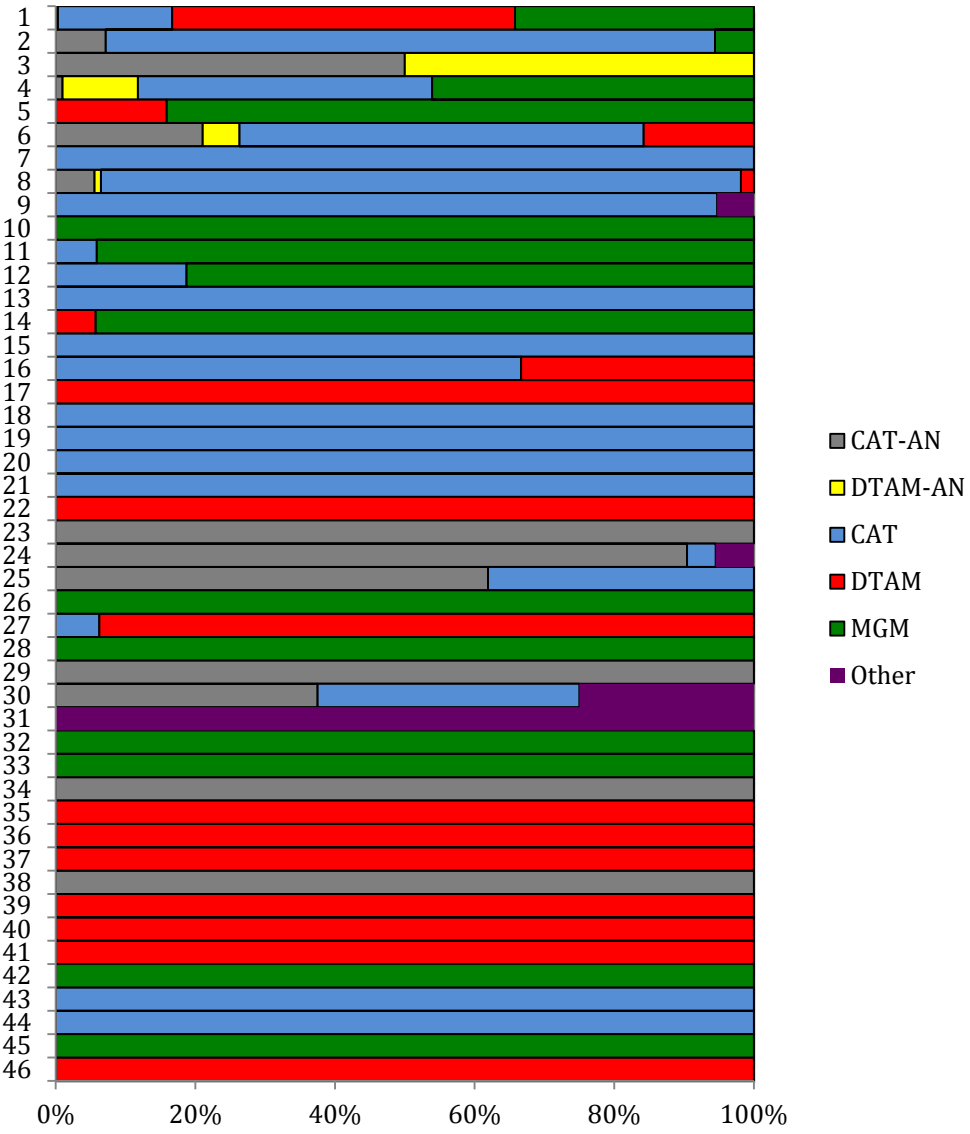


Figure 7. Media and oxygen incubation method associated with each operational taxonomic unit (OTU) identified within the library. Adapted from [4]. Bars indicate the relative proportion of each culture condition for each OTU (labeled by number on the left axis). Abbreviations: cellulose (CAT), acetogen (DTAM), halophile (MGM), and anaerobic chamber incubations (-AN).

Additionally, Maier et al (2004) demonstrated that a *Bacillus pallidus*, now *Geobacillus pallidus,* isolate isolated from textile finishing effluent showed no ability to degrade azo dyes. This aligns well with the results of our study; only 2 isolates out of 21 isolates from the OTU most closely related to *Geobacillus pallidus* decolorized the lignin-like azo dye Congo red. These two isolates did not degrade cellulose; they were from two different sites (E08 and F09) and were isolated using two different media types (DTAM and MGM5). *Bacillus pallidus* is also known to tolerate ~2.4% (v/v) of 2-propanol in MSM and produces solvent stable enzymes (e.g. lipase) [98]. It seems noteworthy, that no isolate from OTU1 or from any other OTU in our screened library subset, was tolerant to 2% (v/v) of the organic solvent *n*-butanol. This result is distinct from the original hypothesis that isolates from sites with high total dissolved solids and/or high salinity might well tolerate higher solvent or product concentrations.

In the BLAST analysis of all representative sequences, OTU 2 was most closely related to *Geobacillus vulcani,* which has been shown to degrade aromatic compounds associated with lignin [99] . There do not seem to be published data from any evaluation of *G. vulcani* for the capacity to degrade cellulose, to degrade lignin, or to tolerate organic solvents. In OTU2 of the 19 isolates screened 79% degraded cellulose and 32% were positive for Congo red decolorization. Published studies of *Geobacillus vulcani* isolates indicate that the growth temperature for this species is between 45°C and 70°C [77], this was consistent with 95% of the OTU2 isolates growing at 55ºC, but not at 37ºC. One OTU2 isolate, H20C009, not only grew at 37ºC, but also, it was positive for all traits screened (Table 4 and Table 5). Subsequent BLAST analysis of a longer,

untrimmed nucleotide sequence for H20C009 indicated it best match is *Geobacillus stearothermophilus*. *G. stearothermophilus* has the demonstrated capacity to degrade azo dyes [100] .

OTU4 was most closely related to *Bacillus thermoamylovorans,* which degrades cellulose in co-cultures [101] and grows at mesophilic temperatures [102] . Similarly, in this study 56% of the 32 OTU4 isolates screened were positive for growth at 37°C and 59% were positive for cellulose degradation. There are no previous published studies concerning the capacity of *B.* thermoamylovorans to tolerate or not tolerate organic solvents and to decolorize Congo red, 28% of the OTU4 isolates screened decolorized Congo red. Like the OTU2 isolate H20C009, one OTU4 isolate, A07M340, was positive for all traits screened.

OTU5 is most closely related to *Bacillus asahii.* OTU8 aligns with *Bacillus smithii.* There are no data on *Bacillus asahii, Bacillus smithii* for the traits screened in this study [103]. Zero isolates from OTU 5, were able to decolorize Congo red, however 86% of the isolates tested were positive for cellulose degradation. The 16 OTU8 isolates, most closely related to *Bacillus smithiii,* were unable to grow at 37ºC and decolorize Congo red, while 26% did degrade cellulose.

Bacillus licheniformis is a known cellulose degrader, [85], tolerant to organic solvents such as toluene and benzene [104], produces lignin degrading enzymes [105] and is known to grow in mesophilic temperatures [106].

Table 5. Vanillin utilization screening by OTU. Vanillin utilization screens were conducted at 37°C using only those isolates that were capable of growth at 37°C. More than half of the isolates screened from OTU 4 were positive for Congo red decolorization and vanillin utilization. National Center for Biotechnological Information (NCBI) BLAST search was conducted on the representative sequence of each OTU using the 16s ribosomal RNA sequences (Bacteria and Archaea) alignment tool which was accessed 24-04-13. The BLAST search did not include uncultured organisms, environmental samples or metagenomes and differed from the OTU assignments generated on the phylogenetic tree. The unique column is the number of unique isolate sequences identified in that OTU out of the total sequences in that OTU.

OTU	NCBI Blast ID	Total Unique in OTU	Total Tested	Vanillin Utilization	
				(+)	(-)
1	*Geobacillus pallidus*	102/1260	1	0	1
2	*Geobacillus vulcani*	26/252	1	1	0
4	*Bacillus thermoamylovorans*	7/102	18	9	9
5	*Bacillus asahii*	4/44	9	3	6
6	*Geobacillus thermoglucosidasius*	3/57	6	0	6
7	*Actinomadura rubrobrunea*	1/1	0	0	0
8	*Bacillus smithii*	6/110	0	0	0
9	*Geobacillus debilis*	8/19	0	0	0
11	*Bacillus licheniformis*	6/17	7	0	7
12	*Geobacillus pallidus*	3/16	0	0	0
14	*Ureibacillus thermospaericus*	2/35	0	0	0
15	*Brevibacillus thermoruber*	2/3	1	0	1
19	*Geobacillus stearothermophilus*	2/2	0	0	0
20	*Geobacillus stearothermophilus*	1/1	0	0	0
21	*Bacillus thermoamylovorans*	1/1	0	0	0
22	*Caldalkalibacillus uzonensis*	1/1	0	0	0
28	*Ureibacillus terrenus*	1/1	0	0	0
32	*Bacillus aeolius*	1/1	0	0	0
36	*Bacillus psychrodurans*	2/2	0	0	0
37	*Luteimonas aestuarii*	1/1	0	0	0
40	*Geobacillus pallidus*	1/1	0	0	0
41	*Bacillus psychrodurans*	1/1	0	0	0
44	*Geobacillus thermoleovorans*	1/1	0	0	0
45	*Pseudomonas gessardii*	1/2	0	0	0
			43	13	30

More than half of the isolates screened from OTU 11, which is most closely related to this *B. licheniformis*, were positive for cellulose degradation and growth at 37ºC; however, none of these isolates decolorized Congo red. These isolates were expected to decolorize Congo red, because *B. licheniformis* is a known laccase producer [105].

Geobacillus debilis has not been shown to degrade cellulose [84], azo dyes or tolerate organic solvents, our library harbors at least 3 isolates of OTU9 that were able to degrade cellulose and 2 that were able to decolorize Congo red. Those two isolates that were able to decolorize Congo red were also able to degrade cellulose, these isolates come from M24 and were isolated on CAT media, and one of the two isolates was classified as having a unique sequence within OTU9. These two isolates are candidates for future studies. This species has been shown to grow between 35°C and 78°C [107], however no OTU9 isolate tested in our library was capable of growth at 37°C.

Ureibacillus thermosphaericus (OTU 14) has been shown to degrade lignin and cellulose in co-culture [108], it grows between 35°C and 65°C, it produces organic solvent tolerant enzymes [109] and tolerates phenols and furans from lignocellulosic hydrolysate [86]. Only one isolate tested in our library from OTU 14 was able to degrade cellulose, all 17 isolates, including that isolate were negative for all other screens.

In summary, the library is diverse and includes a variety of combinations of the traits screened across isolates from different OTUs. With 207 isolates screened for Congo red decolorization, cellulose degradation and growth at 37°C; 40% were not positive for any trait, 30% for a single trait, 29% for a pair of traits, 2% were positive for three traits. It is rare to find an isolate that is positive for more than 2 traits. Very

frequently a stains had 1 or fewer traits. Taking into account the 43 isolates that were screened for vanillin, 2 isolates (1% of 207) were positive for these traits and all other traits screened. As predicted, some OTUs that were closely related, shared similar results. OTUs 1, 36, 40 and 41 were branched closely on the phylogenetic tree (Figure 5) and behaved similarly in screens, very few isolates from these OTUs were positive for any traits. However, OTUs 4 and 5 were closely branched on the tree and OTU4 contained multiple isolates for Congo red decolorization and OTU5 contained none. Thus, there are a variety of phenotypes across different OTUs.

CHAPTER III

IDENTIFICATION OF ISOLATES WITH COMPLEX SUBSTRATE UTILIZATION PHENOTYPES AS CANDIDATES FOR METABOLIC ENGINEERING

III.1 Introduction

The application of industrial biotechnology is increasing for the production of fuels and chemicals such as bioethanol and organic acids [110]. These industrially produced fuels and chemicals must be technically feasible and sustain lower production cost than their counterparts. Substrate costs are considered the most expensive element of production, representing 40-60% of the costs [111]. There is in interest in using inexpensive feedstock such as lignocellulosic hydrolysate and bio-oil for production of valued products. An ideal microbe for production of such fuels and chemicals would be able to completely utilize these feedstock, tolerate inhibitory compounds, and naturally or synthetically produce the end products. Lignocellulosic associated feedstock contain five carbon sugars (i.e. xylose and arabinose), which are difficult to ferment and also contain growth inhibitors. This chapter presents results from screening isolates for tolerance and utilization of complex substrates and five carbon sugars.

Bio-oil

Lignocellulosic biomass is considered a first-rate source of carbon and energy for production of renewable fuels and chemicals. The complex structure of lignocellulosic biomass requires energy to release simple sugars. The deconstruction of biomass can be

biochemical or thermochemical. Biochemical processes utilize enzymes to degrade biomass and microbes to generate products while thermochemical processes use heat to breakdown biomass and chemical catalysis for product formation [112]. A hybrid process developed at Iowa State University employs both thermochemical and biochemical biomass deconstruction. The pyrolysis of the lignocellulose eliminates recalcitrance and makes use of both carbohydrates and lignin. The resulting pyrolysate is biologically utilizable. Pyrolysis requires high temperatures (300°C-500°C) and anoxic conditions for the decomposition of biomass. Fast pyrolysis selects for energy-rich bio-oil production. Fast pyrolysis takes a few seconds and allows for a range of biomass types and structures to be used.

During fast pyrolysis lignocellulose is degraded to 1,6 anhydroβ-D-glucose, known as levoglucosan. Levoglucusan can be used as a carbon and energy source during fermentation. Some fungal species (e.g. *Aspergillus*) are known to degrade levoglucusan. Some soil bacteria are capable of dehydrating levoglucose during metabolism [113]. Bio-oil is packed with microbial growth inhibitors such as, furans, phenols, acids, hydroxymethylfurfural, furfural, formic acid, valeric acid, butyric acid, acetic acid, acetol, and guaiacol.

Bio-oil contains many unknown toxic compounds that have yet to be characterized. Most industrially used biocatalysts are unable to directly metabolize levoglucosan. Levoglucosan can be acid hydrolyzed to glucose, this step is costly, creating the need for microorganisms that are capable of utilization of levoglucosan. In nature, levoglucosan is found near burning biomass (e.g. forest fires) [114].

Bio-oil is rich in fermentable sugar and is an ideal feedstock for the production of biofuels but it contains multiple toxic compounds that are inhibitory to microbial growth. [114] has shown that many fungal species can grow in activated charcoal treated bio-oil, but not in raw bio-oil. Dr. Zhanyou Chi at Iowa State University has shown tolerance to raw bio-oil in engineered E. coli isolates to 0.2%, while algal tolerance is at 0.3%. To mitigate these toxic, inhibitory effects, detoxification steps such as, activated charcoal treatment, solvent extraction, and overliming must precede fermentation or bio-oil can be collected in fractions that contain minimal inhibitory compounds. Another approach is to find naturally occurring microbes that are capable of tolerating the inhibitory compound found in bio-oil. No single compound attributes directly to bio-oil toxicity but act synergistically [115, 116]

Hydrolysate

Lignocellulose consists of lignin, hemicellulose and cellulose. When lignocellulose is hydrolyzed by acid treatment, the resulting hydrolysate can be used as a substrate for bio-product (e.g. renewable fuels) fermentation by microbes (e.g. fungi, yeast bacteria, and algae). Biomass can be degraded by a consortium of microbes (e.g. termite gut) due to the ability of these individual isolates to degrade complex sugars, utilize 5-carbon sugars in the presence of toxic degradation compounds. Lignocellulosic hydrolysate contains glucose, xylose, arabinose, mannose and other saccharides. Not all microbes are capable of fermenting all of these sugars, making some fermentation in adequate [117]. In addition to sugars being produced during lignocellulose hydrolysis, a number of toxic compounds are formed. These toxic compounds have inhibitory effects

on the growth and efficiency of fermentative microorganisms. The range of inhibitory compounds dependent on the lignocellulosic substrate, pretreatment and hydrolysis conditions. The main groups of inhibitors are furans (furfural, 5-hydroxmethyfurfural), phenolics, and aliphatic acids (acetic, formic and levulinic acid) [118, 119]. Furans are produced in the pretreatment and hydrolysis steps from monosaccharides, acetic acid accumulation is a resultant of acetyl groups in hemicelluloses, while phenolics are derived from lignin.

III.2 Methods

HPLC Analysis of Lignocellulosic (Corn Stover) Hydrolysates

Lignocellulosic hydrolysates were provided by Dr. Katy Kao's lab at Texas A&M. Eight-hundred microliters of the samples was added to a Costar® Spin-X® centrifuge tube filter (0.22μm) and centrifuged for 5 minutes at 16, 000 x g to remove cells and debris. This filtrate was then added to a HPLC vial. Sulfuric acid (0.004 M) was used as eluent. The flow rate was 0.6 mL/min at 30°C with a UV detector at 215nm to detect HMF, xylose and furfural, the injection volume was 20μL. The organic acid concentrations were calculated from the peak area using the organic acid analysis standard and from Bio-Rad Laboratories, Inc (Berkley, California).

Growth in Bio-oil, Hydrolysate, Pentose Sugars, and other Carbon Sources

Growth curves were generated in the BioscreenC (Lab systems, Helsinki, Finland). For all the experiments each well was inoculated with 20 μL of an overnight liquid culture in LB broth [66]. The carbon source assay plates were loaded with 330 μL

of MSM containing 1.5% waste glycerol, 1:10 (i.e. in the case of the isolate H20C009, the ratio of corn stover hydrolysate to MSM was 1:20) corn stover hydrolysate, 0.5% arabinose, or 3% xylose, and wells loaded with no carbon source were used as controls. The bio-oil assay plates were loaded with 330 µL of LB containing either, 0.2%, 0.5%, 1.25%, 1.5%, 1.75% or 3% bio-oil the pyrolytic sugar fraction for tolerance screens and 330 µL of MSM containing either 0.5% and 1% bio-oil (pyrolytic sugar) for utilization screens. The optical density measurements were taken using the wide band filter at 450-580nm at 37°C for 48 hours. The plate was shaken for 10 seconds at medium speed before each four-hour measurement interval. The average absorbance at each time point was used to generate the growth curve. The waste glycerol contained 60-100% (w/v) glycerol, 10-30% (w/v) water, 5-10% (w/v) sodium chloride, and <0.1% (w/v) methanol. Dr. Zhiyou Wen at Iowa State University provided a bio-oil fraction (pyrolytic sugar) and it contained, but was not limited to: 5-hydroxymethylfurfural, cyclotene, hydroquinone, vanillin, 3-methylcatechol, syringol and many unknown compounds (data not shown). The same batch of bio-oil was used across all screens.Those isolates that produced an exponential growth curve were considered positive.

Organic Acid Measurements

Two hundred and fifty microliters of the five replicates for each isolate were combined into one Costar® Spin-X® centrifuge tube filter (0.22µm). These samples were then prepared and analyzed by the same methods used in the anaylsis of the lignocellulosic hydrolysate.

III.3 Results

HPLC Analysis of Lignocellulosic (Corn Stover) Hydrolysates

Table 6 describes the contents of the lignocellulosic hydrolysate provided by the Kao lab at Texas A&M.

Table 6. Lignocellulosic hydrolysate content for materials employed in screens. The organic acid, furfural, and HMF contents were analyzed using HPLC.

MA[1]	CA[2]	FA[3]	AA[4]	OA[5]	HMF[6]	Fufural[7]
0.00	131.92	2386.77	62.45	0.00	0.06	0.40

[1]Malic acid (μM) [2]Citric acid (μM) [3]Ferulic acid (μM) [4]Acetic acid (μM) [5]Oxalic Acid (μM)
[6]Hydroxymethylfurfural (g/L) [7]Furfural (g/L)

Growth on Various Carbon Sources

Twenty-one isolates that were capable of growth at 37°C (identified in Chapter 2) were screened for the bio-oil (pyrolytic sugar fraction) tolerance in LB media. No isolates were able to grow in media containing any concentration of bio-oil as the sole carbon source. Eighteen isolates were positive for tolerance in 0.2% bio-oil, 16 in 0.5% bio-oil, 8 in 1.25% and 6 in 1.5%. No isolates tested grew in 1.75% or 3.0% bio-oil (Table 7. The positive isolates from 0.2% were in 6 OTUs and 7 sites, with 10 isolates from OTU4 and 5 from OTU 11. Of the 18 positive isolates, 6 were positive across all bio-oil concentrations up to 1.5%, 5 of these isolates were from OTU 11, and 1 from

OTU 4, the 5 isolates from OTU 11 were all from Big Bend, TX, four of these same isolates: J20M023, J20M027, J11M287, and J11M005 were also tolerant and able to utilize high concentrations of lignocellulosic hydrolysate. Two isolates from OTU 11, J11M017 and J04M004 were only able to tolerate up to 1.25% bio-oil and 1.0% bio-oil, respectively, but were able to tolerate high concentrations of lignocellulosic hydrolysate (1:10 ratio of hydrolysate to MSM). Sixteen isolates were positive in 0.5% bio-oil; the two isolates that were able to tolerate 0.2% bio-oil but not 0.5% bio-oil were from OTU 4, P01M008 and J04M010. Eight isolates were positive in 1.25% bio-oil, half as many isolates as in 0.5% bio-oil. Isolates A07M347 and H20M327, both from OTU 4 were able to tolerate up to 1.25% bio-oil.

Ten isolates had longer exponential phases in 0.5% bio-oil than in 0.2% bio-oil, 7 of these isolates were from OTU 4 and 1 each from OTUs 1, 2 and 11. H20C009, the isolate that was positive for every screen tested in Chapter II was able to tolerate 0.2% and 0.5% bio-oil in LB and tolerate/utilize 1:20 lignocellulosic hydrolysate in MSM.

Due to the capacity to tolerate high concentrations of hydrolysate, isolates J11M017, J04M004, J11M005, J11M287 were studied further for carbon substrate utilization (Figure 8). Isolate H2C009 was also included in the further studies, because it was positive in all traits screened in Chapter II (Appendix C). Isolate J04M004 had a shorter lag phase in arabinose media than in other carbon sources (Figure 8a). J04M004 grew at approximately the same rate in hydrolysate and xylose. J11M005 and J11M017 had the best performances in waste glycerol. J11M017 also grows in arabinose and has a longer lag phase in xylose (Figure8c).

Table 7. Isolates screened for hydrolysate utilization (as the sole carbon source) and bio-oil tolerance. Isolates that showed exponential growth were considered positive, shown by the number 1, and those not exhibiting exponential growth were considered negative, shown by the number 0 in the table. The color gray indicates the isolate was not screened for that particular trait.

Site	Isolate	OTU	1:2 Hydrolysate	1:10 Hydrolysate	1:20 Hydrolysate	0.2% Bio-oil	0.5% Bio-oil	1.25% Bio-oil	1.5% Bio-oil	1.75% Bio-oil	3.0% Bio-oil
S44	C017	1				1	1	0	0	0	0
H20	C009	2	0	0	1	1	1	0	0	0	0
A07	M339	4	0	0	1	1	1	0	0	0	0
A07	M340	4	0	0	1	1	1	0	0	0	0
A07	M345	4	0	0	1						
A07	M347	4	0	0	1	1	1	1	0	0	0
A07	M348	4	0	0	1						
A07	M349	4	0	0	1	1	1	0	0	0	0
A07	M352	4	0	1	1	1	1	0	0	0	0
A07	M355	4	0	0	1	1	1	0	0	0	0
E08	M016	4	0	1	1	1	1	0	0	0	0
H20	C002	4	0	0	1	1	1	0	0	0	0
H20	C027	4				1	1	0	0	0	0
H20	M327	4				1	1	1	0	0	0
J04	M010	4	0	0	1	1	0	1	0	0	0
P01	M008	4	0	0	1	1	1	0	0	0	0
S44	C011	4	0	0	1						
F01	M010	5				1	0	0	0	0	0
F01	M017	5	0	0	0						
F02	D001	5				1	0	0	0	0	0
F05	M393	5	0	0	0						
F05	M396	5	0	0	0						
G08	M102	5				1	0	0	0	0	0
J04	M004	11	0	1	1	1	1	0	0	0	0
J11	M005	11	0	0	0	1	1	1	1	0	0
J11	M017	11	0	1	1	1	1	1	1	0	0
J11	M287	11	0	1	1	1	1	1	1	0	0
J20	M023	11	0	1	1	1	1	1	1	0	0
J20	M027	11	0	1	1	1	1	1	1	0	0
H01	C007	15	0	0	0						

J11M287 grew at the same rate in arabinose and waste glycerol it had a longer lag phase in hydrolysate and xylose (Figure8d). H20C009 was able to utilize 1.5% waste glycerol, 3% xylose, and 1:20 lignocellulosic hydrolysate as sole carbon sources in MSM. Limited growth occurred both in the control media, which contained no carbon source, and in 0.5% arabinose (Figure 9).

Organic Acid Production

Table 8 shows the organic acid produced by each isolate across carbon sources. J04M004 does not completely utilize xylose in hydrolysate media, 0.276% xylose remained while 1.65% xylose remained in xylose media. In MSM media without a carbon source this isolate grew, while producing malic acid.

J11M017 was unable to produce any organic acids from the pentose sugars arabinose and xylose. J11M005 was able to produce 165.98μM of acetic acid the pentose sugar arabinose while only producing 62.51μM in the pentose sugar xylose. J11M005 did not produce any acetic acid in hydrolysate or waste glycerol.

Table 9 shows organic acid organic acid production of H20C009 across carbon sources. H20C009 was the only isolate screened that produced oxalic acid in xylose, lignocellulosic hydrolysate, arabinose, and control media (no carbon). It also produced ferulic acid in all carbon sources, except lignocellulosic hydrolysate where it potentially utilized ferulic acid. It did not produce acetic acid in arabinose or control media.

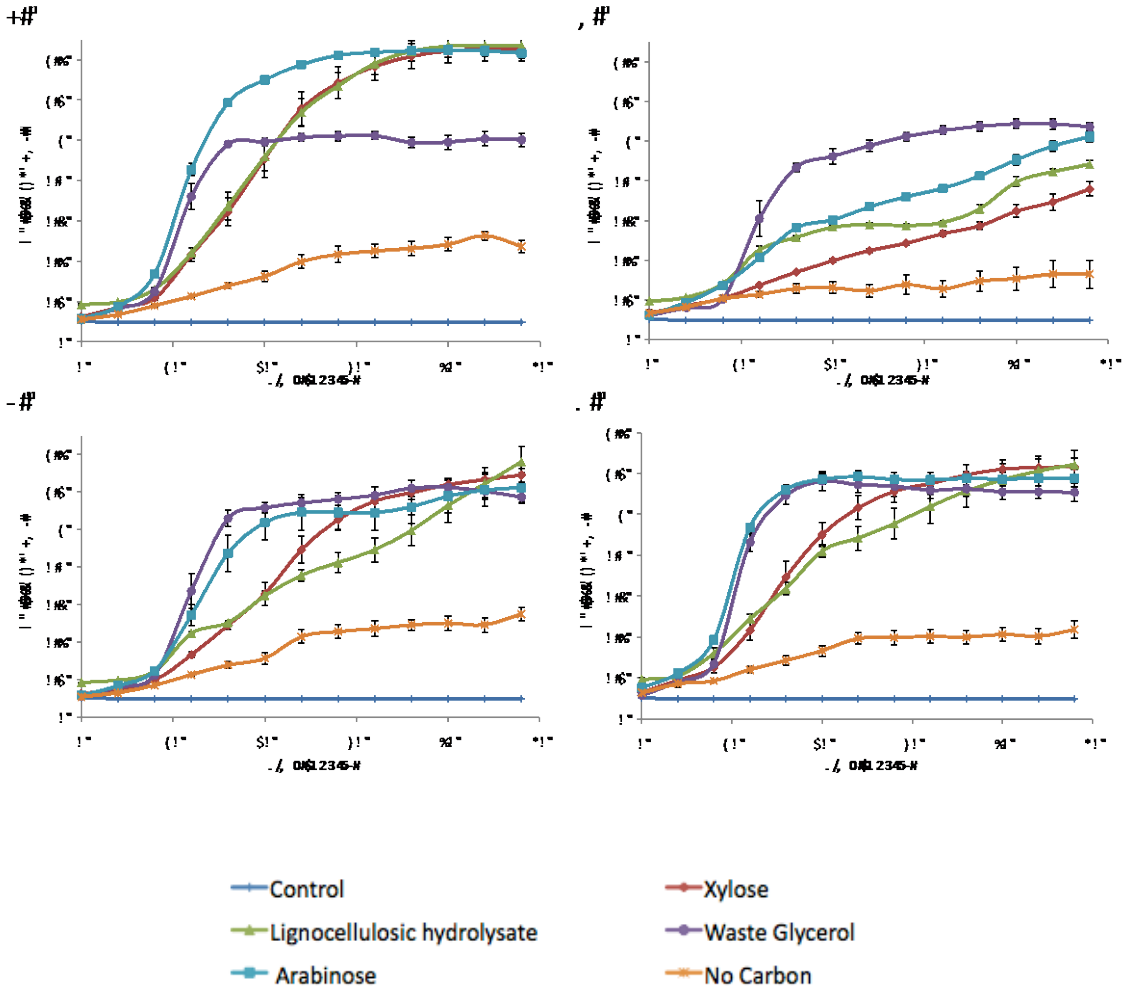


Figure 8. Growth curves for hydrolysate tolerant isolates across different carbon sources. All these isolates originated from the same site, Big Bend, TX, and are members of the same operational taxanomic unit (OTU 11; see Chapter 2). a. J04M004 b. J11M005 c. J11M017 d. J11M287. MSM without any carbon sources was used as a control. Lignocellulosic hydrolysate (1:10) and waste glycerol (1.5%) are complex substrates and xylose (3%) and arabinose (0.5%) are five-carbon sugars. Data are the mean of five replicates across time with error bars indicating standard error.

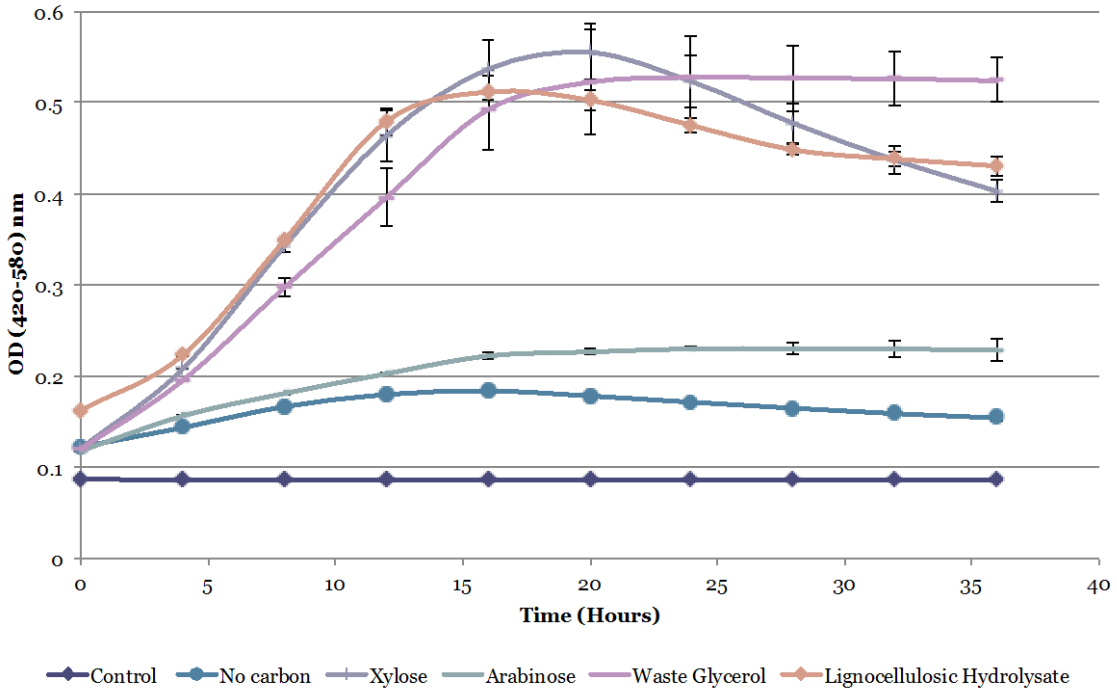


Figure 9. Growth curves of H20C009 in cultures with different carbon sources in minimal salts media. The BioscreenC generated these growth curves. Un-inoculated wells were used as a control. Data are the mean of five replicates across time with error bars indicating standard error.

Table 8. Organic acid production of isolates in varying carbon sources. Isolates were selected to grow under different carbon sources and HPLC measurements were taken after 7 days of growth to measure the production of organic acids. Xylose (3%) and Arabinose (0.5%) were the five carbon sugars used, lignocellulosic hydroloysate (1:10) and waste glycerol (1.5%) were the complex substrates screened. No carbon source was used as a control.

Isolate	Carbon Source	Organic Acid			
		MA[1]	CA[2]	FA[3]	AA[4]
J04M004	Xylose	16.90	0.00	0.00	0.00
	Lignocellulosic Hydrolysate	10.49	7.02	8.85	0.00
	Waste Glycerol	0.00	0.00	15.53	120.99
	Arabinose	0.00	0.00	0.00	149.18
	No Carbon	8.62	0.00	0.00	0.00
J11M005	Xylose	13.88	0.00	0.00	62.51
	Lignocellulosic Hydrolysate	14.46	11.06	0.00	0.00
	Waste Glycerol	13.61	0.00	32.72	0.00
	Arabinose	0.00	0.00	0.00	165.98
	No Carbon	0.00	0.00	0.00	0.00

Table 8. Continued.

Isolate	Carbon Source	Organic Acid			
		MA[1]	CA[2]	FA[3]	AA[4]
J11M017	Xylose	0.00	0.00	0.00	0.00
	Lignocellulosic Hydrolysate	13.32	4.70	0.00	25.18
	Waste Glycerol	21.37	0.00	0.00	0.00
	Arabinose	0.00	0.00	0.00	0.00
	No Carbon	0.00	0.00	0.00	0.00
J11M287	Xylose	10.34	0.00	0.00	0.00
	Lignocellulosic Hydrolysate	14.68	4.19	0.00	0.00
	Waste Glycerol	10.10	0.00	17.80	0.00
	Arabinose	0.00	0.00	0.00	34.77
	No Carbon	0.00	0.00	0.00	0.00

[1]Malic acid (μM) [2]Citric acid (μM) [3]Ferulic acid (μM) [4]Acetic acid (μM)

III.4 Discussion

Industrial bioprocesses are challenged by many factors, among them is the absence of isolates able to ferment all sugars (e.g. hexoses and pentoses) present in lignocellulosic hydrolysates and tolerate the presence of harsh inhibitory compounds [120] This pitfall makes the identification of natural isolates capable of utilizing pentose sugars and tolerating various concentrations of inhibitors a significant advance. These isolates could be engineered with additional traits of interest while using less costly substrates or waste products. Taken together, the results for isolate J04M004 may indicate it can utilize carbon dioxide as a carbon source. J04M004 is limited in its ability to utilize xylose. Yet J04M004 produced malic acid in both the xylose treatment and MSM media without a carbon source. Malic acid is important in carbon fixation at night in plants [121, 122] , thus, one possible explanation for malic acid accumulation in the absence of carbon in the media is that carbon dioxide is serving as the carbon source. J11M005 utilized xylose and arabinose, but with a longer lag phase than in waste glycerol. The longer xylose lag phase for the hydrolysate tolerant isolates (Figure 8) may indicate that xylose utilization is dependent on induction of a pathway. This seems reasonable to expect because other closely related isolates rely on this strategy [123].

Table 9. Organic acid production of isolate H20C009. Organic acid production from various carbon sources. Isolates were selected to grow under different carbon sources and HPLC measurements were taken after 7 days of growth to measure the production of organic acids. Xylose (3%) and Arabinose (0.5%) were the five carbon sugars used, lignocellulosic hydrolysate (1:10) and waste glycerol (1.5%) were the complex substrates screened. No carbon source was used as a control.

Isolate	Carbon Source	Organic Acid				
		MA[1]	CA[2]	FA[3]	AA[4]	OA[5]
H20C009	Xylose	8.33	2.91	24.17	40.36	1.59
	Lignocellulosic Hydrolysate	0.00	9.41	37.53	34.87	3.93
	Waste Glycerol	0.00	0.00	30.24	167.13	0.00
	Arabinose	0.00	3.24	12.85	0.00	2.42
	No Carbon	0.00	0.00	8.89	0.00	1.85

1Malic acid (µM) 2Citric acid (µM) 3Ferulic acid (µM) 4Acetic acid (µM) 5Oxalic Acid (µM) 6Hydroxymethylfurfural (g/L) 7Furfural (g/L)

All of these OTU 11 isolates were able to utilize arabinose, which is expected owing to the improved arabinose isomerase seen in other closely related isolates [124]. These OUT 11 isolates tolerated high concentrations of lignocellulosic hydrolysate and bio-oil, and thus are candidates for metabolic engineering studies.

H20C009, which is most closely, related to *Geobacillus stearothermophilus*, was able to grow in xylose, likely owing to the fact this species can utilize hemicellulose and has a gene that encodes for xylose isomerase [125].

Although this species is known to produce enzymes for xylan and arabinan utilization [126], it showed limited growth in arabinose containing media. H20C009 was also able to utilize waste glycerol; this is expected due to gylcerol dehydrogenases found in this species [127]. This isolate was the only isolate screened to produce oxalic acid, which plays a key role in enzyme regulation in fungi during lignocellulosic degradation, and plant pathogenesis [128]. In wood rotting fungi, oxalic acid has been shown to acidify host tissue and aid in calcium sequestration from host cell walls [129]. H20C009 produced more oxalic acid in lignocellulosic hydrolysate than in any media. H20C009 was also capable of utilizing and tolerating lignocellulosic hydrolysate, and therefore is an isolate of interest for metabolic engineering.

Four of the isolates that turned out to be interesting here were from the same site and fell into the same OTU. Furthermore, that OTU was relatively rare in the collection, 17 isolates out of 1866 (Table 3, Chapter 2), and 15 of the 17 were from Big Bend, while the other two were from two other sites (E08, G13). Isolates from OTU 4 were positive in lignocellulosic hydrolysate and bio-oil screens, it is important to note that OTU 4 and

OTU 11 are located on the same branch in the phylogenetic tree in Chapter II (Figure 5). This may indicate that isolates with the tendency to utilize these complex substrates would be more likely to occur in sites with characteristics similar to those at Big Bend. H20C009 and A07M340, which were positive for all traits screened in Chapter II, was able to utilize/tolerate 1:20 lignocellulosic hydrolysate and tolerate up to 0.5% bio-oil. Isolate H20C002 from OTU 4 was positive for the same traits as H20C009. H20M327 was not screened for hydrolysate, however it was screened for bio-oil and showed tolerance in up to 1.25%. This may indicate that San Francisco Bay, CA (H20) isolates are more likely to utilize the complex substrates.

CHAPTER IV

IDENTIFICATION OF GLYCEROL UTILIZING ISOLATES WITH THE POLYHYDROXYALKANOATE (PHA) ACCUMULATION PHENOTYPE

IV.1 Introduction

Glycerol is a by-product of biodiesel fuel production. Ten pounds of glycerol is produced in the biodiesel industry for every 100 pounds of biodiesel [130]. Rather than disposing of the glycerol, an alternative use should be found. A alternative use for glycerol aid in the reduction of the cost of biodiesel [131]. Glycerol can be used as a microbial fermentation substrate to produce 1,3 propanediol, glycolipid biosurfactants and single cell oils [132]. Glycerol is a highly reduced carbon compound, which allows for higher product yields during fermentation in comparison to simple sugars [133].

Polyhydroxyalkonates (PHAs) are bacterial produced polyesters. They can be produced from various substrates (e.g. sugars, triacylglycerol) by a diversity of bacterial species. PHAs have crystalline thermoplastic properties and amorphous elastomeric properties. They are practical alternatives to petroleum-based plastics due to their recyclable nature and easily degraded to CO_2 and H_2O. They can be easily processed and are biocompatible allowing for relevance in medical applications [134]. The Imperial Chemical Industries in the United Kingdom developed a novel biopolymer, BIOPOL®, which consists of the PHAs 3-hydroxybutyrate and 3-hydroxyvalerate. BIOPOL® has a low crystallinity and a high elasticity. It is used industrially to coat paper, paperboards,

produce shampoo bottles and disposable razors.

PHA is biosynthesized by *Bacillus*, *Pseudomonas* and other bacterial species. PHAs are energy storage compounds in bacterial cells. They are found in cytoplasm inclusions as insoluble granules [135]. PHAs vary in length based on side chain monomers. PHAs are divided into short-chain length PHAs (3-5 carbon atoms) and medium-chain length PHAs (6-14 carbon atoms). Bacteria under environmental stress produce PHA biopolymers (e.g. nutrient limited conditions and low temperatures) with an excess of carbon [136, 137]. PHAs require microorganisms for production they cannot yet be chemically synthesized. PHAs produced industrially by gram-negative bacteria such as *Pseudomonas oleovorans* and *Methylobacteriaum organophillum* [138]. PHAs produced from Gram-negative bacteria contain lipopolysacharide (LPS) endotoxins that purify with PHAs. LPS endotoxins are known to cause fevers and induce immunogenic reactions, which are not conducive for medical applications. Gram-positive bacteria (e.g. *Bacillus*) do not have a LPS and have more potential as industrial PHA producers. Fermentative substrates used in PHA production are costly; efforts are currently in place to explore inexpensive raw materials as substrates (e.g. glycerol). To examine bacterial utilization of glycerol, cell growth is screened overtime in minimal media containing glycerol (concentrations may vary) [133].

This study involved assessing the PHA production capacity of a subset of the bacterial library (Chapter 2) using a sensitive plate screening method [139]. The assay employed Nile red, a lipophilic dye that emits a strong fluorescence when bound to PHAs in bacterial cells. It seemed reasonable to expect the library (Chapter 2) harbored

isolates capable of both growth on glycerol and production of PHAs because other work had established that this diverse resource exhibited variation in utilization of complex substrates (Chapter 3), and also, diversity in a variety of other biofuel associated phenotypes (Chapter 2). Thus, the same subset of the library was used to study variation in PHA production using the established assay.

IV.2 Methods

PHA Agar Plate Assay

Those 43 isolates capable of growth at 37°C in Chapter II were screened in triplicate for 72 hours at 37°C for PHA production on MSM containing: 6.73 g/L of Na_2HPO_4•$7H_2O$, 1.50g/L of KH_2PO_4, 0.20g/L $MgS0_4$•$7H_2O$, 1.00g/L of NH_4Cl, 0.02g/L of $CaCl_2$•$2H_2O$- 0.02g, 0.0012g/L of Fe(III)NH_4 citrate and 1mL/L of trace elements containing: 50.00g/L of EDTA, 8.30g/L of $FeCl_3$, 0.84g/L of $ZnCl_2$, 0.13g/L of $CuCl_2$•$2H_2O$, 0.2g/L of $CoCl_2$•$6H_2O$, 0.014g/L of $MnCl_2$•$4H_2O$ and 0.20g/L of H_3BO_3 and 1.5% bacto-agar (supplemented with 2% NaCl and 1.5% glycerol and 0.5µg/L Nile red. After 72 hours of incubation, plates were exposed to ultraviolet light for the detection of fluorescence as previously described by [139].

Glycerol Utilization and Fluorescent Intensity Measurements

Isolates positive for the agar plate assays (S48C018 was not included because it was more fastidious) were grown from glycerol stocks at 37°C in vacuum-sealed bags gassed with nitrogen. Those isolates were monitored every day for growth. One colony

from each isolate capable of growing at 37°C was used to make liquid cultures in LB broth. Liquid cultures were grown overnight and 20µL of this culture were randomly, inoculated in replicates of four into 330µL of minimal salts media (supplemented with 2% NaCl). Growth curves were determined over 72 hours using the BioscreenC system (Lab systems, Helsinki, Finland) at 37°C with 10 seconds of medium shaking before each three-hour measurement. Growth curves were compared in the presence of 1.5% and 3% glycerol. Samples were immediately transferred to a 96-well plate and centrifuged at 2,250 g for 10 minutes. The Nile red staining and fluorescent measurement was modified from the methods described by [140]. The supernatant was removed and the pelleted cells were re-suspended in 250µL of molecular grade water (HyClone® HyPure™ Molecular Biology Grade Water, HyClone Laboratories, Logan, Utah, United States). Ten microliters of 80µg/mL of Nile red dissolved in dimethyl sulfoxide was added to the suspension. The final concentration of Nile red was 3.1µg/ml. This solution was incubated at room temperature for 30 minutes then centrifuged at 2,250g for 10 minutes. The supernatant was removed and the pellet was re-suspended in a 250µL of molecular grade water (HyClone® HyPure™ Molecular Biology Grade Water, HyClone Laboratories, Logan, Utah, United States). The fluorescence of the re-suspended cells was taken at an excitation wavelength of 485 nm and an emission wavelength of 595 nm using a TECAN SpectraFluor (Tecan Group, Ltd., Switzerland) platereader.

IV.3 Results

PHA Agar Plate Assay

A total of 20 isolates out of 43 tested were positive for PHA production. All isolates from La Sal Del Ray, TX (AO7; Table 2) included in the assay were positive. Similarly all OTU 4 isolates screened were positive for PHA production **(**Figure 10**)**. Figure 11 shows the number of positive isolates within a given site. Brazoria National Wildlife Refuge consists of 4 sites, F01, F02, F05, F09 and isolates from OTU 5. The San Francisco Bay, CA site consisted of sites H01and H20; and isolates from OTUs 2 (1 isolate), 4 (4 isolates) and 15 (1 isolate). Big Bend, TX consists of 3 sites, J04, J11 and J20 with isolates belonging to OTUs 4, 8, and 11. Puerto Rico, PR consists of the site P01 with all isolates from this site belonging to OTU 4. Yellowstone, WY consists of the sites S44 and S48 with isolates included in OTUs 1, 4 and 6.

Isolates from OTUs 4 and 2 were all positive for this screen (Figure 12). Included in the positive isolates from OTU 4 was 1 isolate from Big Bend, TX. Overall, eighteen of the 20 positive isolates belonged to OTU 4. These isolates were distributed across 5 sites. OTU 2 contained one isolate from San Francisco Bay, CA that was positive for PHA production. OTU 6 contained 2 isolates from Yellowstone, WY positive for PHA production.

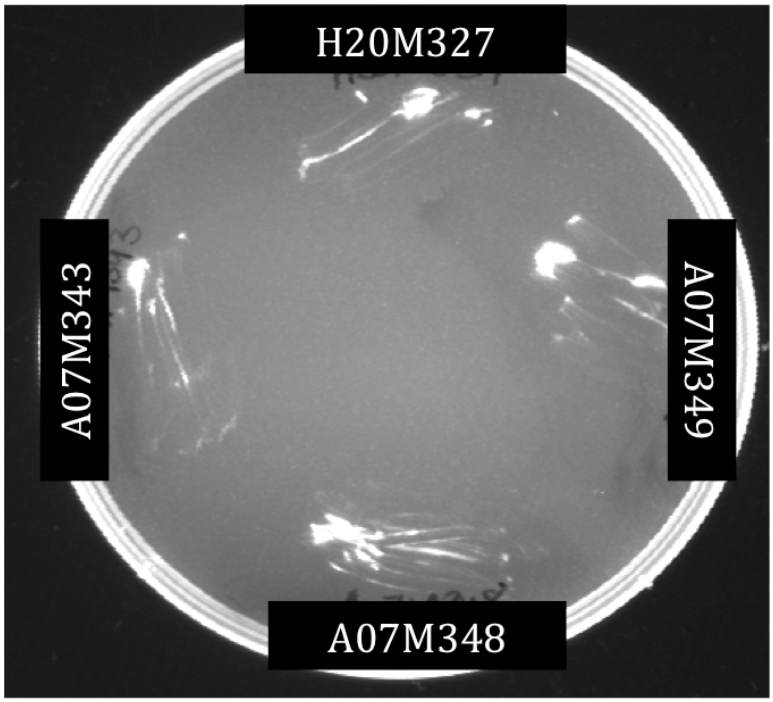


Figure 10. PHA plate assay. Plate in picture was inoculated with isolates from OUT 4 (Chapter II). Notice all isolates fluoresced under UV (312nm) light, indicating PHA accumulation in cells.

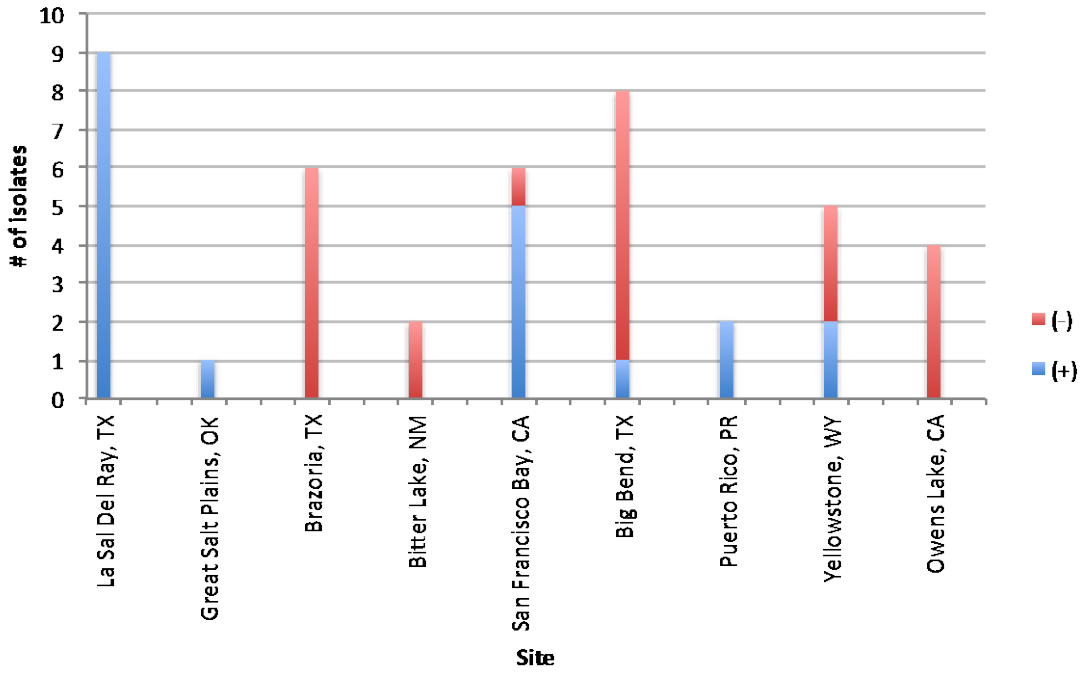


Figure 11. PHA production by site. Data are counts of isolates that did and did not produced PHA within each OTU. Forty-two (S48C018 was not included because it was more fastidious) isolates from the library were screened. Isolates that fluoresced at 312 nm on Nile red were considered positive. All isolates screened from La Sal del Ray, TX were positive for PHA production. Approximately 83% of the isolates tested from San Francisco Bay, CA were positive.

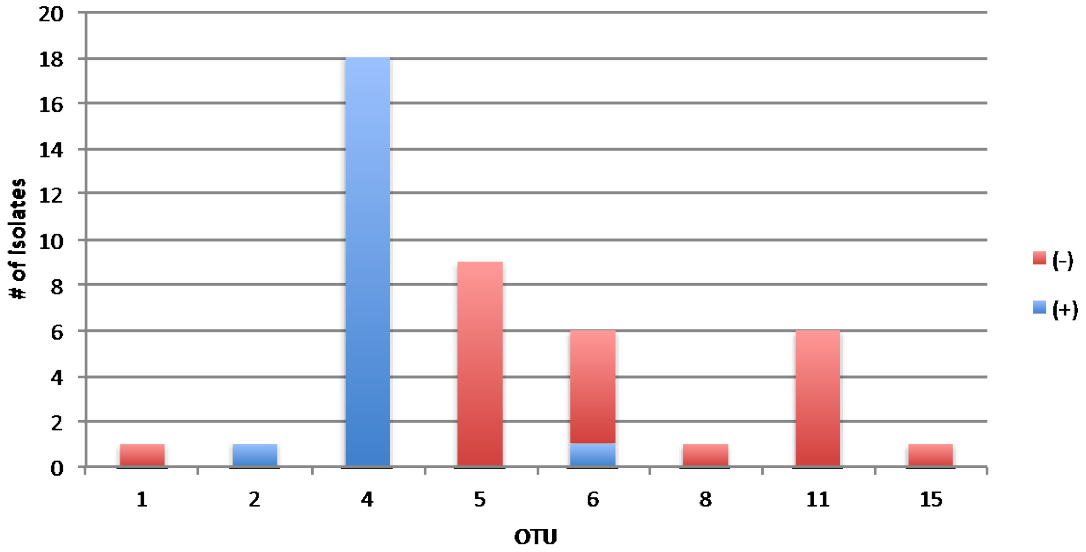


Figure 12. PHA production by OTU. Data are counts of isolates that did and did not produce PHA within each OTU. A total of 42 isolates (S48C018 was not included because it was more fastidious) were screened. Isolates that fluoresced at 312 nm on Nile red in the PHA assay were scored as positive for the trait (+). All isolates screened from OTU 4 were positive.

Glycerol Utilization and Fluorescent Intensity

The growth curves of the 20 isolates screened, showed that most isolates were inhibited by higher concentrations of glycerol. However, isolates E08M016 and H20M327 (all from OTU 4) were inhibited by 1.5% glycerol. S48C017 (OTU 6) was unaffected by the glycerol concentrations (Figure 13). J04M010 from OTU 4 reached exponential phase 50 hours earlier in 3% glycerol.

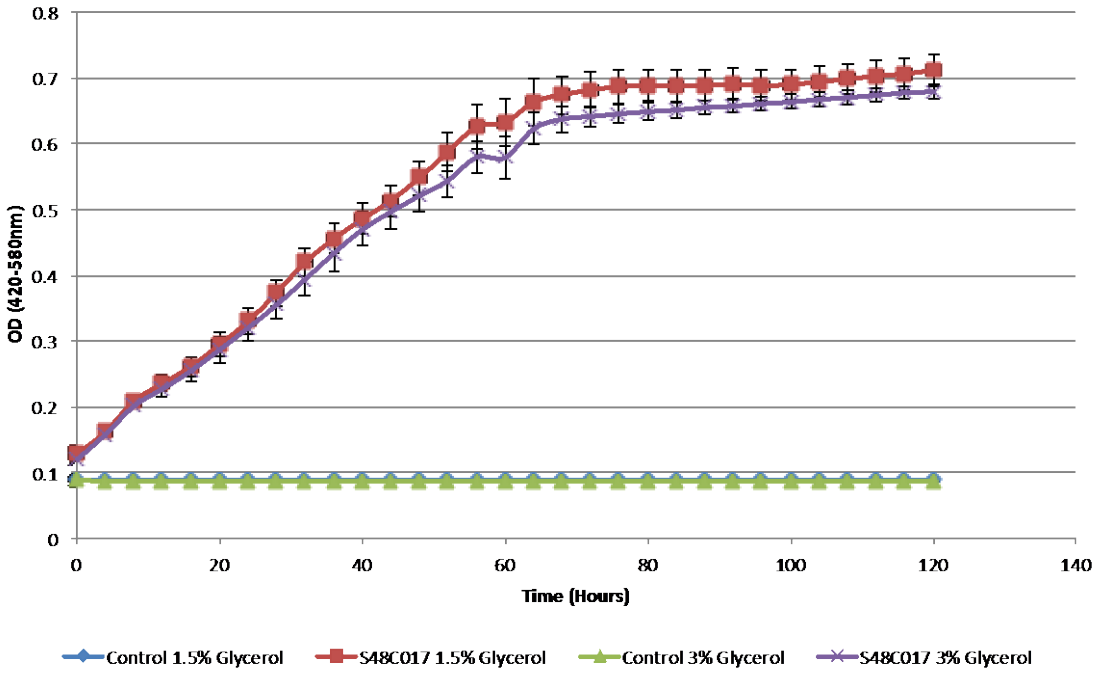


Figure 13. Growth curves of isolate S48C017 in MSM media containing different glycerol concentrations. Glycerol was the sole carbon source in this nutrient-limited media. Un-inoculated media was used as a control. Error bars shown are the standard error of five replicates.

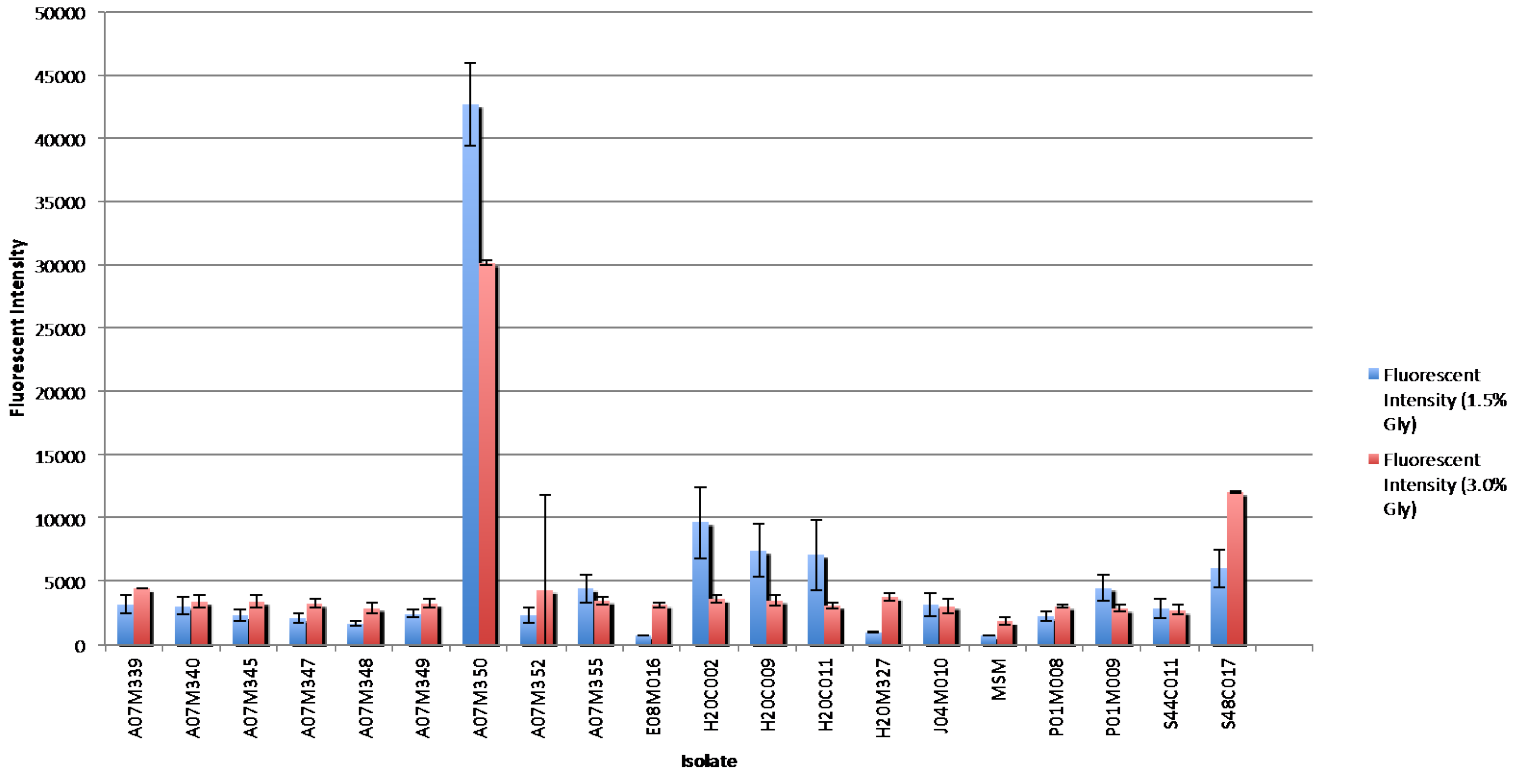


Figure 14. PHA fluorescence intensity. These 19 isolates tested positive in the Nile red agar plate assay. Fluorescence was measured 72 hours after growth. A07M350 fluoresced approximately 9 times higher than any other isolate in both 1.5% and 3% glycerol. Error bars are the standard error of four replicates.

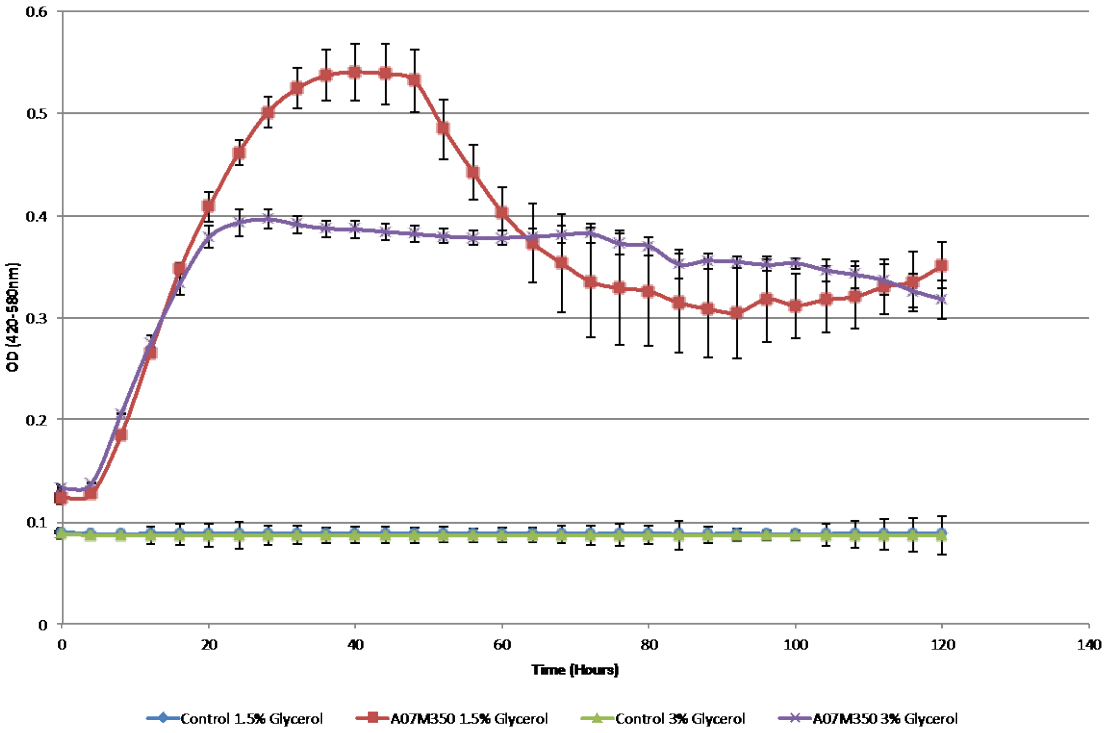


Figure 15. Growth curves of isolate A07M350 in MSM media containing different glycerol concentrations. Glycerol was the sole carbon source in this nutrient-limited media. Un-inoculated media was used as a control. Error bars are the standard error of five replicates.

Figure 14 shows the fluorescent intensity observed for each isolate under two glycerol concentrations. A07M350 an isolate from OTU 4 performed approximately 9 times higher than most other isolates. Its fluorescent intensity was inhibited in 3% glycerol as well as its growth (Figure 15). S48C017 from OTU 6 seemed to fluoresce more under 3% glycerol conditions about more than two times than most other isolates.

Isolates H20C002 (OTU 4), H20C009 (OTU 2) and H20C011 (OTU 4) fluoresced approximately 2 times higher than most isolates under 1.5% glycerol conditions.

IV.4 Discussion

It is expected that most isolates would be inhibited by higher glycerol concentration [141]. Glycerol increases staining efficiency and fluorescent intensity [142]. This could account for the slight increase in intensity in some isolates under 3% glycerol conditions. Isolates from San Francisco Bay, CA (H20) fluoresced 2 times more than most other isolates, this could be due to the sediment characteristics of the site in which these isolates originated, this sediment was characterized as having the second highest conductance (ds/m) and sodium concentration (mg/kg) of all sites utilized in this Chapter. All isolates screened from OTU 4 were positive for PHA production, this OTU described in Chapter II as being most closely related to *Bacillus thermoamylovorans*, which has no data to date on it ability to produce PHA. A07M350 is one of the many isolates from OTU 4 and A07 that was positive for PHA production in the agar plate assay, however, the fluorescence intensity for both 1.5% glycerol and 3% glycerol was approximately 9X higher than any other isolate screened. It is extremely important to note that of all the sites screened in the PHA agar plate assays, H20 and A07 contained the most positive isolates, these sites also contain the highest sodium concentrations, 18,262 mg/kg and 23,861 mg/kg respectively, as well as the highest conductance, 74.2 ds/m and 96.00 ds/m respectively. PHA production has been shown in halophilic

organisms but limited reports are available and mention only a few halophiles: *Haloferax*, *Halomonas*, *Azotobacter*, *Escherichia,* and *Alcaligenes* [143]. The isolate library described in Chapter II contains 24 isolates from A07 (La Sal del Ray, TX) and 43 from H20 (San Francisco Bay, CA). Isolates from these sites are likely to be PHA producers. The sediment from these sites is available and could be re-visited to select for PHA producers. Also, sites such as G08 (Bitter Lake, NM), T02 (Still Water, Nevada) and U22 (Owens Lake, CA) have similar, if not higher conductance and sodium levels, making isolates from these sites candidates to be screened to determine if these sediment characteristics are suggestive of PHA production. G08 contains 70 isolates in the library, while T02 has 64 and U22 contains 101 isolates, these isolates could be an additional source of PHA, as well as, re-sampling the microbial sediment communities of these sites.

CHAPTER V

MICROBIAL COMMUNITIES SCREENED IN A NOVEL MICROBIAL FUEL CELL (MFC) ARRAY TO ASSESS BOTH INSTRUMENT EFFICACY AND VARIATION IN MFC PERFORMANCE AMONG NATURAL COMMUNITIES[1]

V.1 Introduction

A microbial fuel cell is an electrochemical device that utilizes microbial metabolism to convert organic matter into electricity [144]. Bacterial isolates transfer metabolically produced electrons across a cell membrane to an external electrode. The high, direct conversion of organic matter into electricity makes MFC's efficient. MFC's can function at low and ambient temperatures and can expand our sources for energy. They have the potential to power wastewater treatment and bioremediation devices. A MFC consist of an anode and cathode separated by a proton exchange membrane. Electricigens are inoculated into the anode where they release electrons, which are then delivered to the cathode to produce a current. The current determines whether a isolate is capable of energy conversion. There are currently a limited number of organisms known for high performance in a MFC [145]. Performance is measured by current density and

[1] Adapted from Hou H, Li L, Ceylan CU, Haynes A, Cope J, Wilkinson HH, Erbay C, de Figueiredo P, Han A: A microfluidic microbial fuel cell array that supports long-term multiplexed analyses of electricigens. *Lab on a Chip* 2012, **12**(20):4151-4159 with permission from The Royal Society of Chemistry.

maximal output power density.

The microfabricated MFC array is an easy to use, compact device for the identification and characterization of electrochemically competent microbes (Figure 16) [96]. This device was developed at Texas A&M and consists of 24 spatially distinct, integrated anode and cathode chambers. This system utilizes a catholyte and anolyte replenishment to support long-term power generation. It was fabricated using sophisticated microfabrication approaches that can allow scale-up to massively parallel systems [6]. The microbial fuel cell array allows for multiple, reproducible electrochemical screens in parallel with the 24 individual chambers functioning as an individual miniature MFC. High-throughput cell arrays are significant for screening and analyzing a consortium of microbes.

There is an increasing interest in the effective use of natural resources and using microbes to produce energy. To improve MFC output and higher power densities, bacterial isolate selection is a major part of sustainability research. Microbes can use waste and organic matter to generate power and raw materials. Biotechnological industries aim to use a mixed culture of microbes for maximum production yield. Developing bioprocesses using mixed cultures is based on natural and ecological selection by parameter manipulations (e.g. pH, temperature, oxygen conditions) and by varying the natural inoculum source [146]. Using natural inoculum sources could allow for a high performing and enhanced natural microbial population that has the capacity to exceed known power outputs in an MFC. In most studies, Proteobacteria were the dominant phyum in microbial communities. The ratios of alpha, gamma, etc. differed

depending on inoculum source. Waste products such as sewage sludge have been used as organic substrate in MFCs inoculated with microbial communities. These microbial communities were comprise of predominantly (45%) Proteobacteria. Other phyla in the communities include: Bacteriodetes, Actinobactera, Firmicutes and Chloroflexi. These communities were able to produce a maximum power output of 3.2 ± 1.7 W m^{-3} [147].

Though it is not easy to establish the mechanisms and roles of the individual microorganisms contributing to power generation when using microbial communities in an MFC, microbial communities produce more electric output than individual isolates [147, 148]. The MFC array will allow for high-throughput, parallel screening of multiple communities, these communities are from a library of the same soils used as inocula in the carboxylate platform fermentations (Chapter II).

V.2 Methods

Site Selection

The Wilkinson lab has studied variation among sediment microbial communities collected from nature as inocula in carboxylate platform fermentations [4, 5, 36] Frequent collection trips were conducted from October 2008 to May 2010; with samples chosen based on variation in physical and ecological features or presumed gradients (e.g. moisture, salt accumulation, temperature). In total 501 samples from 77 sites were collected.

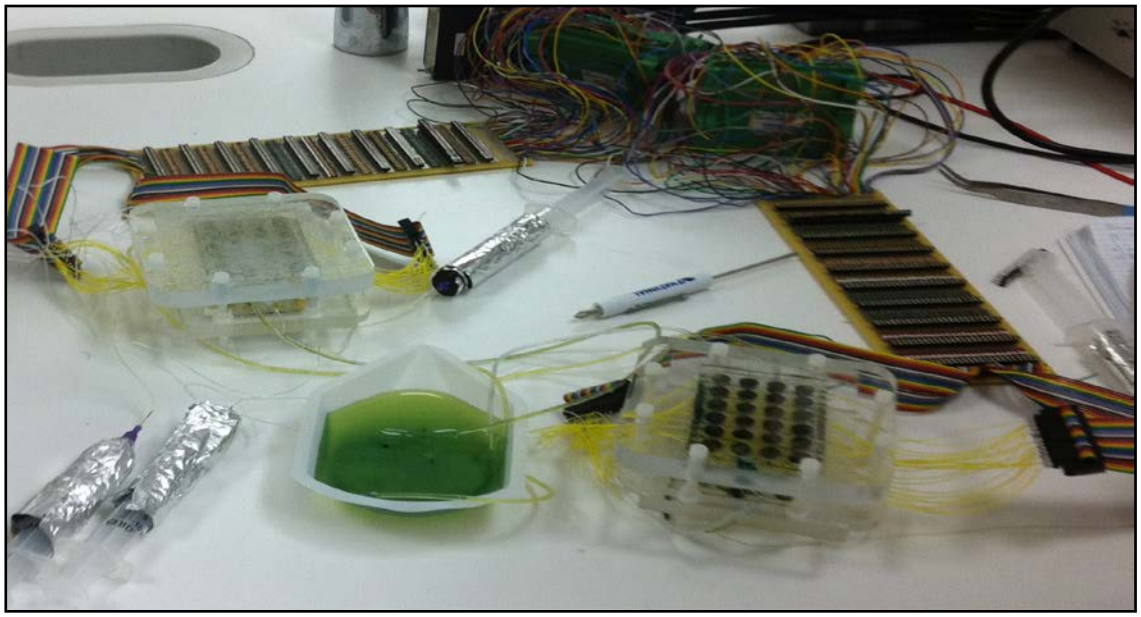


Figure 16. The microfabricated MFC array developed at Texas A&M University.

Natural Sediment Community Collection

Approximately 1.5 L of sediment or soil was collected for each sample. Specifically, a standard stainless steel bulb planter (10-12 cm deep) was used to pull three adjacent cores, which were placed in independent zip-top bags. Subsequently, the zip-top bag was placed into a vacuum seal bag and the air removed using a Foodsaver™ vacuum sealer (Sunbeam Products, Boca Raton, FL). Each of the three cores was handled differently to accommodate different types of processing and analyses in the laboratory at Texas A&M. One core was flash frozen with dry ice and then stored at -80°C, while the other two were placed in a cooler to reach ambient temperatures and subsequently homogenized and used as inoculum in the carboxylate platform

fermentations, used for soil analysis, or stored. Soils were chosen for the screen based on geographical diversity (Figure 17a.)

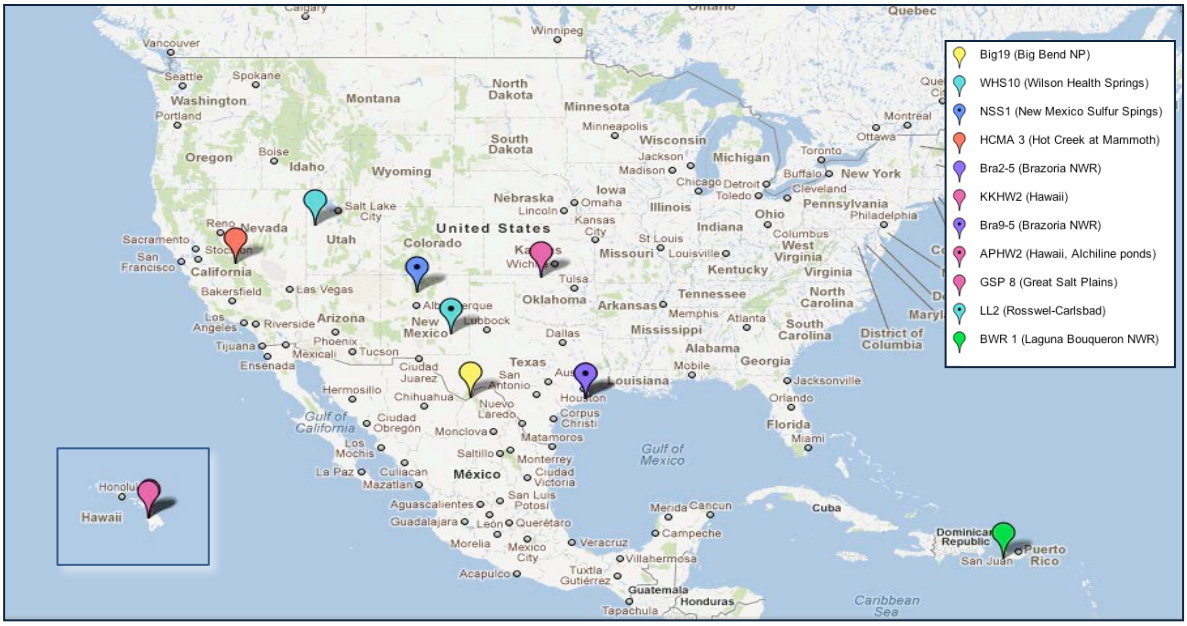
Taxonomic Data for Soils

Eight of the 10 soils used in this screen were retrieved from a sample stored at 4°C while the other two soils were stored at -80°C. Whole genomic DNA was extracted from the soil samples used in the MixAlco™ fermentations using the PowerMax Soil DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA) with a modified protocol [149]. Whole community DNA was quality checked by PCR amplifying the 16s rDNA region, using universal bacterial primers 27F and 1492R [4]. High-quality DNA samples were sent to the Research and Testing Laboratory (RTL, Lubbock, Texas) for tag-encoded pyrosequencing using universal bacterial primers 27F and 519R [4]. Taxonomic distributions (Figure 17b.) were estimated from these sequence data using the Visualization and Analysis of Microbial Population Structures website maintained by the Josephine Bay Paul Center. (VAMPS, http://vamps.mbl.edu/index.php) (Accessed 19 October 2011).

Bacterial Community Isolation

A modified soil fractionation method [150] was used to separate bacteria from soil particles. Five grams of soil (wet weight) was homogenized in 45 mL of phosphate buffered saline (PBS, 100 mM) in a 50 mL Falcon tube. The soil solution was vortexed at maximum speed for 1 minute. The soil homogenate was then centrifuged for 20 minutes at about 2,000 g in a refrigerated centrifuge. The supernatant was collected in a sterile 250 mL Erlenmeyer flask.

a.



b.

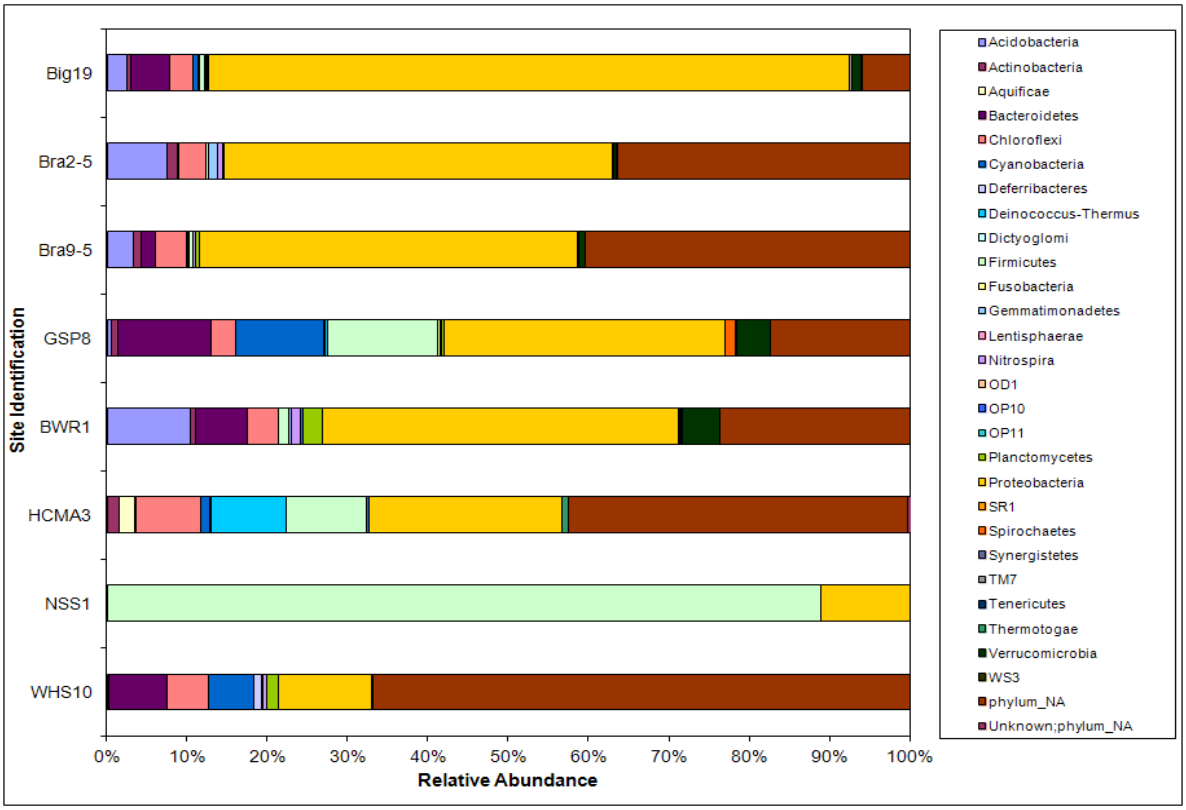


Figure 17. Geographic and bacterial diversity associated with soil samples used as sources for natural communities screened in the MFC array (APHW2, LL2 and KKHW2 data not shown). a. Locations in the United States and Puerto Rico where thermal and/or saline soils were sampled (Google Maps®)(accessed 20 February 2011). **b.** Phylum level diversity for bacterial communities from 8 sites used in this study.

The remaining soil pellet was re-suspended in 45 mL of PBS, homogenized by vortexing, and centrifuged again.

This step was repeated twice and the supernatants from the low speed centrifugations were combined and centrifuged at 10,000 g for 30 min.

The supernatant from the combined centrifugation was discarded, and the final pellet contained live bacterial cells. The pellet was re-suspended in fresh TSB, grown overnight, and diluted to an optical density between 0.8-1.0.

Electrochemical Activity Measurements

Each isolated bacterial community was loaded in triplicate into the MFC array device with environmental isolate *Shewanella* MR-1 used as a positive control. Power output for each well was recorded over a 330-hour time course. The maximum power output was obtained for each well and averaged for each community at the end of the time course.

V.3 Results

The maximum power output of each sample was normalized to the control isolate MR-1. All environmental soil samples showed electricity generation except for NSS1 from -80°C (Figure 18). Four samples: BWR1 (Puerto Rico), WHS10 (Wilson Hot Springs,UT), WHS10 sediment taken from -80°C and BIG19 (Big Bend, TX), showed higher power outputs than the positive control. WHS10 from -80°C showed the highest

power output of 6.6 mW $^{m-2}$ at 50 h after inoculation, which was 1.78-fold higher than that of the control isolate.

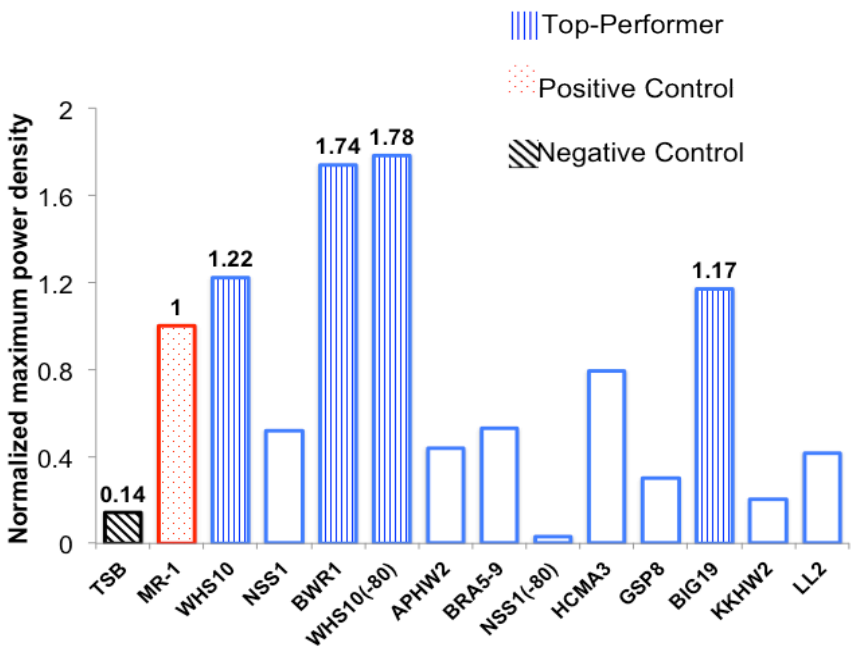


Figure 18. Environmental soil sample screening with the microfluidic MFC array (Bra2-5 data not shown). Soil samples were loaded into batch-mode operated anode chamber of two MFC array devices with *Shewanella oneidensis* MR-1 as positive control and TSB as a negative control on each device. Power outputs of all MFC units were measured twice a day. Maximum power outputs of all soil samples over 300 hours' operation were calculated and normalized to *Shewanella oneidensis* MR-1 on the same device and were plotted together (n = 3).

V.4 Discussion

Bacterial communities and compositions that can be sustained in MFCs are just beginning to be studied [151] studies thus far have focused on system architecture. Diverse ranges of microorganisms from environmental inocula have been found to be associated with MFCs [152]. Pure cultures of isolates belonging to, Firmicutes, Acidobacteria, and Proteobacteria have been found to produce electrical current in MFCs [153]. As expected, Wilkinson laboratory soil communities containing those phyla: Big19, WHS10, and BWR1, were able to generate current in the MFC array. Big 19 was comprised of approximately, 75% Proteobacteria, BWR1 of approximately 70% Proteobacteria and 10% Acidobacteria, and WHS10 contained 10% Proteobacteria and 65% Unknown phyla (Figure 17b).

Though it is predicted that microbial communities produce more current than single isolates [148], 6 communities in our screen produced less electric current than the positive control isolate *Shewanella* MR-1. This could be due to community composition, as mentioned before communities with a dominant presence of Proteobacteria were able to exceed known maximum power outputs (≥45%)[147]. Communities NSS1, HCMA3 and GSP8 were all communities that produce less current than MR-1, and as suspected contain less than 45% Proteobacteria. However, Bra 5-9 had a lower output than MR-1 but contains approximately 45% Proteobacteria and 3% Acidobacteria.

These results show the MFC array as a screening device for efficiently detecting electricity-generating microbes in environmental samples and that the best performing

communities in the MFC screens are candidates for defining the optimal species compositions for power production. Nine out of 10 soil communities maintained the generation of detectable power on the MFC array device. The microbial diversity of the soil communities varies across communities. The most abundant phylum was Proteobacteria, except in HCMA3, GBS3, and WHS10. These data support the hypothesis that soil communities similar to communities in Logan (e.g. dominant in Proteobacteria) can contribute to power generation in MFC devices. The utility of the device was demonstrated in an environmental microbe screen. During the screen, the MFC array allowed for parallel analysis of power generation of diverse microbes while operating with 380 times less reagents than conventional MFC's. In this screen, electricity generation was profiled using the reference isolate *Shewanella oneidensis* MR-1 along with environmental isolates. The electricity profiles displayed analogous performances in replicates and revealed an environmental isolate with a higher power output than the reference isolate.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Fossil fuels are limited, non-renewable resources that are unevenly distributed throughout the world. The increasing price of petroleum, the diminishing supply of fossil fuels, and negative environmental impacts (e.g. greenhouse gas emissions) will ultimately require a shift to new, preferably renewable, energy sources. Renewable liquid biofuels are sustainable, reduce negative environmental impacts, and are biodegradable. Lignocellulosic biomass is an important substrate for biofuel production. Most biomass used in energy is derived from wood and wood waste. This waste is made of organic compounds such as lignin, cellulose, and hemicellulose. Microbes can hydrolyze biomass to sugar and in turn the sugar fermented into a biofuel product.

It is important that microbes used for production of alternative energy sources and other bioproducts (e.g. biofuel and bioplastics) are able to: grow with minimal nutrients, produce high yields of end products, and tolerate products and by-products of the process. Accomplishing all of these phenotypes is difficult and requires not only the discovery of environmental strains that have evolved these traits but, further optimization of these strains [154] .

Chapter II of this dissertation describes the discovery of environmental strains with multiple biofuel process related traits. The library assembled from isolates in the best performing carboxylate biofuel platform fermentation communities, using a variety of media and oxygenation conditions yielded a diverse group of OTUs, most of which spanned the Phylum *Firmicutes*. In the screens for Congo red, cellulose degradation and

mesophilic growth, 207 isolates were screened and 125 were positive for at least one trait (Figure 19). Forty-three isolates grew at 37°C, some of those isolates were used in vanillin utilization screens, in which 13 isolates were positive, and the complex substrate screens discussed in Chapter III. Chapter III involved a search for robust environmental isolates from the library to produce valued products (e.g. organic acids) from complex substrates and biofuel byproducts. This included assessing isolates for tolerance/utilization of lignocellulosic hydrolysate, bio-oil, and fermentation of pentose sugars (e.g. xylose and arabinose). Four isolates from OTU 11 were proved to be robust in this screen, while the isolatesA07M340 and H20C009, which were positive for traits screened in Chapter II were able tolerate and utilize complex compounds at lower concentrations.

Chapter IV describes an evaluation of 43 isolates capable of mesophilic growth for the ability to produce PHA from the bio-diesel byproduct, glycerol. A little less than half of the isolates screened were positive for PHA production and growth in the lower concentration of glycerol. The PHA positive isolates originated from sediments that had higher conductance and sodium levels than other sediments for isolates evaluated in the screen. Thus this site characteristic effect may well indicate sites with similar characteristics harbor more isolates with potential for this trait. Also, all the isolates screened from OTU 4 were positive for PHA production making this OTU a target for future additional studies. Once again, isolates A07M340 and H20C009 were at some level positive for the all traits screened including, PHA production.

Microbial fuel cells (MFCs) are a novel approach to generate energy, specifically

electricity. Chapter V describes tests of the efficacy of natural communities in and MFC array developed at Texas A&M [6].

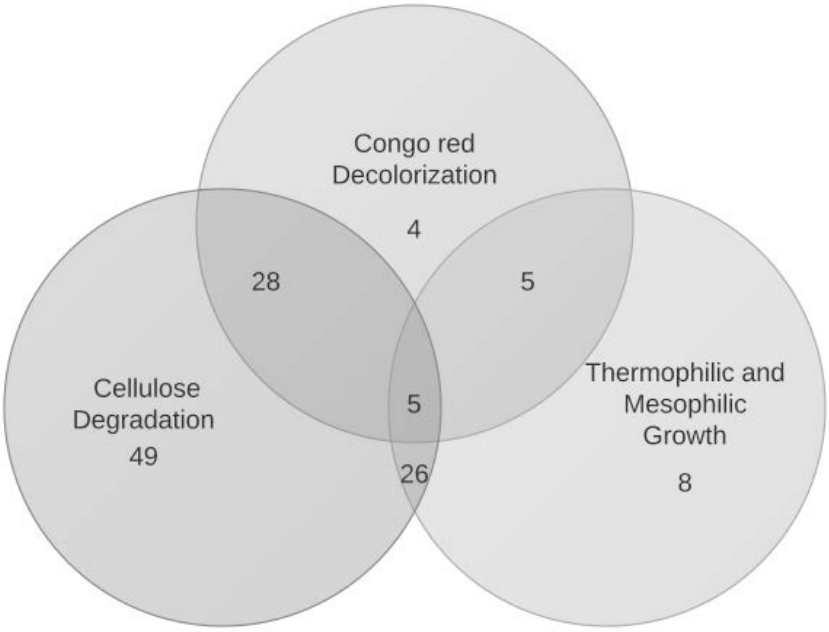


Figure 19. Venn diagram displaying the number of positive isolates (n= 125 positive for at least one trait) identified during a screen of a diverse set of 207 library isolates. Congo red decolorization and cellulose degradation were screens to determine the ability of strains to degrade biomass in biofuel processes. Thermophilic growth was conducted at 55°C and mesophilic growth was conducted at 37°C.

This study involved direct isolation of entire communities from a library of the same soils used as inocula in the carboxylate biofuel platform fermentations. Nine out of 10 of the communities screened maintained detectable amounts of power generation. It was also shown that microbial communities isolated from extreme environments containing greater than 45% Proteobacteria were able to produce power beyond the known standards (e.g. *Shewanella* MR-1) used in industry.

Characterizing species for application in industrial bioprocesses requires substantial efforts [155]. This research has proved to be a valuable resource in discovering multiple environmental strains with biofuel related traits. These isolates are a reservoir for microbial host to be utilized in bioprocesses.

This library was very efficacious in showing the vast intrageneric and intraspecific diversity of *Bacillus* species and their abilities to perform multiple biofuel related traits (Figure 19). Multiplicity is important in bioprocesses such as the production of lignocellulosic biofuels which is a multi-step process consisting of biomass degradation, sugar fermentation and product accumulation. Various approaches have been employed for this process but are not limited to: engineering one industrial strain with most traits, simple engineered communities, and the use of complex communities. Our library offers opportunities for all of the above, providing a resource of isolates with ideal microbial traits used in biofuel bioprocesses (Figure 20).

The research in this dissertation capitalizes on initial investments in an interdisciplinary project to screening natural communities for performance in a biofuel platform to further characterize particular isolates and communities for a variety of traits

relevant to advancing microbial technologies in the energy industry.

Table 10 includes a list of candidates for further study in the Wilkinson lab. Efforts in the Wilkinson lab will include genome sequencing for isolates of interest to catalog the gene and pathway potential in the isolate library. As well as, continued attempts to enrich and characterize the library for isolates with industrially relevant traits.

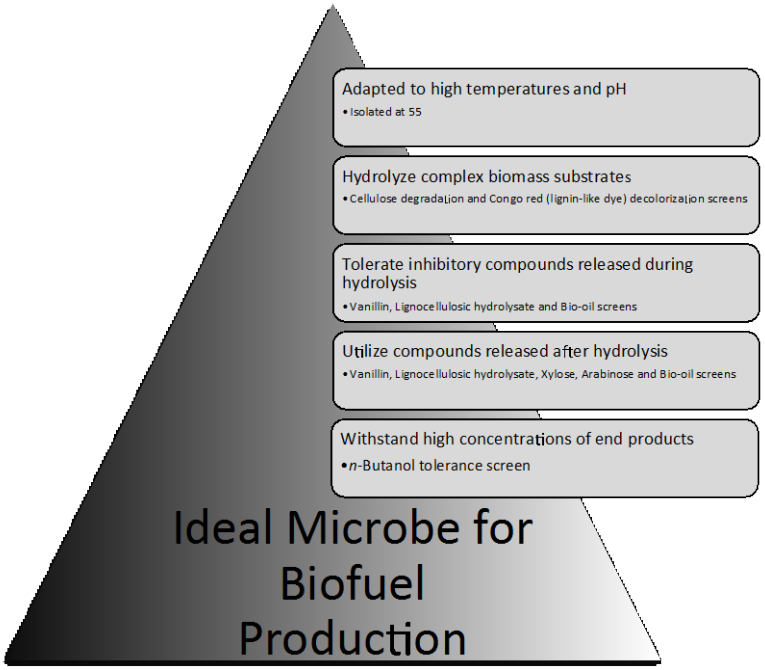


Figure 20. Model for the ideal biofuel microbe. This model was adapted from [1]. The ideal microbe for the production of biofuels would be able to withstand high temperatures and extreme pH, hydrolyze complex biomass substrates and compounds released during hydrolysis, and tolerate inhibitory compounds produced during biomass hydrolysis and high concentrations of end products. The efforts in this dissertation assessed an assembled library for these traits.

Table 10. Isolates of interest for further studies.

Isolate	OTU	Rationale
S44C017	1	from the largest OTU only capable of cellulose degradation, growth at 37°C and growth in low concentrations of bio-oil
S44D010	1	negative for all traits tested (was not tested in screens that were at 37°C)
E08M013	1	capable of Congo red decolorization along with F09D005, but not capable of cellulose degradation; a unique sequence
F09D005	1	capable of Congo red decolorization along with E08M013, but not capable of cellulose degradation; a unique sequence
H20C002	2	is positive for the same traits as H20C009 except for the ability to decolorize Congo red; from the same site, media and OTU as H20C009
H20C009	2	positive for every trait screened (tolerant to lower concentrations of the bio-oil and hydrolysate); a unique sequence
A07M350	4	has the highest fluorescent intensity in PHA screens
A07M352	4	does not fluoresce as high at A07M350, but is from the same site, media and OTU
M24C029	9	only strain along with M24C030 capable of cellulose degradation and Congo red decolorization in OTU 9; a unique sequence
M24C030	9	only strain along with M24C029 capable of cellulose degradation and Congo red decolorization in OTU 9
J04M004	11	positive for the same traits as J11M005 except in its ability to tolerate higher concentrations of bio-oil; it is from the same media, OTU, and geographic region as J11M005 but from a different site; a unique sequence
J11M005	11	capable of growth in high concentrations of bio-oil

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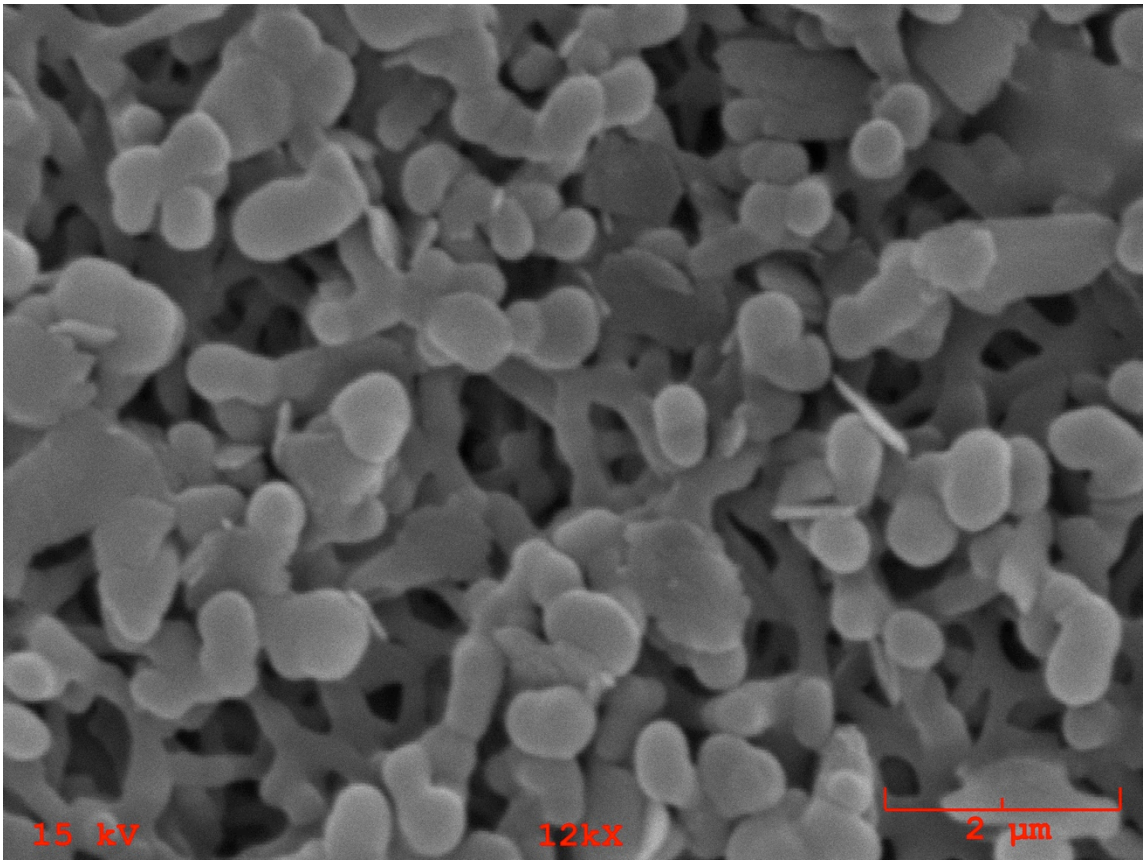
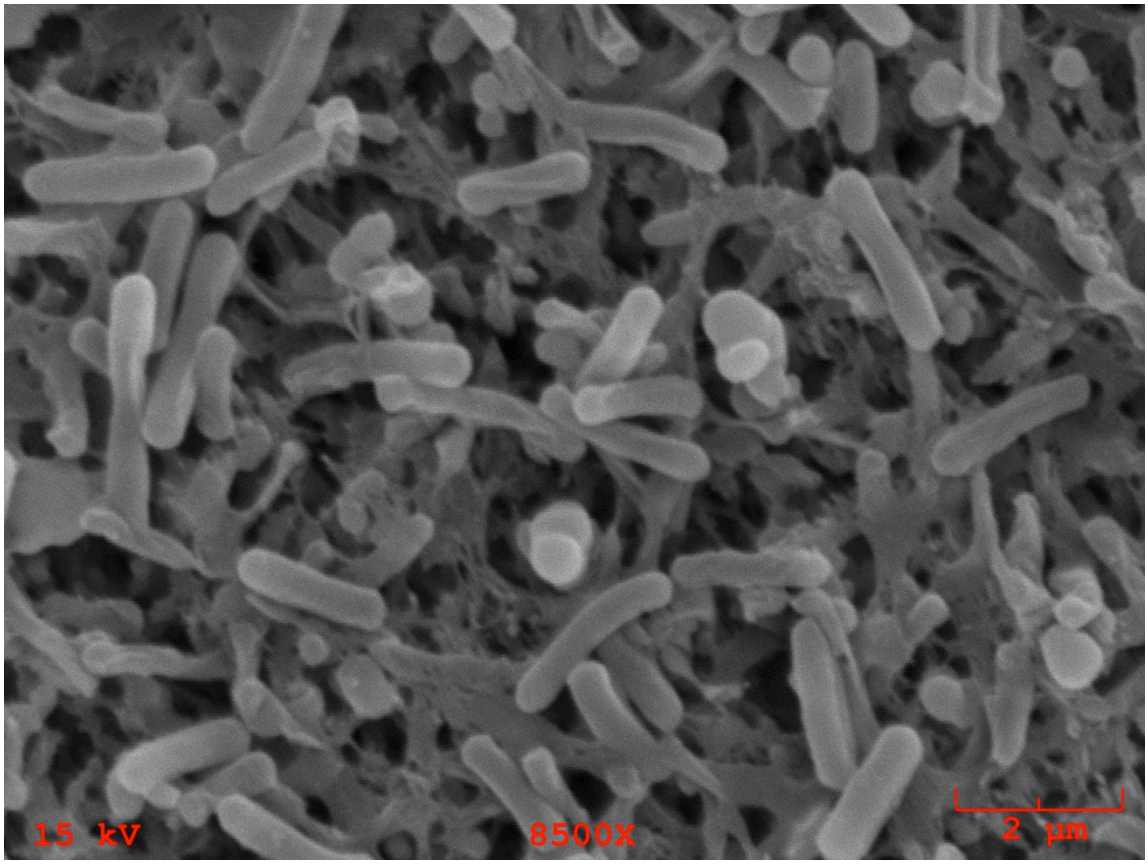
APPENDIX A

SEM (scanning electron microscopy) image of isolate H20C009 from OTU 2.



APPENDIX B

SEM (scanning electron microscopy) image of isolate S48C018 from OTU 6.



APPENDIX C

A table of all 207 isolates screened from the library, 1 indicates the isolate was positive for the traits screened and 0 indicates the isolate was negative. Gray filling in the table indicates the isolate was not screened for that trait. Traits are listed at the top of the column.

SITE	ISOLATE	OTU	Cellulase	CR Decolorize	37C	PHA	Vanillin	1:2 Lignocellulosic Hydrolysate	1:10 Lignocellulosic Hydrolysate	1:20 Lignocellulosic Hydrolysate	0.2% Bio-oil	0.5% Bio-oil	1.25% Bio-oil	1.5% Bio-oil	1.75% Bio-oil	3.0% Bio-oil
E08	M013	1	0	1	0											
F01	M011	1	1	0	0											
F09	D004	1	0	0	0											
F09	D005	1	0	1	0											
F09	D026	1	0	0	0											
G08	C012	1	0	0	0											
G23	C002	1	0	0	0											
G23	C015	1	0	0	0											
G24	C010	1	0	0	0											
H01	M191	1	0	0	0											
J04	D008	1	0	0	0											
J19	M020	1	0	0	0											
J20	M006	1	0	0	0											
N09	M024	1	0	0	0											
S44	C017	1	0	0	1	0	0				1	1	0	0	0	0
S44	D010	1	0	0	0											
U22	C020	1	1	0	0											
U22	D007	1	0	0	0											
U22	D011	1	0	0	0											
U22	D017	1	1	0	0											
U22	M431	1	0	0	0											
E07	C003	2	1	0	0											
E08	M128	2	0	0	0											
F02	C011	2	0	0	0											
F02	C012	2	1	1	0											
F02	C026	2	1	0	0											
F02	M176	2	0	0	0											
G08	C009	2	1	0	0											
G19	C012	2	1	1	0											

SITE	ISOLATE	OTU	Cellulase	CR Decolorize	37C	PHA	Vanillin	1:2 Lignocellulosic Hydrolysate	1:10 Lignocellulosic Hydrolysate	1:20 Lignocellulosic Hydrolysate	0.2% Bio-oil	0.5% Bio-oil	1.25% Bio-oil	1.5% Bio-oil	1.75% Bio-oil	3.0% Bio-oil
G23	C023	2	0	0	0											
H01	C001	2	1	1	0											
H20	C009	2	1	1	1	1	1	0	0	1	1	1	0	0	0	0
H20	M325	2	1	0	0											
J04	C004	2	1	0	0											
J04	C028	2	1	1	0											
J20	M030	2	1	1	0											
S44	C007	2	1	0	0											
S44	C008	2	1	0	0											
S44	C015	2	1	1	0											
S44	C021	2	1	0	0											
A07	M338	4	1	1	0											
A07	M339	4	0	1	1	1	1	0	0	1	1	1	0	0	0	0
A07	M340	4	1	1	1	1	1	0	0	1	1	1	0	0	0	0
A07	M341	4	0	1	0											
A07	M345	4	0	1	1	1	0	0	0	1						
A07	M347	4	0	1	1	1	1	0	0	1	1	1	1	0	0	0
A07	M348	4	0	1	1	1	0	0	0	1						
A07	M349	4	0	0	1	1	1	0	0	1	1	1	0	0	0	0
A07	M350	4	0	0	1	1	0									
A07	M352	4	0	0	1	1	0	0	1	1	1	1	0	0	0	0
A07	M355	4	1	0	1	1	1	0	0	1	1	1	0	0	0	0
A07	M360	4	0	1	0											
E08	M016	4	1	0	1	1	0	0	1	1	1	1	0	0	0	0
F22	M504	4	1	0	0											
H01	C005	4	1	0	0											
H01	C006	4	1	0	0											
H01	C012	4	0	1	0											
H20	C002	4	1	0	1	1	1	0	0	1	1	1	0	0	0	0

SITE	ISOLATE	OTU	Cellulase	CR Decolorize	37C	PHA	Vanillin	1:2 Lignocellulosic Hydrolysate	1:10 Lignocellulosic Hydrolysate	1:20 Lignocellulosic Hydrolysate	0.2% Bio-oil	0.5% Bio-oil	1.25% Bio-oil	1.5% Bio-oil	1.75% Bio-oil	3.0% Bio-oil
H20	C011	4	1	0	1	1	1									
H20	C027	4	1	0	1	1	1				1	1	0	0	0	0
H20	M301	4	1	0	0											
H20	M327	4	0	0	1	1	0				1	1	1	0	0	0
J04	M010	4	1	0	1	1	1	0	0	1	1	0	1	0	0	0
J04	M015	4	1	0	0											
N09	M001	4	0	0	0											
N09	M022	4	1	0	0											
P01	M008	4	1	0	1	1	0	0	0	1	1	1	0	0	0	0
P01	M009	4	1	0	1	1	0									
R08	M006	4	0	0	0											
R08	M020	4	1	0	0											
S44	C003	4	1	0	0											
S44	C011	4	1	0	1	1	0	0	0	1						
E08	M436	5	1	0	0											
F01	M009	5	0	0	0											
F01	M010	5	1	0	1	0	0				1	0	0	0	0	0
F01	M017	5	1	0	1	0	1	0	0	0						
F01	M018	5	0	0	0											
F02	D001	5	1	0	1	0	0				1	0	0	0	0	0
F02	D002	5	1	0	0											
F02	D003	5	1	0	0											
F02	D005	5	1	0	0											
F02	D026	5	0	0	0											
F02	D032	5	1	0	0											
F02	M034	5	1	0	0											
F02	M038	5	1	0	0											
F02	M402	5	1	0	0											

SITE	ISOLATE	OTU	Cellulase	CR Decolorize	37C	PHA	Vanillin	1:2 Lignocellulosic Hydrolysate	1:10 Lignocellulosic Hydrolysate	1:20 Lignocellulosic Hydrolysate	0.2% Bio-oil	0.5% Bio-oil	1.25% Bio-oil	1.5% Bio-oil	1.75% Bio-oil	3.0% Bio-oil
F05	M393	5	1	0	1	0	0	0	0	0						
F05	M396	5	1	0	1	0	1	0	0	0						
F05	M397	5	1	0	0											
F09	M435	5	1	0	1	0	0									
G08	M102	5	1	0	1	0	1				1	0	0	0	0	0
J20	M010	5	1	0	0											
U22	M429	5	1	0	1	0	0									
U22	M430	5	1	0	1	0	0									
G08	C001	6	1	0	1	0	0									
G08	C025	6	0	0	0											
J19	C002	6	1	0	0											
S48	C002	6	1	0	0											
S48	C003	6	1	0	0											
S48	C004	6	1	0	1	0	0									
S48	C005	6	1	0	0											
S48	C006	6	1	1	0											
S48	C011	6	1	1	0											
S48	C012	6	1	1	0											
S48	C013	6	1	1	0											
S48	C015	6	1	0	0											
S48	C016	6	1	0	0											
S48	C017	6	1	1	1	1	0									
S48	C018	6	1	1	1	1	0									
S48	C019	6	1	1	0											
S48	C020	6	1	1	0											
S48	C021	6	1	1	0											
S48	C022	6	1	1	0											
S48	C023	6	1	1	0											

SITE	ISOLATE	OTU	Cellulase	CR Decolorize	37C	PHA	Vanillin	1:2 Lignocellulosic Hydrolysate	1:10 Lignocellulosic Hydrolysate	1:20 Lignocellulosic Hydrolysate	0.2% Bio-oil	0.5% Bio-oil	1.25% Bio-oil	1.5% Bio-oil	1.75% Bio-oil	3.0% Bio-oil
S48	C024	6	1	0	0											
S48	C024	6	1	1	0											
S48	C027	6	1	1	0											
S48	C029	6	1	1	0											
U22	C009	6	0	0	0											
U22	C015	6	1	1	0											
U22	C021	6	0	0	1	0	0									
U22	C025	6	1	1	0											
U22	C026	6	1	0	1	0	0									
U22	C027	6	1	1	0											
U22	C028	6	1	1	0											
U22	C029	6	1	0	0											
G08	C011	7	0	0	0											
E08	C033	8	1	0	0											
G08	C016	8	0	0	0											
G08	C017	8	0	0	0											
H20	C004	8	0	0	0											
H20	D002	8	0	0	0											
J18	C028	8	1	0	0											
J18	C029	8	1	0	0											
J18	C030	8	1	0	0											
K49	C001	8	1	0	0											
K49	C002	8	0	0	0											
K49	C003	8	0	0	0											
K49	C008	8	0	0	0											
K49	C010	8	0	0	0											
K49	C020	8	0	0	0											
K49	C023	8	0	0	0											
S44	D026	8	0	0	0											

SITE	ISOLATE	OTU	Cellulase	CR Decolorize	37C	PHA	Vanillin	1:2 Lignocellulosic Hydrolysate	1:10 Lignocellulosic Hydrolysate	1:20 Lignocellulosic Hydrolysate	0.2% Bio-oil	0.5% Bio-oil	1.25% Bio-oil	1.5% Bio-oil	1.75% Bio-oil	3.0% Bio-oil
J18	C007U	9	0	0	0											
J18	C009	9	0	0	0											
J18	C018	9	0	0	0											
J18	C022	9	1	0	0											
J19	C005U	9	0	0	0											
J19	C022	9	0	0	0											
M24	C019	9	0	0	0											
M24	C029U	9	1	1	0											
M24	C030	9	1	1	0											
J04	M004	11	1	0	1	0	0	0	1	1	1	1	0	0	0	0
J11	M005	11	1	0	1	0	0	0	0	0	1	1	1	1	0	0
J11	M011	11	0	0	0											
J11	M017	11	1	0	1	0	0	0	1	1	1	1	1	1	0	0
J11	M287	11	1	0	1	0	0	0	1	1	1	1	1	1	0	0
J20	M023	11	1	0	1	0	0	0	1	1	1	1	1	1	0	0
J20	M027	11	0	0	1	0	0	0	1	1	1	1	1	1	0	0
J20	M029	11	1	0	0											
J20	M031	11	0	0	1	0	0									
J04	M017	12	0	0	0											
K49	M002	12	0	0	0											
K49	M005	12	0	0	0											
K49	M305	12	0	0	0											
K49	M307	12	0	0	0											
N09	M018	12	0	0	0											
P01	M011	12	0	0	0											
P01	M013	12	0	0	0											
P01	M018	12	0	0	0											
T02	M006	12	0	0	0											
T02	M020	12	0	0	0											

SITE	ISOLATE	OTU	Cellulase	CR Decolorize	37C	PHA	Vanillin	1:2 Lignocellulosic Hydrolysate	1:10 Lignocellulosic Hydrolysate	1:20 Lignocellulosic Hydrolysate	0.2% Bio-oil	0.5% Bio-oil	1.25% Bio-oil	1.5% Bio-oil	1.75% Bio-oil	3.0% Bio-oil
U22	C014	12	1	0	0											
U22	C019	12	1	0	0											
F02	M031	14	0	0	0											
G19	D003	14	0	0	0											
G19	D013	14	0	0	0											
G19	D021	14	0	0	0											
G19	D026	14	0	0	0											
G19	D028	14	0	0	0											
G24	D003	14	0	0	0											
G24	D006	14	0	0	0											
G24	D015	14	0	0	0											
G24	D020	14	0	0	0											
K49	D009	14	1	0	0											
S48	D007	14	0	0	0											
S48	D015	14	0	0	0											
S48	D017	14	0	0	0											
S48	D025	14	0	0	0											
S48	D028	14	0	0	0											
E08	C019	15	0	0	0											
G23	C013	15	0	0	0											
H01	C007	15	0	1	1	0	0	0	0	0						
E08	C017	19	1	0	0											
S44	C019	20	1	1	0											
S44	C024	21	1	1	0											
F08	D029	22	0	0	0											
F02	M041	28	1	0	0											
R08	M008	32	0	0	0											
H01	D011	36	0	0	0											

SITE	ISOLATE	OTU	Cellulase	CR Decolorize	37C	PHA	Vanillin	1:2 Lignocellulosic Hydrolysate	1:10 Lignocellulosic Hydrolysate	1:20 Lignocellulosic Hydrolysate	0.2% Bio-oil	0.5% Bio-oil	1.25% Bio-oil	1.5% Bio-oil	1.75% Bio-oil	3.0% Bio-oil
H20	D004	36	0	0	0											
G13	D029	37	0	0	0											
S44	D013	40	0	0	0											
H01	D012	41	0	0	0											
G08	C006	44	1	1	0											
K49	M013	45	0	0	0											
K49	M015	45	0	0	0											