CHEMICAL TREATMENT OF LIGNOCELLULOSIC BIOMASS USING IONIC LIQUID AND THE DEVELOPMENT OF A COMPATIBLE HYPER-TOLERANT BACTERIAL STRAIN FOR LIPID PRODUCTION

A Thesis

by

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ABSTRACT

Lignocellulosic biomass is an abundant and potentially valuable source of organic substrate for biofuel-producing microorganisms. Various physical, chemical, and biological treatment methods have been established to alleviate the recalcitrance of lignocellulose for substrate utilization. The objective of this study is to evaluate the potential of another chemical treatment method, utilizing an ionic liquid [Emim][Cl] pretreatment coupled with dilute acid treatment, to convert spent coffee grounds, corn husk, and bermuda grass into fermentable sugars. The second objective was to develop a highly tolerant bacterium to minimize the need for detoxification. Fermentable sugar yields were quantified and compared to enzymatic treatment methods. Results indicated that the chemical treatment method was able to produce sufficient sugar yields to be considered a prospective alternative to enzymatic hydrolysis. *R. opacus* PD630 was able to utilize the sugar released from this chemical treatment for growth. Additionally, a compatible hyper-tolerant strain PD630^{V4} was developed using an adaptive evolution approach. Inhibitory concentrations were quantified for the PD630 and used as an adaption benchmark. The adapted strain was capable of growth on inhibitory levels of pH, [Emim][Cl], and HMF. Moreover, PD630^{V4} strain showed significantly less inhibition than the wild-type strain on crude hydrolysate. The PD630^{V4} strain was capable of reaching stationary phase and accumulating lipids in approximately 5 days when cultivated in the crude spent coffee grounds, corn husk, and grass hydrolysates.

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Contributors

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1. INTRODUCTION AND OBJECTIVES

1.1 Introduction

There are many advantages for use of biofuel including: (i) reducing US dependency on foreign oil, (ii) development of environmentally sustainable and renewable sources, (iii) compatibility with most gasoline engines, and (iv) reducing greenhouse pollutants such as carbon dioxide and nitrogen oxides emissions [1]. In recent years, there has been a renewed interest toward the development of biofuels derived from lignocellulosic biomass. This is partly due to society's response to limited fossil fuel reserves, increasing energy costs and emissions of carbon dioxide.

However, the recalcitrance of lignocellulosic biomass represents a major obstacle towards wide commercialization of this energy source. In general for biofuel production, raw lignocellulosic biomass typically goes through three primary phases: pre-treatment, saccharification, and fermentation [2] (Figure 1.1). These phases are typically implemented through a physical/chemical treatment system, followed by a microbiological treatment [3].

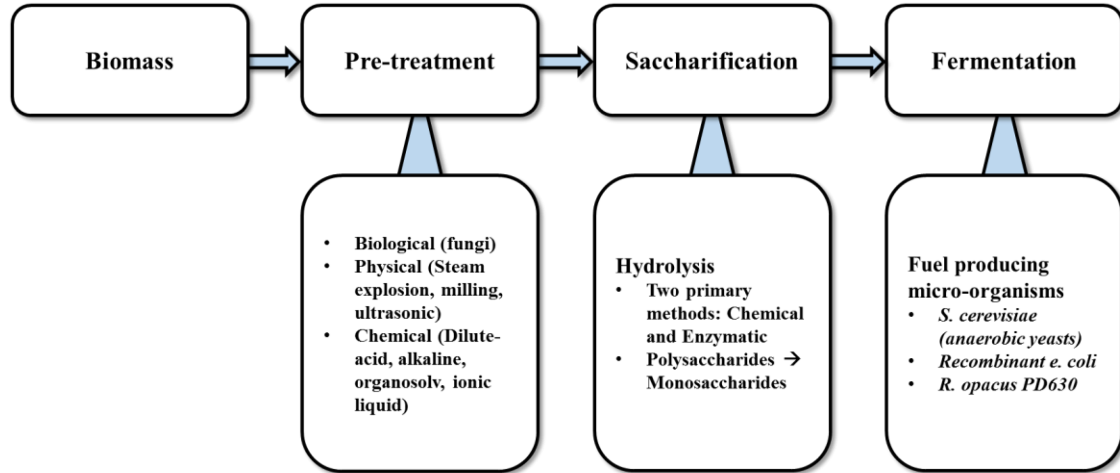


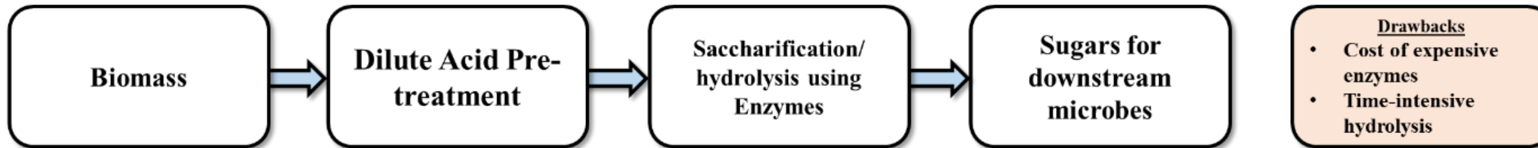
Figure 1.1 Three-stage biorefinery process to convert biomass to biofuel.

The physical/chemical treatment systems are used for the pre-treatment phase. The system is used to catalyze the crude biomass into a suitable substrate that can be utilized by downstream biological catalysts. There is a plethora of physical/chemical treatment systems that have been investigated in the last few decades, including, but not limited to steam explosion, acid, alkali, organosolv, and ionic liquids [4-7]. Downstream microbiological systems take place in the saccharification and fermentation stage. Microbiological systems are used as a catalysts for converting polysaccharides into monomers, and monomers into valuable products, such as biofuels [8]. Microbiological systems have a niche in these processes because of their unqiue biological mechanisms that help to convert a particular substrate into valuable products [9]. These mechanisms are considered unique for microbials and are difficult to reproduce by other means.

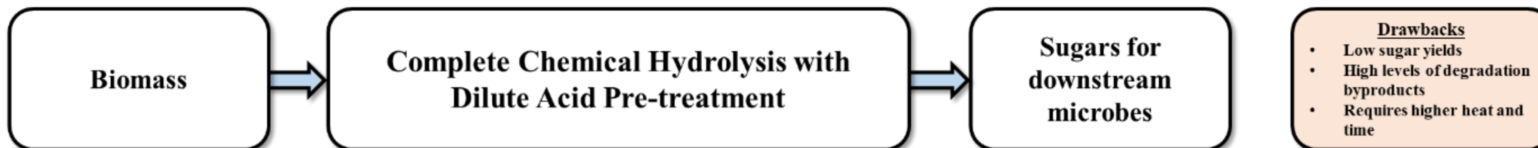
Examples of microbiological catalysts include *Saccharomyces cerevisiae* (yeasts) [10], recombinant *E. coli* [11], microalgae [12], and *Rhodococcus opacus* PD630 [13].

However, these biofuel processes can exhibit disadvantages that limit its potential for wide commercialization. In particular, a well-known biofuel process is the dilute acid/enzyme method [14]. In this method, the pre-treatment phase consist of biomass treated by dilute acid at high temperature. Saccharification consists of enzymatic hydrolysis that break the substrate into monomeric sugars. The fermentation phase consists of microorganisms that can convert sugars into biofuel. In this particular process, the acid treatment can yield unwanted by-products that reduce the overall sugar yields [7, 15, 16]. In addition, enzymes and microbial fermentation are inhibited by compounds and conditions in the prior chemical system. Because of this, the process requires additional complications, such as pH neutralization, compound removal, and/or genetically tolerant enzymes and microorganisms, all of which are costly [9, 17, 18]. To be considered an economically viable, the process must be streamlined and cost-effective. Thus, this study will investigate the potential of an alternative chemical treatment process using ionic liquids (ILs) on different biomass species (Figure 1.2).

1. Biomass treatment process: using dilute acid and enzymatic hydrolysis



2. Biomass treatment process: using chemical hydrolysis with dilute acid



3. Proposed chemical treatment process: using ionic liquids

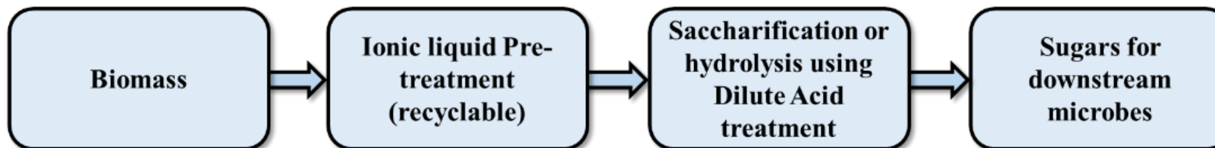


Figure 1.2. Established treatment trains using chemical and enzymatic hydrolysis and their drawbacks, and proposed chemical treatment using ionic liquid (blue).

1.2 Goals, objectives and hypothesis

The overall **goal** of this research is to investigate an alternative chemical treatment process and develop compatible tolerant bacteria for lipid production. To achieve this goal, two specific objectives are proposed and explained. The hypothesis and tasks for each objective are outlined below.

Objective 1. Evaluate the potential of an alternative bio-refinery treatment for downstream fermentation: ionic liquid pretreatment and dilute acid treatment

Hypothesis: An ionic liquid (IL) pre-treatment will aid in improving total sugar yields by the dilute acid hydrolysis treatment. Sugar yields will increase if ionic liquids are used instead of acid for pretreatment. Sugar yields will be sufficient so that enzymatic hydrolysis may not be required

Task 1-A. Determine the reducing sugar yield from the proposed chemical treatment on commercial cellulose and compare the yields to established treatments such as acid + enzymes, and IL + enzymes.

Task 1-B. Evaluate the lignin removal ability of IL pretreatment compared to dilute acid treatment on spent coffee grounds (SCG), corn husk, and Bermuda grass (BG).

Task 1-C. Determine the total sugar yields from the proposed chemical treatment on these three biomass species and compare the yields to established treatments such as acid + enzymes, and IL + enzymes.

Task 1-D. Evaluate the ability of PD630 to grow on each biomass hydrolysate.

Objective 2. Develop a hyper-tolerant lipid-accumulating bacteria that is compatible with the alternative chemical treatment

Hypothesis: Using an adaptive evolution method, an improved PD630 strain can be developed to tolerate acidic conditions, residual amounts of IL, and 5-Hydroxymethylfurfural (HMF) formation from the chemical treatment process. This improved strain will be able to grow more quickly than the wild-type strain in hydrolysate.

Task 2-A. Determine the original levels of each inhibitory factor (pH levels, IL concentrations, and HMF concentrations) in each hydrolysate.

Task 2-B. Determine the actual concentrations PD630 can tolerant for each inhibitory factor.

Task 2-C. Using the adaptive evolution strategy, adapt the wild-type PD630 strain to acidic conditions (low pH), residual IL concentrations, and HMF concentrations. Compare growth of the wild-type PD630 and the evolved PD630 in corresponding media.

Task 2-D. Test the growth of the evolved strain and the wild-type on crude and untreated hydrolysate and compare lipid accumulation.

1.3 Thesis overview

This thesis consists of five sections. **Section 1** describes the steps of biomass treatment for biofuel production as well the goals and objectives for this research. **Section 2** reviews the recent literature on treatment techniques and more specifically the ionic liquid pretreatment for better understanding on the advantages and disadvantages of these treatments. The model strain used in these experiments and the adaptive evolution strategy is also described. **Section 3** shows the results of the proposed treatment obtained in this research. The sugar yields obtained from the proposed treatment and comparative treatments are displayed in this section for the three biomass species. Sugars yields were shown to be sufficient for a growth substrate for PD630. Lignin removal was also investigated in this section to show that the model IL was able to remove significant lignin from the biomass. Growth of wild-type PD630 was also examined in this section and found to be inhibited if the hydrolysate is not detoxified or neutralized. **Section 4** shows the results obtained from quantifying the inhibitory factors found in each hydrolysate and their inhibitory effects they have on PD630. This section also shows the results of adaptive evolution strategy. A triple tolerant mutant strain, termed $PD630^{V4}$ was developed and successfully able to overcome inhibition and grow on each crude hydrolysate. **Section 5** summarizes the findings in this study and implications of the work done in this research.

2. LITERATURE REVIEW

2.1 Biomass processing

Biomass is a term used to describe organic matter that is typically derived from plants or other organisms [19]. Lignocellulosic biomass is a complex plant-based composite material that primarily comprises three polymeric compounds: lignin, cellulose, and hemicellulose (Figure 2.1). Examples of lignocellulosic biomass include agricultural residues (straws, bagasse, stover), herbaceous energy crops (switchgrass, alfalfa), woody crops (softwood, hardwood), and industrial wastes (spent coffee grounds, paper and pulp waste, forestry residues) [20].

In recent years, there has been a renewed interest toward the development of biofuels derived from lignocellulosic biomass [21]. This is partly due to society's response to limited fossil fuel reserves, increasing energy costs and emissions of carbon dioxide. More specifically, the US has been making an effort to combat the dependency on foreign oil and environmental deterioration [8]. This includes sustainability efforts that are dedicated toward researching renewable energy production. It has been noted that the utilization of biomass has a remarkable potential to be used as a supplementary, or even alternative, source to non-renewable fossil fuel-based energy. Lignocellulosic biomass is the one of the most abundant resource in the world, taking on many different forms such as industrial wastes, agricultural residues, and dedicated energy crops. It is estimated that only 5 - 8% of biomass produced every year could be enough to satisfy the current consumption rate of petroleum through proper biomass conversion

technologies [22]. Biomass feedstocks are renewable, environmentally friendly, and available in diverse forms all over the world. Furthermore, current generation lignocellulosic feedstocks are not utilized for mainstream food production, and therefore do not having competing interests with food crops. Depending on the conversion technique, lignocellulosic biomass can be utilized to produce many types of renewable products including but not limited to bio-ethanol, bio-diesel, bio-plastics, methane, hydrogen gas, syngas, and lignin-based products [10, 23-26]. Bio-fuel production is currently the most ubiquitous practice using lignocellulosic biomass.

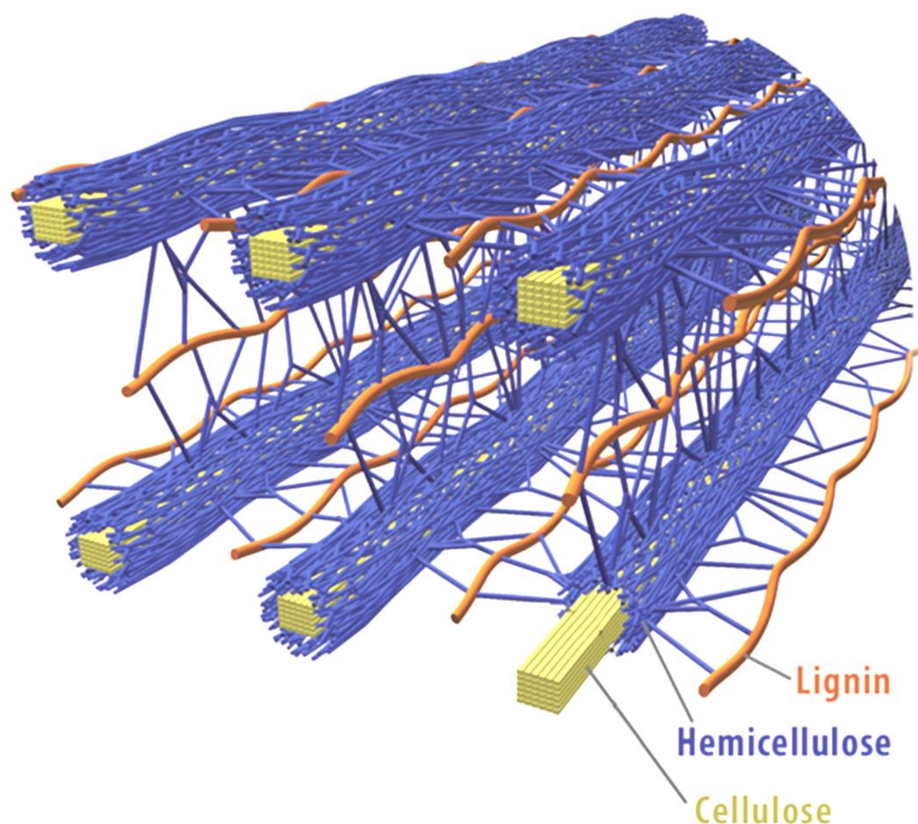


Figure 2.1. Model structure of a lignocellulosic polymer separated into cellulose, hemicellulose and lignin fractions [27].

Cellulose is a linear polymeric carbohydrate that is made up of monosaccharide glucose units. The cellulose portion of lignocellulosic biomass can be considered its skeleton core, making up approximately 30 – 50% of the overall biomass [27]. The D-glucose monomeric units of cellulose have a β-(1, 4) - glycosidic bond that covalently links the two units together, allowing them to form a linear chain structure, which is referred to as the oligosaccharide glucan unit. Two D-glucose units linked by a singular β-(1, 4) - glycosidic bond is identified as cellobiose. The chains of repeating cellobiose are the primary components that make up the entire cellulose molecule. These intramolecular bonds gives this molecule a flat sheet configuration. Van der Waal forces allow these flat sheet structures to interact to remain stable. Each molecule is also linked to its neighboring molecule by a single intermolecular hydrogen bond to form a cellulose strand. The linear configuration of each cellulose strand allows it to be tightly packed together to form a stabilized cellulose fibril. Also because of its length and high molecular weight, it is insoluble in water and many organic solvents. This long, block-like polymer configuration makes cellulose distinctly crystalline and stable. Cellulose is categorized in multiple polymorphs [28, 29]. Cellulose I is the naturally occurring form of cellulose, which can be divided into cellulose I-α and cellulose I-β. Cellulose I is considered the native form of cellulose and is stable in nature. It characteristically linked by an intermolecular hydrogen bond between each cellulose molecule on the same sheet. Cellulose II is formed through dissolution and regeneration or swelling treatment of Cellulose I. Cellulose II has a distinct hydrogen bond linking together the parallel flat sheets, making this polymorph form more thermodynamically favorable. Since cellulose

II is more thermodynamically stable than cellulose I, the transformation from cellulose I to cellulose II is considered irreversible.

Hemicellulose is an amorphous polymeric carbohydrate that is constructed from monosaccharides, pentose (five-carbon) sugars (i.e. xylose and arabinose) and hexose (six-carbon) sugars (i.e. glucose, galactose, and mannose). Additionally, hemicellulose branches may also contain sugar acid groups. In most grasses and hardwood, the primary sugar in hemicellulose is xylose. Due to its amorphous matrix form, hemicellulose can be considered the lattice that holds the cellulose structure in place, making up approximately 15 – 35% of the overall biomass [27]. Hemicellulose is also is non-covalently bonded to cellulose. The amorphous properties of hemicellulose allow it to be more prone to deconstruction than lignin and cellulose.

Lignin is an aromatic polymer that consists of a combination of coniferyl, sinapyl, and p-coumaryl phenolic monomers which form into guaiacyl, syringyl and p-hydroxyphenyl subunits, respectively. Lignin makes up approximately 10 – 30% of the overall biomass. The arrangement of the monomers that form lignin differs between the species of plant. For example, grass types will have small amounts of p-hydroxyphenyl subunits, and softwoods may contain only guaiacyl subunits. The delignification process is vastly dependent on the configuration of the lignin structure. The Carbon–Oxygen (C-O) bonds of α- and β-arylalkyl ethers (β-O-4) ether bonds are the most common linkages that hold the aromatic rings and overall lignin structure together, although there are other carbon-carbon (C-C) and C-O linkages that are present in lesser quantities. The lignin component of a plant can be described as a supporting barrier that protects the cellulose-

hemicellulose complex from physical or chemical damage. In terms of biochemical protection, lignin also reduces enzymatic hydrolysis by forming a physical barrier that reduces enzymatic accessibility and by adversely binding onto cellulase enzymes. The lignin complex is also covalently bonded to hemicellulose. The resilient nature of lignin makes it difficult to chemically or physically break down its structure to access the cellulose-hemicellulose complex.

The recalcitrance of lignocellulosic biomass represents a major obstacle towards commercialization of this energy source [30, 31]. In conventional bio-fuel production, raw lignocellulosic biomass typically goes through three primary phases: pre-treatment, saccharification, and fermentation.

Saccharification occurs when a polysaccharide is broken down into shorter chains. In many cases of lignocellulosic biomass, saccharification is also referred to as hydrolysis because of the cleavage of polysaccharide linkages occurs with the uptake of a water molecule. In the case of biofuel production, the saccharification/hydrolysis step occurs when cellulose is depolymerized to smaller molecules (polysaccharides and oligosaccharides). Complete hydrolysis occurs when polysaccharides are converted into monosaccharides (e.g. glucose).

The physio-chemical properties of lignocellulosic biomass vary depending on the type of biomass [32]. Regardless, pre-treatment methods should alters physical and chemical properties of the material. The pre-treatment phase is vital to the downstream efficiency of a bio-fuel production process. In nature, plants have developed resilience to external damage, such as could be caused by physical or microbial processes. Because of

this, most types of biomass have a particularly low decomposition rates, ranging from months to years before their molecular structure is broken down, so that the sugars can be utilized as substrate for soil microorganisms. The pre-treatment step represents a significant fraction of the cost of the biofuel production process [4]. In an efficient pre-treatment process in a bio-refinery, decomposition must be completed within hours or days. Some specific goals of pre-treatment should include:

i) Breaking the covalent bonds between lignin and hemicellulose, which is required to separate lignin from the cellulose-hemicellulose complex.

ii) Removal or deconstruction the lignin structure that is protecting the cellulose-hemicellulose complex, which allows access to the cellulose skeleton. Moreover, lignin molecules can inadvertently provide additional binding sites for enzymes to attach onto, reducing the amount of enzymes that can attach onto cellulose. The presence of residual lignin would require higher enzyme loadings in the hydrolysis step to achieve sufficient saccharification.

iii) Reduction of the crystallinity of cellulose through cleaving hydrogen bonds. Breaking down the long-chain polysaccharides (glucan) in cellulose fibrils into shorter chains reduces the crystallinity of the entire structure. Decreased crystallinity allows for easier digestibility and deconstruction. Additionally, shorter polymer chain molecules become more soluble, allow enzymes to more easily attach.

iv) Increasing porosity of the biomass in order to allow for better enzymatic digestibility by increasing the amount of surface area available to bind onto. The larger the area of the cellulose, the more enzymes can attach onto the surface substrate.

Effective pre-treatments should be able to achieve the goals mentioned above. There is no pre-treatment method that is completely superior to others, since lignocellulosic biomass type and technological availability vary dramatically all over the world. There is a plethora of pre-treatment methods that have been investigated in the last few decades. Some of these technologies have increasing potential to be commercialized, as the processes are optimized and costs of materials become cheaper. These methods can be generally categorized into physical, chemical, biological, or a combination of the former three pre-treatments. Each of these pre-treatment methods have their own advantages and caveats summarized in Table 1. It should be mentioned that for a pre-treatment method to be considered for commercialization, the pre-treatment should also take into account: the cost of materials and energy, processing time, biomass type, hazardous chemicals neutralization, catalyst recycling, and formation of inhibitory by-products.

Physical pre-treatment can also be sub-classified as mechanical treatment. Milling, chipping, and extrusion are examples of mechanical methods that have the goal of size reduction. The raw material, such as straw or wood chips, are crushed to allow for better handling and transportation. This method also increases the surface area of the

material. It has also been widely reported that crushing biomass prior to another pre-treatment step will increase the effectiveness of that same pre-treatment by reducing the cellulose crystallinity.

Thermo-physical pre-treatments use high temperature and pressure to decompose the lignocellulose. Steam explosion pre-treatment is a common method that is used. The biomass is exposed to high-pressure steam and the pressure rapidly reduced to create an explosive decompression. During the high pressure steam phase, hemicellulose hydrolysis and lignin breakage occur.

Dilute acid pre-treatment of biomass is a classic technique that has been used in biomass pretreatment for decades. It can be used as a singular treatment step (complete hydrolysis) to convert biomass to glucose. However, this requires high energy and results in the formation inhibitory by-products. Acid hydrolysis happens as a reaction between lignocellulose and the protons occurring from the dissociation of strong acids to yield various molecules such are monosaccharides and oligosaccharides. Dilute acid can also be used as a pre-treatment step, prior to enzymatic hydrolysis. Dilute acid is a common method and relatively inexpensive because of the low cost of materials such as HCl and H_2SO_4. However, the process usually requires high temperatures, which can be energy intensive. Also, acid pretreatment can produce degradation by-products from cellulose, such as 5-hydromethylfurural (HMF) (Figure 2.2), and from lignin, such as phenols, which can inhibit growth of microbes in the downstream processes and this can result in decreased biofuel yields. In treatment cases similar to this, these degradation by-products have to be removed prior to inoculation of microorganisms or enzymes,

adding to the cost of the process. Downstream enzymes, such as cellulase and β-glucosidase, break down the biomass even further, from polysaccharides to monosaccharides. This stage is called enzymatic hydrolysis. Treatment with enzymes can provide an advantage, because of their specificity to carbohydrate chains and conversion into monomeric sugars (glucose). They are not inhibited by most by-products and have high potency, but enzymes do require specific pH ranges, temperatures, and buffer media. Enzymes also require long incubation periods and currently are costly for mass production.

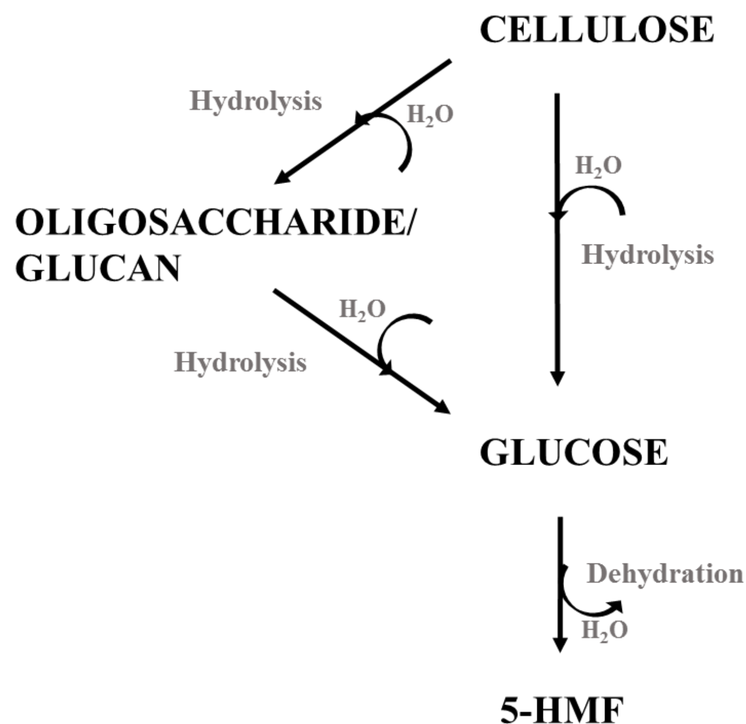


Figure 2.2. Degradation pathway of cellulose to 5-Hydroxymethylfurfural/HMF

2.2 Ionic liquids for biomass treatment

Ionic liquids (ILs) are salts that remain in the liquid phase at below 100^0C. ILs have been popularized because of their desirable characteristics including: negligible vapor pressure (e.g. no emissions of volatile organic compounds (VOCs)), high thermal stability, non-caustic reactions, and versatility. It is estimated that there are over a million different ionic liquids that have been synthesized for various applications, including separation, material synthesis, batteries, bio-catalysis, pure solvents, and co-solvent. ILs contain a large organic cation and an inorganic anion. By modifying the characteristics of the cation and anion, the properties the solvent can be tuned to varying polarity, viscosities, hydrophobicity, and reactivity. This allows ILs to be synthesized for task-specific purposes. Because of their extreme versatility, inert nature, and recyclability, ILs have been called “green solvents of the future” or “designer solvents”. In summary, ILs are considered superior solvents due to:

- Environmental benignity compared VOCs,
- Ability to be tailor-made and designed for specific purposes,
- Non-flammability,
- Unique solubilization potential

ILs can be classified by their cation structure, including the common quaternary ammonium, N-alkyl-pyridinium, and imidazolium [33-35]. ILs are typically viscous liquids and can be acidic, basic, or neutral depending on their composition. Many studies

show inconsistent findings on the polarity of ionic liquids, although it is generally considered that ionic liquids are relatively polar. As it seems, ILs as solvents are able to dissolve both polar and nonpolar molecules because of their ability to possess varying polarities throughout their entire molecular structure [36]. Although ILs are considered "green solvents", the most widely used form of ILs (water stable imidazolium cation-based) are considered toxic. However, a growing trend of cholinium or amino acid cation-based ILs are considered to be less toxic.

Replacing small symmetric cations, as found in solid NaCl, with large asymmetrical organic cations, such as found in ILs, can significantly reduce their melting point temperatures. In addition to symmetry, melting point is also dependent on the hydrogen-bonding, charge distribution, and van der Waals forces of the IL. Most ILs have higher density than water and will decrease if the length of the alkyl chain increases.

Lignocellulosic biomass consists of bonds between lignin, hemicellulose, and cellulose. ILs ability to disrupt the molecular bonds in lignocellulose gives it an innate position in the pre-treatment process stage [37, 38]. Initially, ILs were found to successfully solubilize cellulose. Many studies within the last decade have reported IL effectiveness for swelling cellulose and reduced its crystallinity. This appropriately led to research on effectiveness of ILs in lignocellulosic biomass processing.

Ionic liquids have gained popularity as a promising lignocellulosic pretreatment method because their versatility, delignification efficiency, and ability to promote extremely high yields of digestible sugars. However, ionic liquid conditions are known

to have inhibitory effects on enzymatic digestion and presumably on overall microbial activity, thus requiring significant post-treatment (washing) prior to downstream digestion [37, 39-43]. The post-treatment process is a major complication in the overall lignocellulosic degradation process, which undermines the effectiveness of ionic liquids as a pre-treatment technique [44]. A major advantage of ionic liquids is that the chemicals can be recycled and reused after pre-treating the biomass [45]. The treatment process typically follows the following steps: treatment of biomass with ionic liquid at moderate-to-high temperature, adding an anti-solvent (water) which precipitates the biomass, collecting and recycling the liquid portion, and taking the solid portion, which consists of de-stabilized cellulose (short length polysaccharides), as a valuable product (Figure 2.3). Ionic liquids are not typically used as a singular pre-treatment process. It is usually followed by enzymatic hydrolysis (cellulases and beta-glucosidases enzymes) that breakdown the polysaccharides into monosaccharides (glucose monomers). IL combined with enzymatic hydrolysis has been found to be extremely effective for various biomass types, in many cases, having glucose yields from 92 – 99 % in cellulose and lignocellulose [37, 46-50].

ILs have strong hydrogen-bonding capabilities, depending on the anion (e.g. chloride, phosphates, and acetates). It is inferred that ILs consisting of anions with high hydrogen-bond basicity typically have a correlation with better ability to solubilize cellulose. Of the anions, chloride-based ILs have shown significant cellulose dissolution abilities [51, 52]. It is inferred that chloride ions present in the IL greatly disrupt the

hydrogen bonding between the cellulose chain structure [53] and the $\pi - \pi$ interactions between the lignin complex [54].

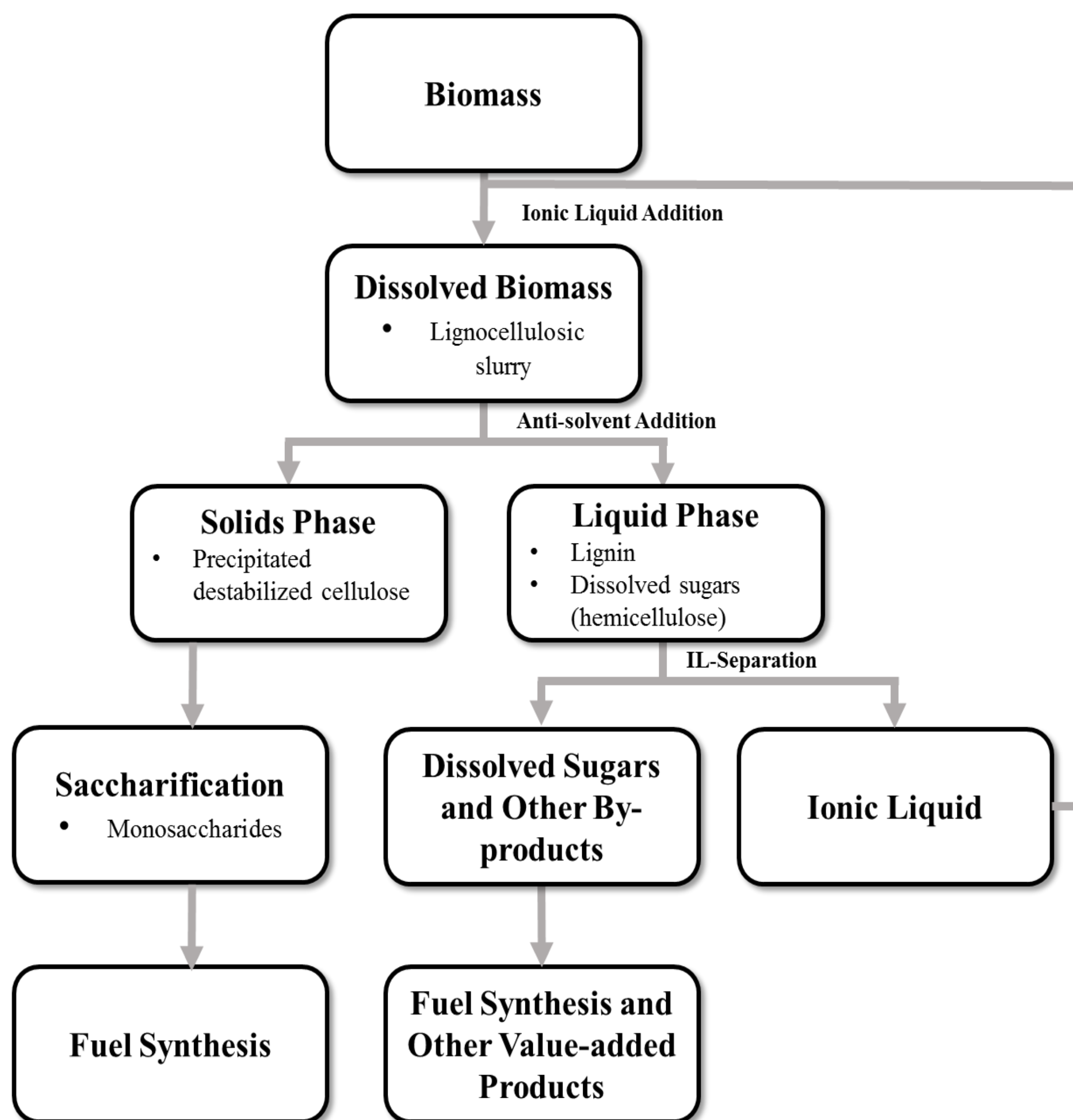


Figure 2.3. Process flow of ionic liquid pretreatment for biomass conversion

2.3 *Rhodococcus opacus* strain PD630

Many organisms in the past few decades have been engineered to produce biofuels from various substrates [55, 56]. Recombinant organisms have poor stability of enzymatic expression and relatively low yields [11, 57]. However, organisms that are natively capable of converting biomass to biofuels have an advantage because only a slight modification to their native pathway could potentially improve the product yield without needing to excessively engineer foreign genes into the organism. The oleaginous microorganisms, *Rhodococcus opacus* strain PD630, can natively accumulate intracellular triacylglycerol (TAG) up to ~87% of its cell dry weight (CDW) [58]. TAG is a vital precursor to common bio-diesel. Moreover, this bacterium can grow rapidly with diverse organics, including lignocellulosic biomass [59, 60]. This bacteria has potential to be used as a biocatalyst for the conversion of lignocellulosic biomass into valuable biofuel. PD630 has been studied extensively in the field of biofuel [61-63]. Although, the bacteria can utilize many carbon sources, it does not innately possess certain cellulolytic enzymes, rendering it incapable of hydrolyzing cellulose singularly [64, 65]. Attempts have been made to engineer cellulolytic genes into the strain [65]. The bacteria has been subjected to engineering xylose and arabinose metabolism via gene insertion [13, 66]. PD630 has also been subjected to adaptive evolution to improve tolerance to lignocellulosic-derived inhibitors and utilization of glycerol [67, 68]. Studies have also been conducted on the behavior of PD630 with different biomass types [59].

2.4 Adaptive/directed evolution

Adaptive evolution is a simple methodical approach to take advantage of the spontaneous mutations that occur during microbial growth [70, 71]. Adaptive evolution has also been referred to as directed or accelerated evolution. Adaptive evolution can be defined as actively inducing specific phenotypic expressions in the microorganism in a stress environment to ultimately allow for only those daughter cells with that specified phenotype to grow and tolerate that stress. By introducing a non-lethal dose of stress, e.g. inhibitory concentrations, temperature, or pH levels, and repeatedly selecting only the adapted or mutant cells that thrive in that stress condition, over a length of time, the overall bacteria culture will exhibit tolerance to the stress. The adapted cell can mutate to acquire different genotypes for stress tolerance [72]. The adapted cell can also respond to the stress conditions by rapid phenotypic changes by triggering different pathway functions to adapt to the stress, such as alterations to the cell envelope wall or increasing appropriate catabolic pathways to rapidly utilize the stress substrate.

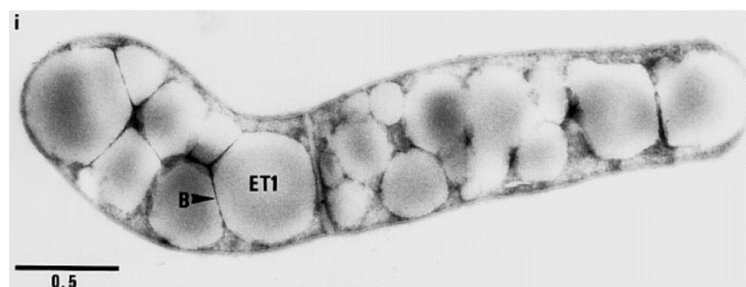


Figure 2.4. *Rhodococcus opacus* strain PD630 exhibiting high intercellular lipid [69]

Justification of this approach can be found in the many studies that have applied the adaptive evolution approach to advantageous effects. Horinouchi *et al.* [73] demonstrated *E. coli* adaptive evolution to ethanol stress and observed its genotypic and phenotypic differences. The Kurosawa research group has also released some studies that show successful *R. opacus* tolerance to glycerol and specific lignocellulosic-derived inhibitory concentrations for higher TAG yields [61]. Adaptive evolution of *Saccharomyces cerevisiae* was also used successfully to tolerate inhibitors and temperature [74]. Ionic liquid tolerance adaption has also been studied in other microorganisms [75].

3. INVESTIGATING THE POTENTIAL OF A CHEMICAL TREATMENT PROCESS FOR DOWNSTREAM FERMENTATION: IONIC LIQUID PRETREATMENT AND DILUTE ACID TREATMENT

3.1 Introduction

Ionic liquids have wide industrial applications because of their tunable characteristics and excellent solvent capabilities. A popular and common ionic liquid, 1-Ethyl-3-methylimidazolium chloride ([Emim][Cl]), has been chosen as the model IL because of its effectiveness towards biomass [76, 77]. ILs are known to have excellent fractionation properties in terms of biomass. [Emim][Cl] will be utilized as pretreatment step, to explore the delignification and destabilization properties on SCG, corn husk, and BG. The biomass will be dissolved in the IL and regenerated with the addition of DI water. Dilute acid hydrolysis will substitute for enzymatic hydrolysis as the secondary treatment in this process to allow for a compatible treatment for downstream PD630 and to emphasize the cost-effective advantage of exclusively using chemical hydrolysis [78].

3.2 Materials and methods

3.2.1 Biomass preparation

Bermuda grass clippings and corn husk were obtained locally (College Station, Texas US). Spent coffee grounds were obtained through a local vendor coffee shop. Each biomass was thoroughly washed with DI water, autoclaved, and dried in a 90 °C oven for 3 days to remove any residual interference (e.g. extractives, waxes, tannins,

soil, and other contaminants). Grass and corn husk were blended and grinded through fine steel-mesh to obtain appropriate particulate size. Spent coffee ground size was used as obtained. Each biomass sample was stored at 4°C in a sealed plastic bag for use in later experiments. Extra pure microcrystalline cellulose (MCC, average 90 µm particle size) was purchased from Fisher Scientific (Fair Lawn, NJ) and used as acquired.

3.2.2 Biomass pretreatment and treatment

Ionic liquid 1-Ethyl-3-methylimidazolium chloride (≥ 95% purity) was purchased from Sigma-Aldrich (St. Louis, MO), was used as received and was maintained in a desiccator until used. A 15 % (w/w) sample solution was prepared by the addition of 1 g of MCC with 5.65 g of [Emim][Cl] in a 10 mL borosilicate glass test vial and covered with a rubber stopper. The same procedure was followed for each biomass species by the addition of 0.5 g of each biomass sample with 2.82 g of [Emim][Cl]. All experiments were performed in duplicates. The samples were heated and manually stirred in a Hach DRB200 reactor block at 120 °C for 15 min to 24 hours. MCC samples were removed from heat at specific time points 0.25, 0.5, 1, 3, 10, and 24 hours. Biomass samples were removed from heat at 0.5, 1, 3, 10, and 24 hours. After each heat incubation, 30 mL of deionized water was added to the sample slurry and a precipitate was recovered immediately. The sample mixture was then centrifuged at 10,000 g for 10 min. The supernatant was separated from the recovered solids by a fine-mesh filter. The supernatant (herein called pre-hydrolysate) was lyophilized at -20 °C before sugar and lignin analysis. The solid portion was placed in a 50 °C oven for 48 hours prior to dilute acid treatment.

Sulfuric acid (97% purity) was purchased from Fisher Scientific (Fair Lawn, NJ). The [Emim][Cl]-pretreated samples (amount depending on TDW after pretreatment) were placed in a glass test vial with 10 mL of 0.1 % sulfuric acid and heated at 120 °C using the reactor block and manually stirred. The supernatant (herein called hydrolysate) was then filtered through a 0.45 µm filter and stored in 4 °C until further experimentation. The residual solids were placed in a 50 °C oven for 48 hours and stored in 4 °C for additional analysis.

Cellulases were purchased from Tokyo Chemical Industry Company (Japan) and β-glucosidase was purchased from Sigma-Aldrich (St. Louis, MO). Pretreated MCC and biomass were washed with DI water at least five times to remove residual acid in dilute acid pretreatment, and washed five times to remove residual [Emim][Cl] in the IL-pretreatment. The pretreated MCC samples were dried at 50 °C overnight. Enzymatic hydrolysis was then conducted in 100 mL of 0.1 M sodium acetate adjusted to pH = 5. Cellulase (35 mg/g solids) [57] and β-glucosidase (64 U/g solids) [32], and the mixture was incubated and magnetically stirred at 50 °C over a period of 48 h. Aliquots were taken at specific time points for sugar analysis.

3.2.3 Sugar analysis

Pre-hydrolysate and hydrolysate samples from the pretreatment and treatment were taken for measurement for total reducing sugars. The reducing sugar analysis method was adapted from the DNS colorimetric assay [79]; Briefly, 0.5 mL of DNS reagent was added to 0.5 mL of sample in a 10 mL borosilicate glass test vial and covered to prevent evaporation. All samples were performed in duplicate unless

otherwise stated. The vials were heated in a Hach DRB200 reactor block at 90 °C for 10 minutes to allow the reaction to take place. Color intensity of the each sample was measured by spectrophotometer (Agilent Technologies 8453 UV-Vis) at 540 nm. The reducing sugar yields were calculated based on a calibration curve using D-glucose standard concentrations (0.05 – 1 g/L). The total reduced sugar (TRS) yield was calculated as,

$$W_{RS} = TRS\ concentration\ (g/L) \times volume\ of\ sample\ (L)$$

$$TRS\ \%\ Yield = \frac{W_{RS} \times 0.9}{TDWi \times F} \times 100$$

where TRS concentration is the calculated sample concentration obtained from the DNS assay, W_{RS} is the mass of the reducing sugars, 0.9 is correction ratio of the glucose conversion factor when a water molecule is added to each broken glucosidic bond during hydrolysis [30]. TDW_i is the initial dry weight of the sample, and F is the mass fraction of total holocellulose contained in each biomass species. For SCG, corn husk, grass, and MCC, F values are 0.515 [80], 0.879 [81], 0.556 [82], and 1.0, respectively.

Glucose was detected using a glucose oxidase (GO) colorimetric assay kit purchased from Sigma-Aldrich (St. Louis, MO). D-glucose was purchased from Sigma-Aldrich (St. Louis, MO). 3,5-dinitrosalicylic acid was purchased from Acros Organics (Geel, Belgium). Phenol, sodium sulfite, sodium hydroxide were purchased from J.T. Baker and VWR.

3.2.4 Lignin determination

Lignin content analysis was adapted from a two-step acid hydrolysis procedure by NREL analytical procedure LAP-004 [83]. Briefly, the 100 mg of prepared samples were placed in a 10 mL borosilicate glass test vial along with 1.5 mL of 72% sulfuric acid solution and stirred frequently for 2 hours in 30 °C. The mixture was transferred to a 250 mL flask and 56 mL of DI water was added to dilute the sample to 3% sulfuric acid. The flask was autoclaved for 30 minutes at 121 °C. The mixture was vacuum filtered to separate the liquid from the solid portion.

The liquid filtrate portion was taken for acid-soluble lignin analysis by spectrophotometer (Agilent Technologies 8453 UV-Vis) at 205 nm with a 1 cm path length cuvette. The acid-soluble lignin (ASL) was calculated as,

$$ASL\ concentration\ (g/L) = \frac{A}{110} \times DF$$

$$W_{Lignin} = ASL\ concentration\ (g/L) \times volume\ of\ sample\ (L)$$

where *A* is the absorbance reading obtain from the spectrophotometer and 110 is the general absorptivity or extinction coefficient (L/g ·cm) for lignin biomass at 205 nm. *DF* is the dilution factor. The solid portion was collected for acid-insoluble lignin or Klason lignin analysis. Klason lignin was gravimetrically determined by the difference of measuring the initial weight and final weight of the solid portion after extreme heat treatment. Total lignin was determined by the addition of ASL and Klason lignin weight.

Aliquots of pre-hydrolysate samples were taken and diluted by DI water to determine lignin content and lignin removal through spectroscopy with the same procedure.

3.3 Results and discussion

3.3.1 Sugar yields from ionic liquid pretreatment and dilute acid treatment of MCC

ILs have been reported to be effective in reducing crystallinity and depolymerization of cellulose [84]. To study the effects of [Emim][Cl] pretreatment on reduced sugar release, MCC was used as a representative model for lignocellulosic biomass to determine sugar yields. 15 % solid loading of MCC was pretreated with [Emim[Cl] over a period of 24 h. Complete dissolution of MCC in [Emim[Cl] was observed after 1 h. MCC immediately precipitated after the addition of DI water as the antisolvent. The liquid pre-hydrolysate was analyzed for reducing sugar release by [Emim][Cl]. The regenerated MCC solids were dried and subjected to dilute acid hydrolysis for final sugar release as shown in Figure 3.1. The data indicates that longer pretreatment time for MCC in [Emim[Cl] result in higher TRS yields in the pre-hydrolysate, which explain the low final TRS yields in the acid hydrolysate as pretreatment time increases. In the scope of this study, it should be noted that any reducing sugar released during the IL pretreatment stage is considered sugar loss, as the [Emim][Cl] and sugar solution would require addition separation methods to utilize the pre-hydrolysate sugar. For an efficient treatment process, most sugar release should occur in the hydrolysate phase. The regenerated MCC were dried and subjected to the

same dilute acid treatment conditions and TRS yields were measured. The data indicates that the TRS yield peaks at approximately 77 % when MCC was exposed to 1 h of pretreatment. Final TRS yield decreases as pretreatment time increases. Final TRS yield was significantly lower at 24 h of pretreatment, as most likely, significant reducing sugars were released in the pre-hydrolysate. For MCC, an IL-pretreatment time of 1 h indicates the optimal amount of TRS yield by dilute acid hydrolysis.

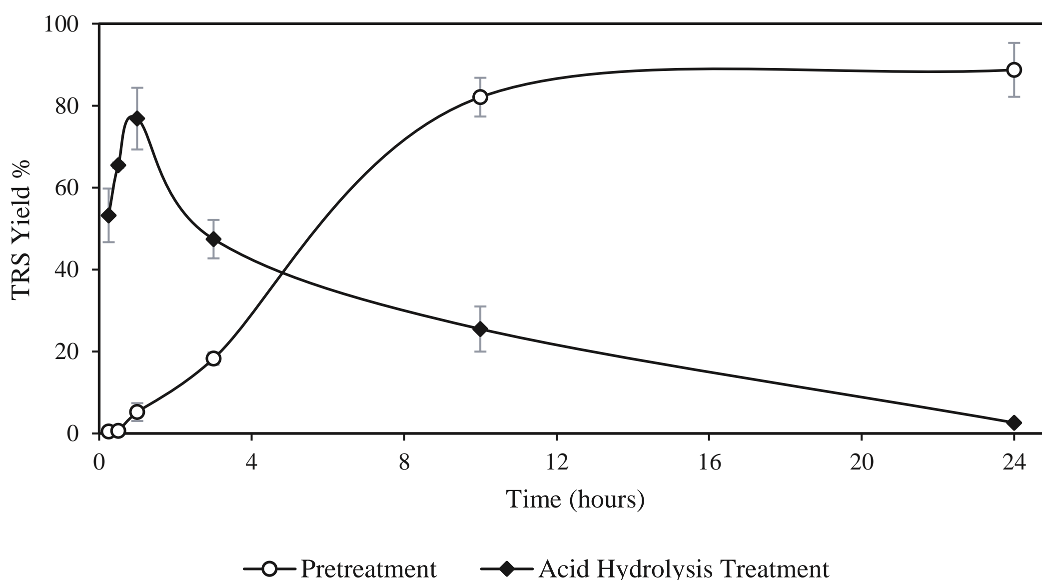


Figure 3.1. TRS yields after 0.25, 0.5, 1, 3, 10, and 24 h of [Emim][Cl] pretreatment (white markers), and corresponding TRS yields after dilute acid treatment (5 h, 120 °C, 0.1 % sulfuric acid) for each regenerated solid (black markers).

Figure 3.2 compares the glucose yield of the alternative treatment method with other treatment methods. Results indicate that sugar yield peaked at approximately 52 % between 3 and 8 h for the alternative treatment method. Extended treatment times show sugar yields slowly decreasing, most likely due to the dehydration of sugar into HMF and other degradation products. The well-established treatment method, dilute-acid pretreatment followed by enzymatic hydrolysis, was also tested. As expected, enzymatic saccharification yields were high at approximately 70 % of reducing sugar in 48 h. IL-pretreatment followed by enzymatic saccharification was also tested. Results showed a remarkable sugar yield of 90 % in 24 h, indicating sugars were released at a higher rate and magnitude than the dilute acid - enzymatic treatment. This is consistent with recent studies that show the capabilities of ILs as superior pretreatment agents in cellulose and lignocellulosic biomass [85-87]. Table 3.1 shows the total time required for each method to reach 52 % reducing sugar release. Although both enzymatic methods yielded higher sugars overall compared to the alternative method, both require longer treatment times, with the IL-pretreatment method requiring approximately 13 h, and dilute acid method requiring even longer at 35 h. In contrast, the alternative method only required 8 h, although it did not reach the magnitude of the latter two methods.

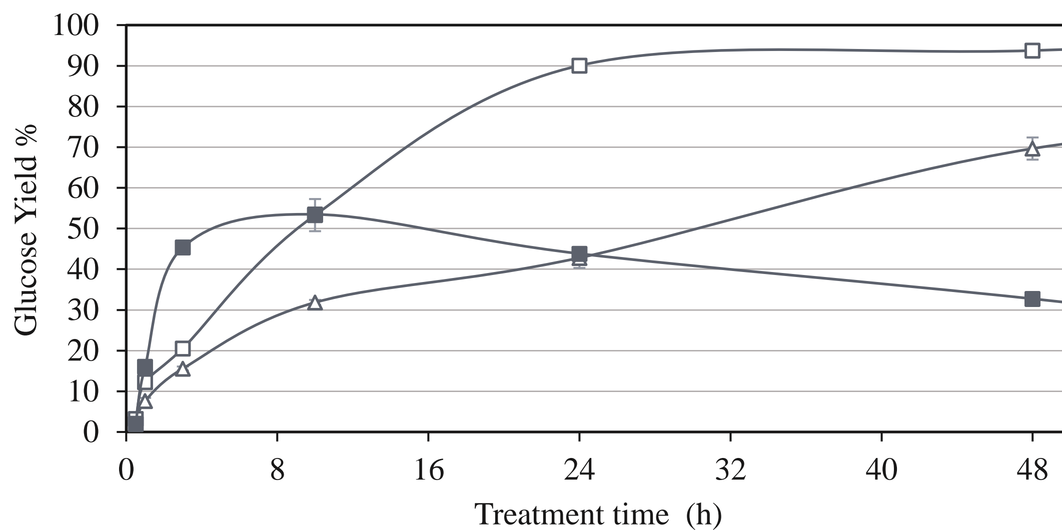


Figure 3.2. Time course of glucose yields over 48 h on SCG, comparing three treatment methods on SCG, Conditions: Acid + enzymes (white triangles) – 3 h, 120 °C, 0.1 % sulfuric acid, IL + enzymes (white squares) – 3 h, 120 °C, 15 % (w/w), IL + Acid (black squares) – 3 h, 120 °C, 15 % (w/w), Treatment conditions: Enzyme treatment – cellulase (70mg/g biomass), β-glucosidase (64 U/g biomass), sodium acetate buffer 100 mL, pH 5, Dilute-acid treatment – 120 °C, 0.1 % sulfuric acid

Table 3.1. Total treatment time required to yield 52% glucose yield for each treatment method

Treatment Method	Pretreatment time (h)	Treatment time (h)	Total Time (h)
Dilute acid pretreatment + enzymatic treatment	3	~32	35
IL-pretreatment + enzymatic treatment	3	~10	13
IL-pretreatment + dilute acid treatment	3	~5	8

3.3.2 Lignin removal by ionic liquid pretreatment of crude biomass

For the alternative process, [Emim][Cl] is also utilized as a pretreatment step for delignification. Figure 3.4 illustrates the total lignin removal of each biomass measured over a period of 24 hours by [Emim][Cl] pretreatment at 15 % (w/w) solid loading. Lignin removal has been shown to decrease as solid loading increases [88] . The percentage of lignin removal after 3 hours is shown to be significant for each biomass even at a higher solid loading, indicating that at 15 % solid loading, lignin removal is still feasible. However, it was observed that the high viscosity of each biomass slurry can be an issue for handling and mixing due to the higher solid loading. It is also demonstrated that the lignin removal after 3 h slowly tapered to approximately 76%, 70%, and 62% for SCG, corn husk, and grass, respectively, over a 24 h period. Complete lignin removal of each biomass did not occur, most likely attributed to longer retention

requirements, the viscosity of the slurry, and the further degradation of the lignin complex through the extensive heat treatment.

Lignin removal efficiency by ionic liquid pretreatment was also compared to an established dilute acid pretreatment method. Figure 3.5 summarizes the lignin removal by both methods over 3 h for each biomass species. This shows that delignification by [Emim][Cl] is increased by a general factor of 3 – 4 compared to the dilute acid delignification when undergoing the same pretreatment duration. This finding is consist with similar studies that show significant delignification of various biomass species by IL treatment [37].

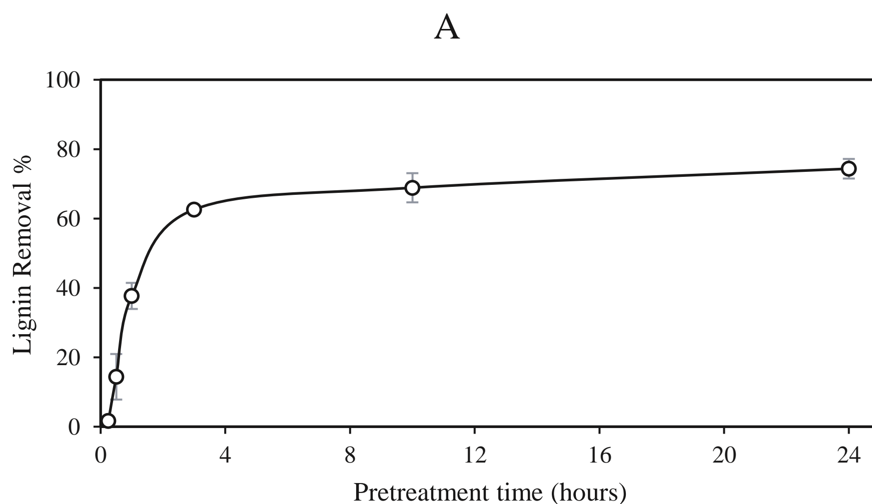
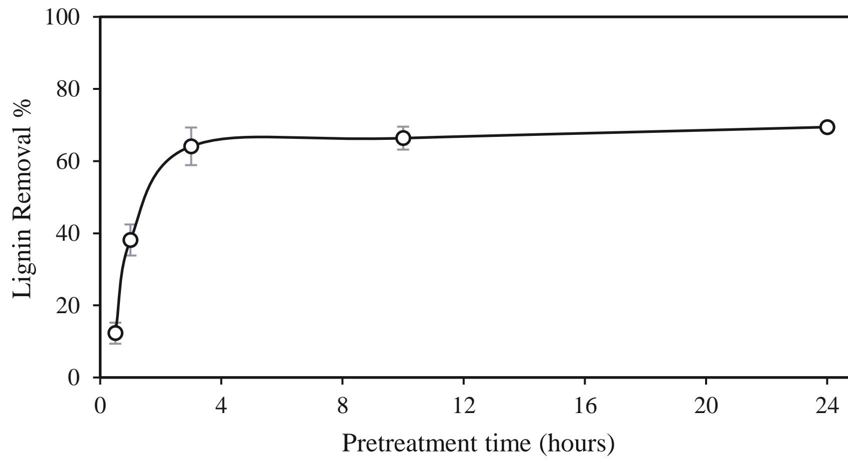


Figure 3.4. Lignin removal (%) by [Emim][Cl] pretreatment (120 °C, 15 % (w/w)) over 24 hours. A) SCG, B) corn husk, and C) BG



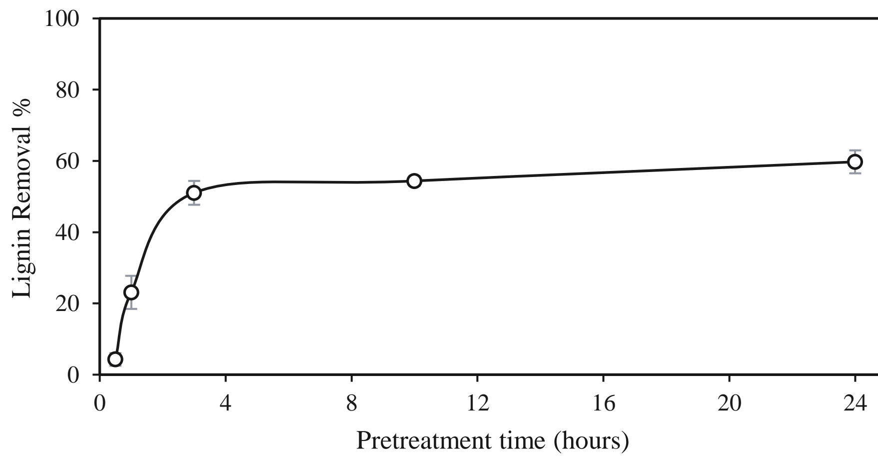
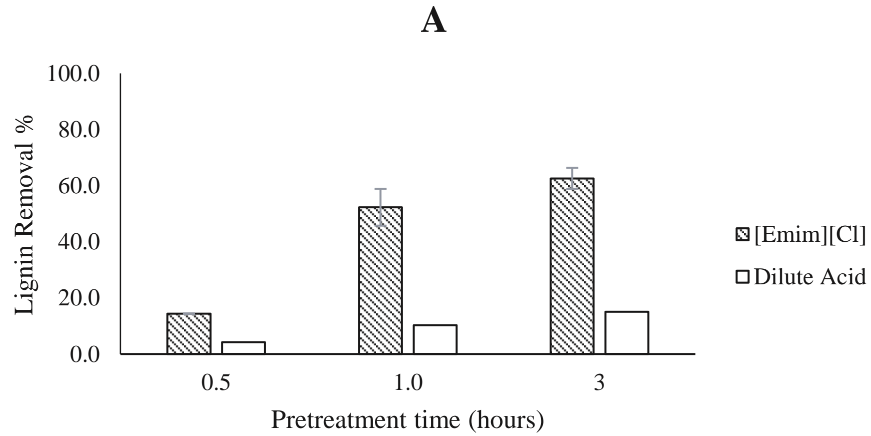
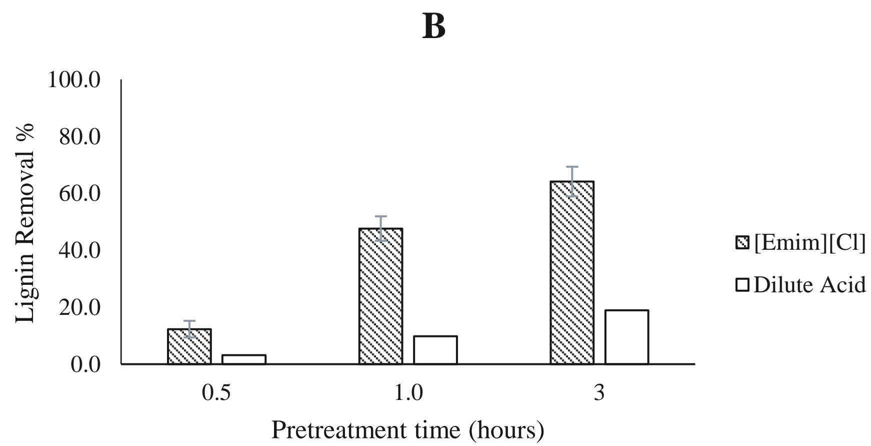


Figure 3.4. Continued





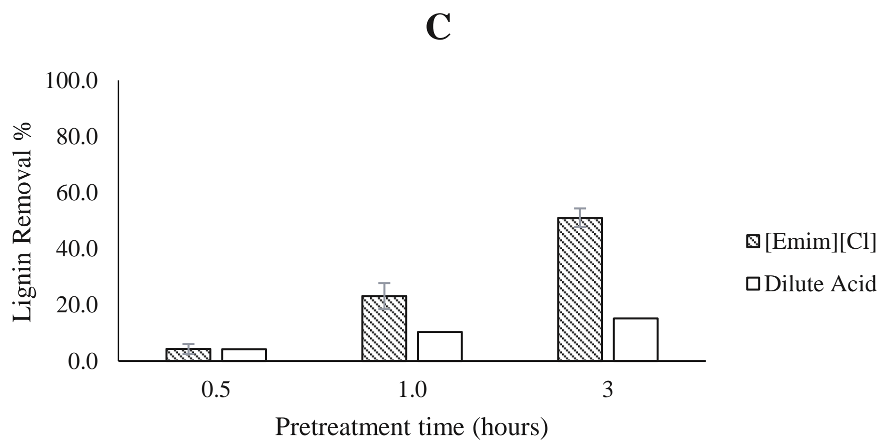


Figure 3.5. Lignin removal (%) comparison between dilute acid pretreatment and IL pretreatment at 0.5, 1, and 3 h. A) SCG, B) corn husk, and C) BG

3.3.3 Sugar yields from ionic liquid pretreatment and dilute acid treatment of crude biomass

Experiments conducted for MCC and lignin removal provided insight on the ideal pretreatment duration for exploiting lignin removal and cellulose destabilization by [Emim][Cl]. Although the optimal pretreatment time for TRS yield was found to be approximately 1 h for MCC, a biomass pretreatment time of 3 h was chosen to optimize for lignin removal. Each biomass type was subjected to 3 h of [Emim][Cl] pretreatment and 5 h of dilute acid treatment. The pre-hydrolysates and hydrolysates were analyzed for glucose and total sugar content as shown in Figure 3.6. After the 3 h of [Emim][Cl] pretreatment, a notable amount of total sugars were released for each biomass type. However, only approximately 9.9 %, 9.8 %, and 9.8 % of the sugar yield was detected as glucose for SCG, corn husk, and grass, respectively. This suggests that a large portion of the sugar yields using [Emim][Cl] were in the form of other reducing carbohydrates, such as hexose and pentose sugars. After dilute acid treatment, overall reducing sugar yields increased significantly to 60.5 % and 68.9 % for SCG and corn husk, respectively. However, grass sugar yields were comparatively low at 35.9 % with only about half yielding glucose. In all biomass types, a substantial portion of the total sugar yield was in the form of glucose after dilute acid treatment. In contrast, each biomass type was subjected to dilute acid treatment for the equivalent time of the alternative treatment duration at 8 h. Total sugar yields showed that a significant portion of sugar was released during the duration of the dilute-acid only treatment. However, most of the sugar yields were in the form of other reduced sugars, while only marginal yields were glucose

sugars. This suggests that using [Emim][Cl] as pretreatment step can significantly increase the glucose yields, likely by allowing the acid to more easily access and cleave the β-1,4-glycosidic bonds in the cellulose structure by removing the lignin-cellulose complex.

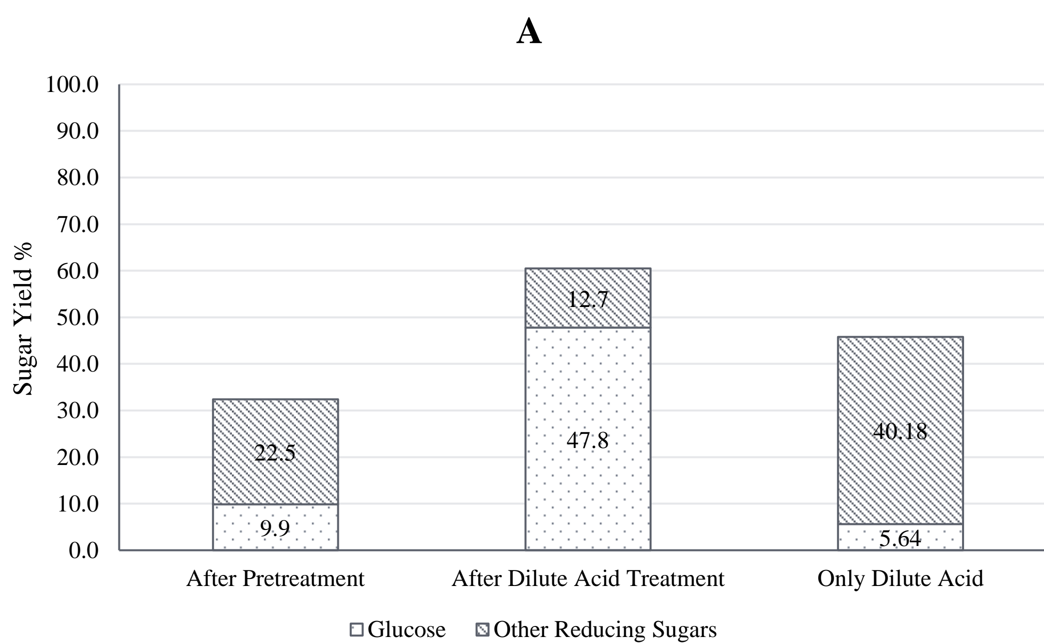
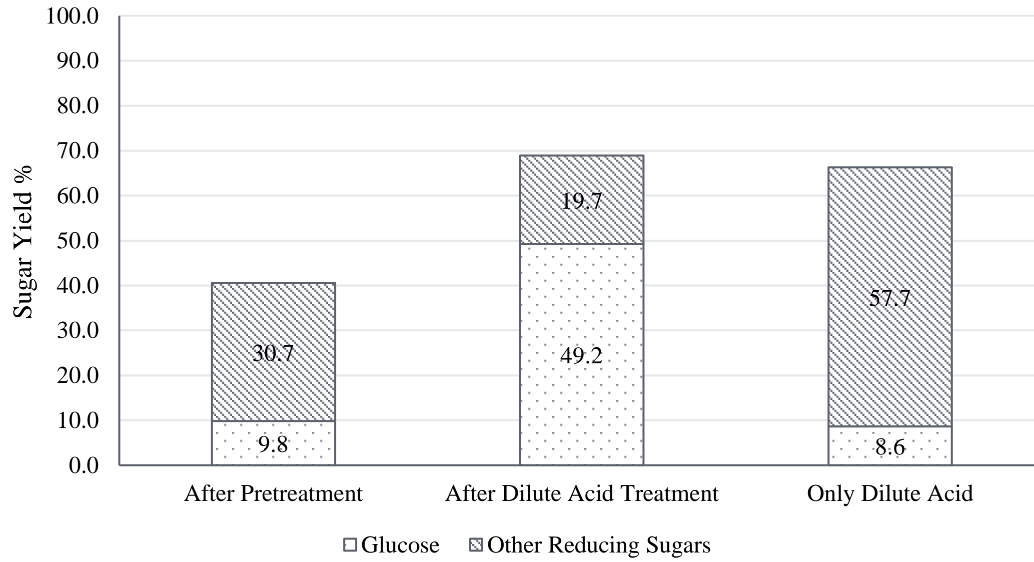


Figure 3.6. Sugar yields (%), After pretreatment: [Emim][Cl] treatment at 120 °C, 3 h, 15 % (w/w). After dilute acid treatment: (120 °C, 5 h, 0.1 % sulfuric acid). Only dilute acid: 120 °C, 8 h, 0.1 % sulfuric acid. A) SCG, B) corn husk, and C) BG



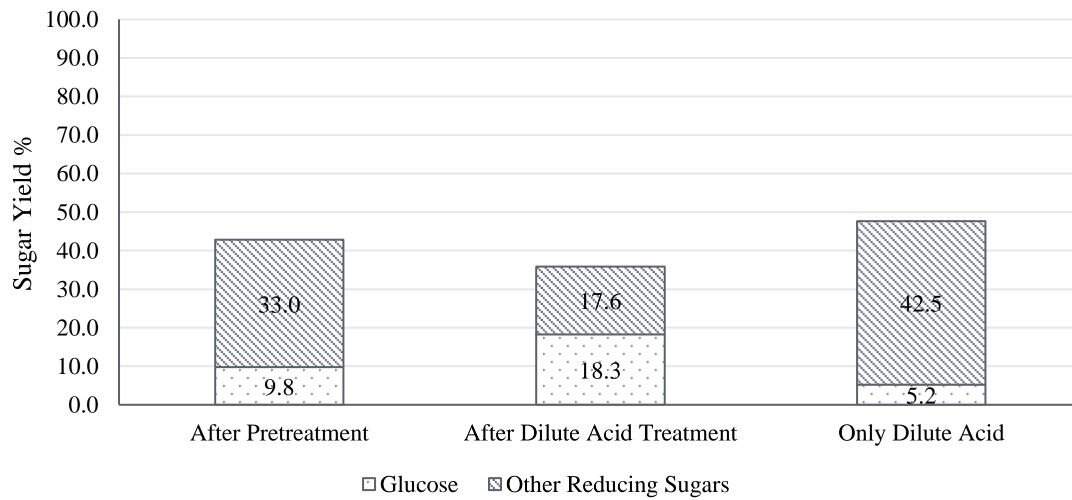


Figure 3.6. Continued.

3.3.4 Growth of *R. opacus* PD630 on biomass hydrolysates

Growth of bacterial strain PD630 was tested on each biomass hydrolysate. Figure 3.7 demonstrates the growth of PD630 on biomass that has been subjected to the alternative treatment. The hydrolysates were detoxified and neutralized by washing the regenerated biomass with DI water prior to dilute acid treatment to remove residual [Emim][Cl]. The final hydrolysate was neutralized to pH 7 prior to growth tests. The PD630 growth data shows stationary phase was reached in approximately 3 days for all hydrolysates. Cell turbidity was observed to be relatively low for all biomass hydrolysates compared to growth on traditional media, indicating that the carbon source in the hydrolysate media may have been exhausted before full growth. Notably, PD630 growth was found to reach the highest in OD for SCG. PD630 was also tested for growth on crude hydrolysate, without detoxification or neutralization. The growth of PD630 in each crude hydrolysate was found to be inhibited for multiple days, having not reached the comparative stationary phase after 5 days of cultivation.

Downstream microorganism growth was observed on biomass using this alternative treatment method after a detoxification step, indicating that this treatment has potential to be used in a bio-refinery process utilizing catalytic microorganisms.

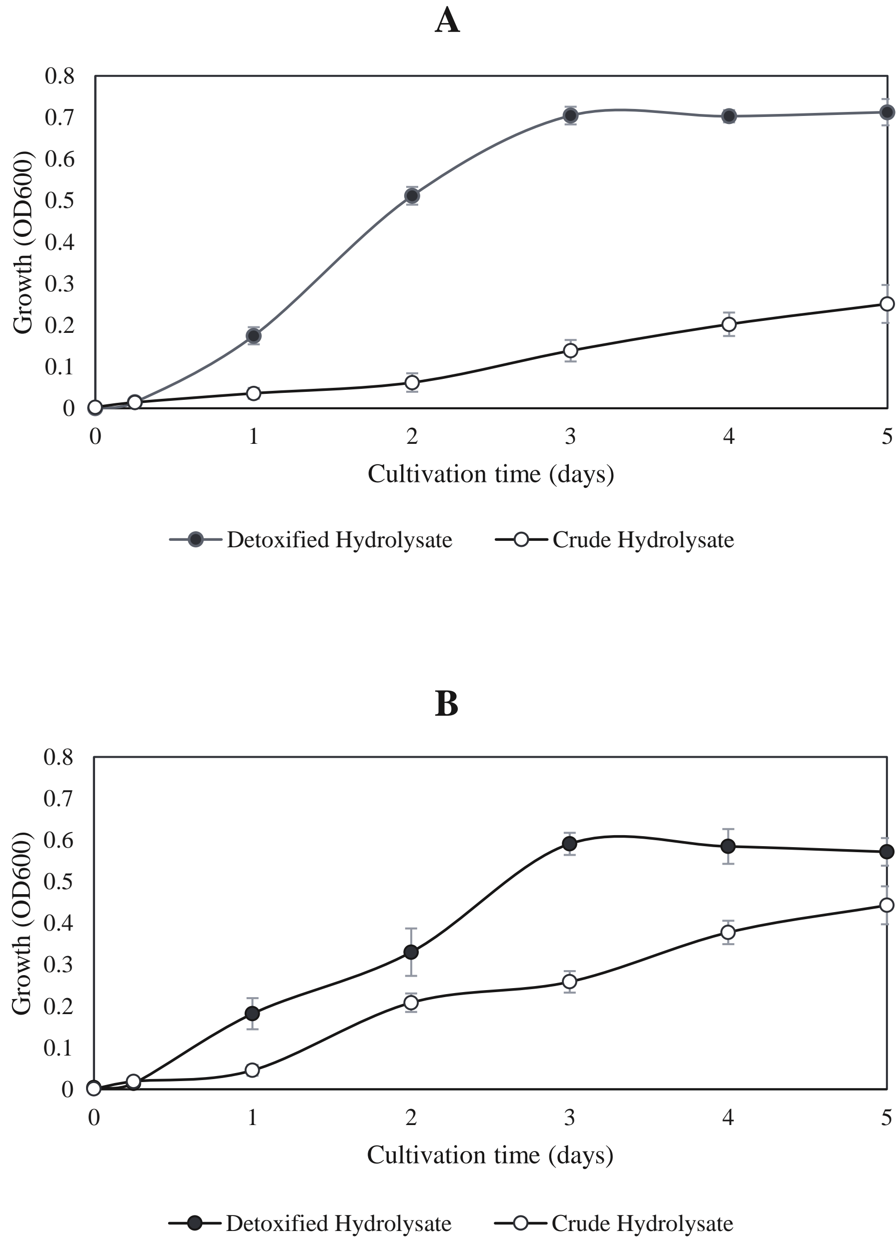


Figure 3.7. Growth curve of PD630 on detoxified hydrolysate (detoxified – washed 5 times after pretreatment, and neutralized to pH 7) (black circles), and crude hydrolysate (crude – unadjusted, raw hydrolysate) (white circles), A) SCG, B) corn husk, and C) BG

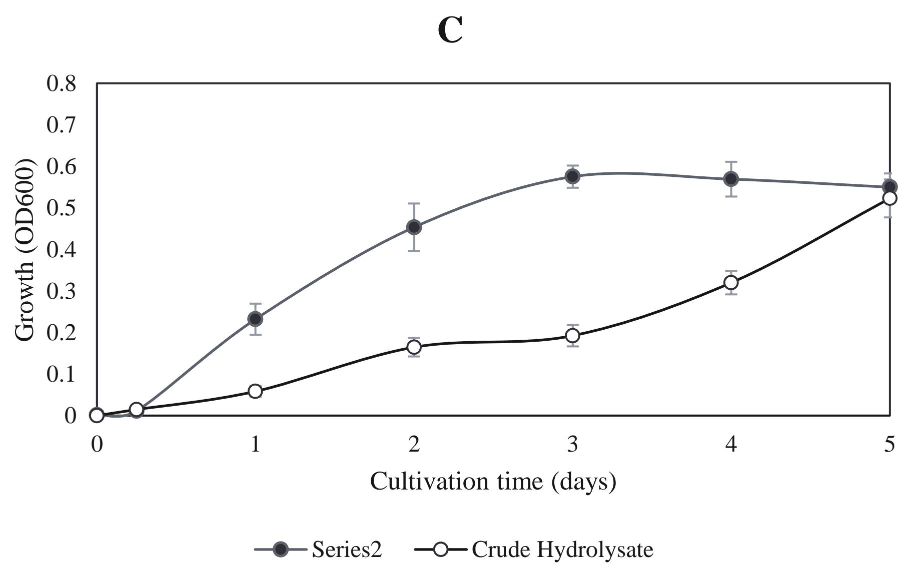


Figure 3.7 Continued.

4. DEVELOPMENT OF A HYPER-TOLERANT LIPID-ACCUMLUATING BACTERIA FOR DOWNSTREAM ULTILIZATION OF SUGARS

4.1 Introduction

Using the results obtained in the previous experiments, it was found that the alternative chemical treatment was able to produce sufficient sugars for PD630 to utilize for growth. However, PD630 growth results indicated severe inhibition if the hydrolysate was not detoxified and neutralized. All treatments that require a detoxification step are considered detrimental for and not economically viable and effective treatment methods [89]. Therefore, PD630 will be subjected to adaptive evolution to minimize or eliminate the need for washing. The adaptive evolution is a strategy to take advantage of the spontaneous mutations that occur during microbial growth. Adaptive evolution can be defined as actively inducing a specific phenotypic expression in the microorganism in a stress environment to ultimately allow for only those daughter cells with that specified phenotype to grow and tolerate that stress. By introducing a non-lethal dose of stress and selecting only the adapted or mutant cells that thrive in that stress condition, over a length of time, the overall bacteria culture will exhibit tolerance to the stress by vertical gene transfer. The adapted cells can respond to the stress conditions by phenotypic changes by triggering different pathway functions to adapt to the stress (such as alterations to the cell envelope wall or increasing appropriate catabolic pathways to rapidly utilize the stress substrate). This approach has been applied in many studies to advantageous effects.

4.2 Materials and methods

4.2.1 Media, bacterial strains and cultivation

Lysogeny broth (LB) and Reasoner's 2A (R2A) media was purchased from Teknova and BD Difco VMR (Radnor, PA). D-glucose, noble agar, and [Emim][Cl] was purchased from Sigma-Aldrich (St. Louis, MO). Biomass was obtained and prepared as described in Section 3.2.1. Extra pure microcrystalline cellulose (MCC, average 90 µm particle size) was purchased from Fisher Scientific (Fair Lawn, NJ). 5-(Hydroxymethyl) furfural (HMF, 98% purity) and Glycerol (>99% purity) were purchased from ACROS Organics (Geel, Belgium). Minimum ammonium mineral salts (AMS) medium was created in the laboratory as described [90] containing: 18.57 mM NH_4SO_4, 0.98 mM K_2SO_4, 0.15 mM $MgSO_4·7H_2O$, 0.07 mM $CaSO_4·2H_2O$, 0.08 mM $FeSO_4·7H_2O$, 3.9 mM KH_2PO_4, 6.1 mM Na_2HPO_4, 0.001 mM KI, 0.002 mM $ZnSO_4 · 7H_2O$, 0.002 mM $MnSO_4·H_2O$, 0.002 mM H_3BO_3, 0.004 mM $CoSO_4$. The pH of the AMS medium is adjusted to a value of 7.5 using H_2SO_4 and autoclaved at 121 °C for 20 minutes. Agar medium was made by the addition of 1.5 % (w/v) noble agar.

The *Rhodococcus opacus* PD630 (DSM 44193) was purchased from DSMZ, Germany. Throughout the experimentation process, the parental PD630 strain was maintained in R2A [91] agar plates at 4 °C for short term preservation and routinely re-streaked every 2 weeks and lyophilized in LB media and 20% glycerol in -80 °C for long term preservation as frozen stock.

For short term preservation, all evolved PD630 strains were maintained in LB agar and supplemented with 10 % of the MIC as stock, excluding pH-tolerance, in which

the growth media is maintained at a neutral pH of 7. For long term preservation, evolved strains were lyophilized in LB media and 20% glycerol in -80 °C as frozen stock.

All cultivation experiments were carried out in at 30 °C. Unless otherwise stated, liquid cultures were grown in a media volume of 50 mL in 250 mL flasks on a rotary shaker at 150 rpm. Stock cultures were taken and streaked onto R2A agar plates until cell colonies formed in 3 days. Seed culture was prepared by inoculating cell colonies into R2A media and allowed to incubate for two days to late exponential phase prior to inoculation into a defined inhibitory R2A media.

4.2.2 Determination of [Emim][Cl], HMF, and acidic conditions in treated cellulose and biomass

Acidic conditions were determined using an Accumet pH probe and compatible pH-meter. Levels of pH were measured and recorded from the final hydrolysate (after sulfuric acid treatment) without any prior neutralization for each biomass samples. These values were used as a benchmark for acidic condition adaption.

Residual [Emim][Cl] concentrations were determined as [Cl^-] anion concentrations using an ion-selective chloride probe and voltmeter. An aliquot of the final hydrolysate was diluted using DI water and [Cl^-] concentrations were determined through a [Cl^-] standard curve. These [Cl^-] concentrations were used as a benchmark for [Emim][Cl] adaption.

HMF concentrations were measured using a technique adapted from the UV-spectroscopy method [92]. Briefly, 5 % (w/v) activated charcoal was added to an aliquot of the final hydrolysate in a 10 mL test vial and boiled at 100 °C for 1 minute to

eliminate spectral interference. The sample was vacuum filtered. The filtrate was measured by spectrophotometer at 284 nm with a 1 cm path length cuvette. HMF content was calculated as,

$$W_{HMF} = C_{HMF}\left(\frac{mmol}{L}\right) \times M\left(\frac{g}{mol}\right) \times K_{HMF} \times DF$$

where W_{HMF} is the concentration of HMF in the sample in mg/L, C_{HMF} is determined from the absorbance reading relative to the calibration curve, M is the molecular weight of HMF, K_{HMF} is 69.3, which is the calibration coefficient to correct for adsorbed HMF, and DF is the dilution factor. The HMF concentrations were used as a benchmark for HMF concentration adaption.

4.2.3 Minimum inhibitory concentration analysis

Cultures were grown in LB media at a pH 7 in 25 mL glass culture tubes with a media volume of 10 mL until mid-exponential phase ($OD_{600} \approx 0.5$) on a Glas-Col Culture Vial Rotator. All samples were performed in duplicate.

For acidic inhibitory effects, seven culture tubes were adjusted to a pH of 4, 4.5, 5, 5.5, 6, 6.5, and 7. Cultures were incubated with 100 µL of seed culture over a period of 24 hours and growth was routinely visually observed and OD was recorded after 24 h. MIC was defined as the pH at which little or no turbidity was visually observed.

For ionic liquid MIC, seven culture tubes were adjusted to a [Emim][Cl] concentration of 0, 1, 2, 3, 5, 10, and 20 g/L. Cultures were incubated over a period of 24 hours and growth was routinely visually and spectrophotometrically recorded after 24 h. MIC was defined as the [Emim][Cl] concentration at which little or no turbidity was visually observed.

For HMF MIC, seven culture tubes were adjusted to a HMF concentration of 0, 0.1, 0.2, 0.5, 1, 2, and 3 g/L. Cultures were incubated over a period of 24 hours and growth was routinely visually and spectrometrically recorded after 24 h. MIC was defined as the HMF concentration at which little or no turbidity was visually observed.

4.2.4 Tolerant strain construction

Electro-competent cells were prepared by growing in nutrient broth supplemented and 0.85 % and 1 % glycine and sucrose, respectively. The cells were collected at mid-exponential phase and pelletized by washing multiple times with DI water through centrifugation at 8000 g for 10 min.

Electroporation was achieved by Bio-rad MicroPulser (Hercules, CA) at 2.5 kV for 1 pulse in a Bio-rad Gene Pulser 0.2 cm cuvette. The cells were immediately inoculated in LB media at 30 °C at 150 rpm for 3 hours. The cells were transferred to R2A agar medium adjusted to an inhibitory pH of 5. Cells were observed daily for 7 days, and six isolate colonies that indicated rapid and robust growth were transferred and re-streaked on new agar medium with the same inhibitory conditions for purification. This was step repeated a third time.

The isolate strains were then used for serial transfer cultivation. The isolates were inoculated in separated flasks with R2A liquid medium flask adjusted to pH 5 with a 50 mL working volume. After reaching approximately mid-exponential phase, 5 mL was transferred to a fresh R2A liquid medium with a step-wise decrease in pH value. This serial transfer was repeated for a total of four serial transfers. After the final generation, cells were plated for single colonies on R2A agar medium adjusted to pH of 5, and re-streaked and purified on LB agar medium. Colonies were therein identified as strain $PD630^{V2}$ and lyophilized until the next adaptation phase.

Acid tolerant strain $PD630^{V2}$ was subjected to electroporation and the adaptive evolution procedure was repeated similarly for [Emim][Cl] tolerance with a stepwise increase in [Emim][Cl] concentration. The final isolate strain was therein identified as strain $PD630^{V3}$ and lyophilized until the next adaption phase.

Acid and [Emim][Cl] tolerant strain $PD630^{V3}$ was subjected to electroporation and the adaptive evolution procedure was repeated similarly for HMF tolerance with a stepwise increase in HMF concentration. The final isolate strain was therein identified as strain $PD630^{V4}$ and lyophilized for further experimentation.

4.2.5 TAG analysis

10 mL of bacterial culture was centrifuged at 5000 g for 15 min. The cell pellet was washed with DI water and resuspended in 1 mL of deionized water. The mixture was transferred to a 20 ml culture tube containing 10 mL of chloroform/methanol mixture (2:1, v/v). The culture tubes were incubated at 37 °C, 200 rpm overnight, the mixture was centrifuged at 2500 g for 20 min to achieve phase separation. 5 mL of the

bottom chloroform layer was transferred new glass vial. An air valve tube was used to evaporate the solvent in the vial to dryness. The lipids were reconstituted in 2 ml of hexane before TLC analysis. This allowed for a better resolution to be developed on the TLC plate. 10 μL of lipid samples and glyceryl trioleate standards (ranging from 0.2 to 100 μg) were applied to silica gel TLC plates (Product No. 4850-820, Whatman, Piscataway, NJ) and separated in a solvent system of hexane: diethyl ether: acetic acid (80:20:1, v/v) [69]. After separation, the TLC plate was dried at 105 °C for 5 min, rinsed with 1 M sodium chloride solution for 15–20 min, and then stained with 0.2% (w/v) amido black solution for 15–20 min. After color was developed, the TLC plate was immersed in the 1 M sodium chloride solution to remove residual dye before drying at 105 °C for 10 min. The TAG content of each sample was determined by analyzing the image of the TLC plate using Fiji/ImageJ software (National Institutes of Health, Bethesda, MD).

4.3 Results and discussion

4.3.1 Original levels of pH, [Emim][Cl] and HMF found in hydrolysates

Inhibition factors were defined as acidic conditions, residual ionic liquid concentration, and HMF formation found in the growth substrate. Inhibitory factors was quantified for each hydrolysate.

Acidic conditions were measured as pH levels in each hydrolysate (Figure 4.1). After dilute acid treatment, the hydrolysate pH values were found to be approximately between 2.0 to 3.0 for each hydrolysate. SCG and BG hydrolysate exhibited the lowest

pH values at 2.05 compared to a pH value of 2.25 for corn husk. For reference, the pH values were also recorded for pre-hydrolysates, after [Emim][Cl] pretreatment. The pH values ranged from 6.65 to 7.05, with SCG having the lowest value and corn husk having the highest pH values. These measurements indicate that the pH values drops significantly after dilute acid treatment of biomass. [Emim][Cl] pretreatment of biomass shows slightly acidic changes in each hydrolysate.

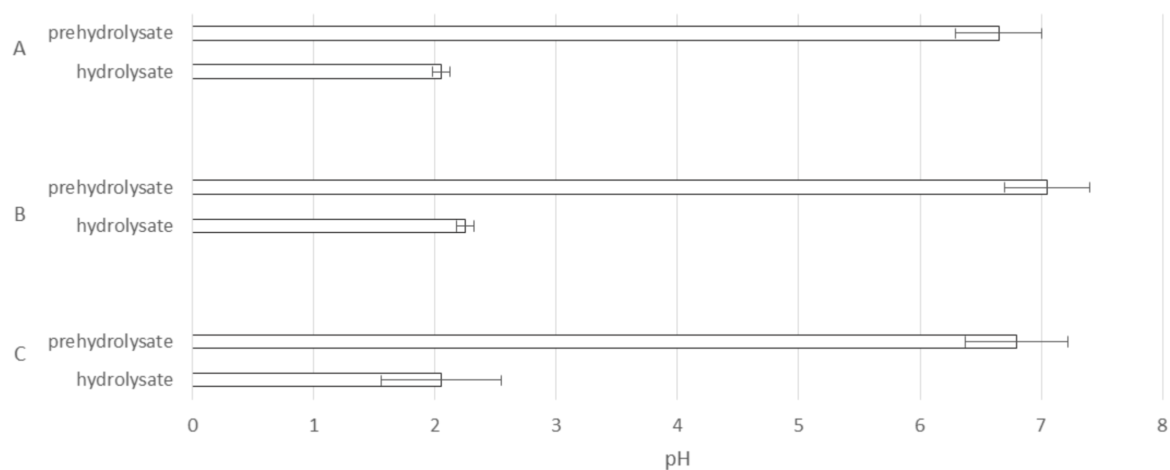


Figure 4.1. pH values in each pre-hydrolysate and hydrolysate. A) SCG, B) corn husk, and C) BG

Residual [Emim][Cl] concentration was measured as [Cl^-] concentration remaining in solution after dilute acid treatment. Table 4.1 summarizes the residual [Emim][Cl] concentrations found in each hydrolysate. SCG was found to have the lowest [Emim][Cl] concentration at 28.1 g/L of residual [Emim][Cl] still remaining in the hydrolysate solution. Corn husk was found to have 34.4 g/L of residual [Emim][Cl]. BG showed the highest concentrations of residual [Emim][Cl] at 37.2 g/L of [Emim][Cl]. The relatively high concentrations of [Emim][Cl] remaining in the solution can be explained by the theoretical mass fraction of [Emim][Cl] remaining on the regenerated solids after pretreatment. Theoretical mass fractions were calculated from the total dry weight of the samples. The theoretical mass fraction of IL accounted for approximately 30 to 60% of the regenerated solids, indicating that a significant amount of IL remains in the regenerated solid portion, regardless of the biomass species. The residual concentrations of IL remaining on the biomass can account for major inhibition of downstream microorganisms and enzymes [93]. These concentrations of IL are associated with the requirement for extensive washing of the regenerated biomass prior to saccharification.

HMF formation was measured by recording the HMF concentration after dilute acid treatment for each biomass. Overall, HMF concentrations were found to be relatively low compared to [Emim][Cl] concentrations (Table 4.2). SCG exhibited the lowest HMF formation after 5 hours of dilute acid treatment at 389.7 mg/L of HMF. BG had 517.4 mg/L of HMF in the hydrolysate and corn husk displayed the most HMF formation at 641.5 mg/L of HMF. Formation of HMF in cellulose and lignocellulosic

biomass is typically associated with mineral acid hydrolysis through the inadvertent dehydration of the glucose monomer by the mineral acid. Longer durations or high treatment temperatures tend to increase glucose yields, however continued treatment may result in HMF formation. Glucose and HMF yields can be optimized by varying treatment time and temperature.

Table 4.1 Residual [Emim][Cl] concentrations remaining in hydrolysate solution, measured as [Cl^-] concentration

Biomass Hydrolysate	**Residual IL Concentration (as Cl^-), g/L**
SCG	28.1 ± 2.3
Corn Husk	34.4 ± 3.1
BG	37.2 ± 3.6

Table 4.2 HMF concentrations in hydrolysate solution

Biomass Hydrolysate	HMF Concentration, mg/L
SCG	389.7 ± 61.4
Corn Husk	641.5 ± 47.0
BG	517.4 ± 84.7

4.3.2 Growth behavior of ionic liquid, pH, and HMF on *R. opacus* PD630

Growth behavior of PD630 on each inhibitory factor was studied by subjecting the strain to varying levels of inhibition and determining the minimum inhibitory concentration (MIC) for each factor. Visual inspection and absorptivity are presented in Figure 4.2 for each inhibitory factor. PD630 was subjected to 0 – 3 g/L of HMF in LB media overnight to determine the inhibitory concentration, defined herein as the concentration where little or no turbidity is observed and the OD is marginal. HMF concentrations were found to be inhibitory for PD630 at relatively low concentrations. At 0.1 g/L, a minor reduction in OD was observed compared to the control sample. At 0.5 g/L, a dramatic reduction in OD was observed from 0.2 g/L, although turbidity was still apparent. 2 g/L of HMF was found to be the MIC, as little to no turbidity and OD were observed.

PD630 was subjected to varying levels of acidic conditions, from pH values of 7 to 4. A gradual reduction in OD was observed between a pH of 6.5 to 5.5. However, a more significant reduction in OD was observed when pH was at 5.5 and 5.0. The pH MIC for PD630 was found to be pH of 4, as little to no turbidity and OD were observed in the sample.

PD630 was subjected to 1 – 20 g/L of [Emim][Cl]. Interestingly, it was found that the OD was higher compared to the control sample when cultured in 1, 2, and 3 g/L of [Emim][Cl]. However, at 5 g/L the OD was less and significantly reduced at 10 and 20 g/L. The MIC of PD630 in [Emim][Cl] was found to be at 20 g/L.

The higher OD observed at lower concentrations of [Emim][Cl] was supported by an additional time course growth experiment on the behavior of PD630 at different concentrations of [Emim][Cl] (Figure 4.3). The growth curve shows an apparent shortened lag phase for cultures supplemented with 1 and 3 g/L of [Emim][Cl] compared to the control culture. However, at 5 and 10 g/L, the growth was unstimulated and even detrimental to the growth of PD630. Other ILs have also been found to have a hormetic effect on certain bacteria, acting as a pH buffering agent during the growth of the bacteria [94]. This hormetic effect of [Emim][Cl] on PD630 has implications to be investigated further.

A

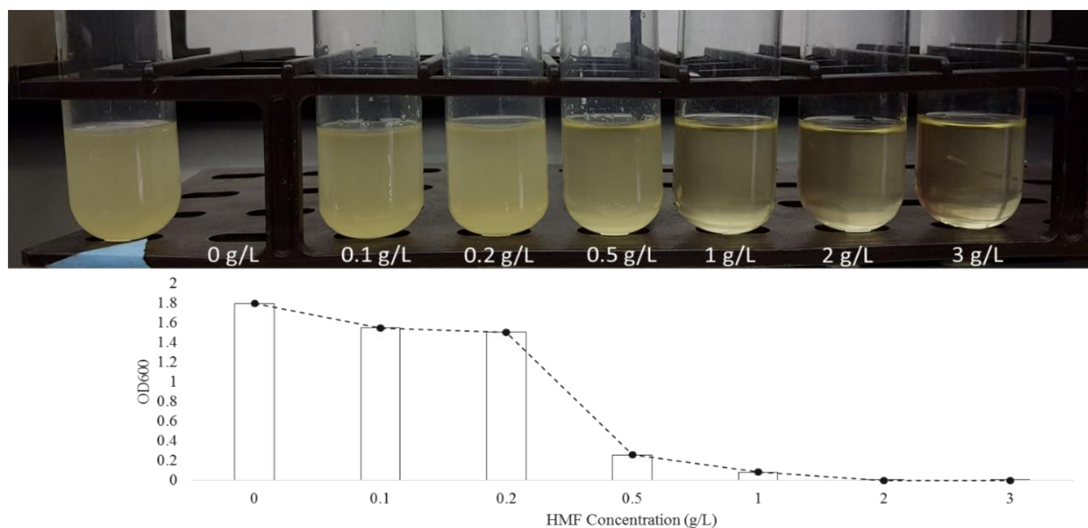
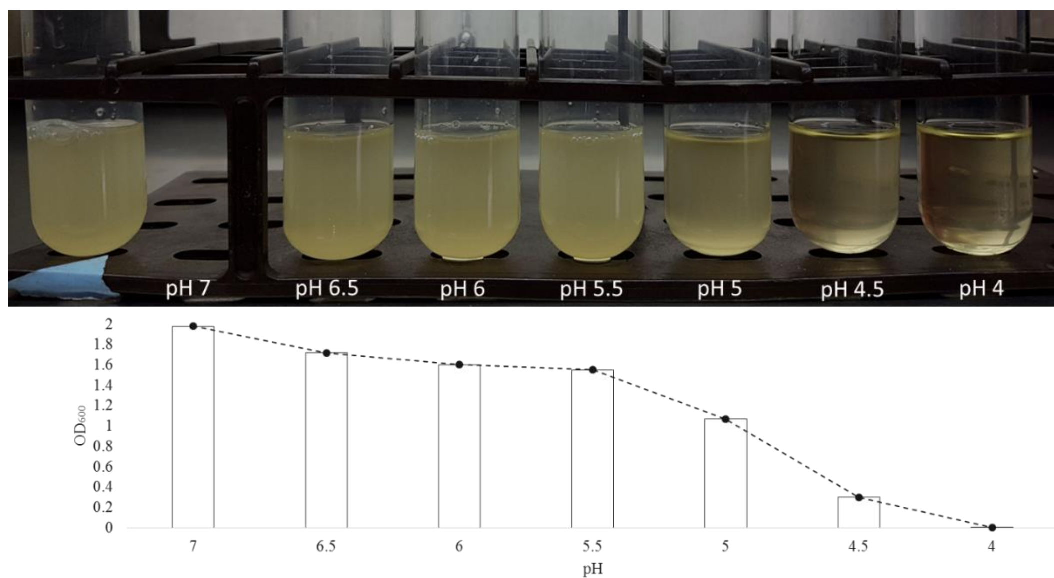


Figure 4.2. Visual inspection and optical density measurements of PD630 for varying A) HMF concentrations (0.1 – 3 g/L), B) pH levels (4 – 7), and C) [Emim][Cl] concentrations (1 – 20 g/L) in LB media grown overnight

B



C

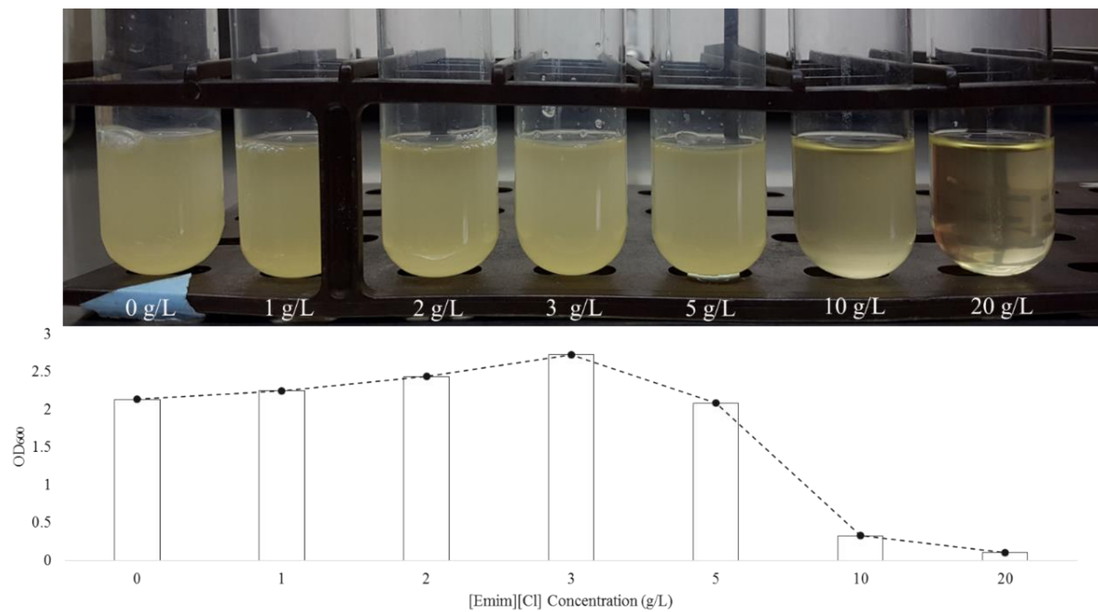


Figure 4.2. Continued.

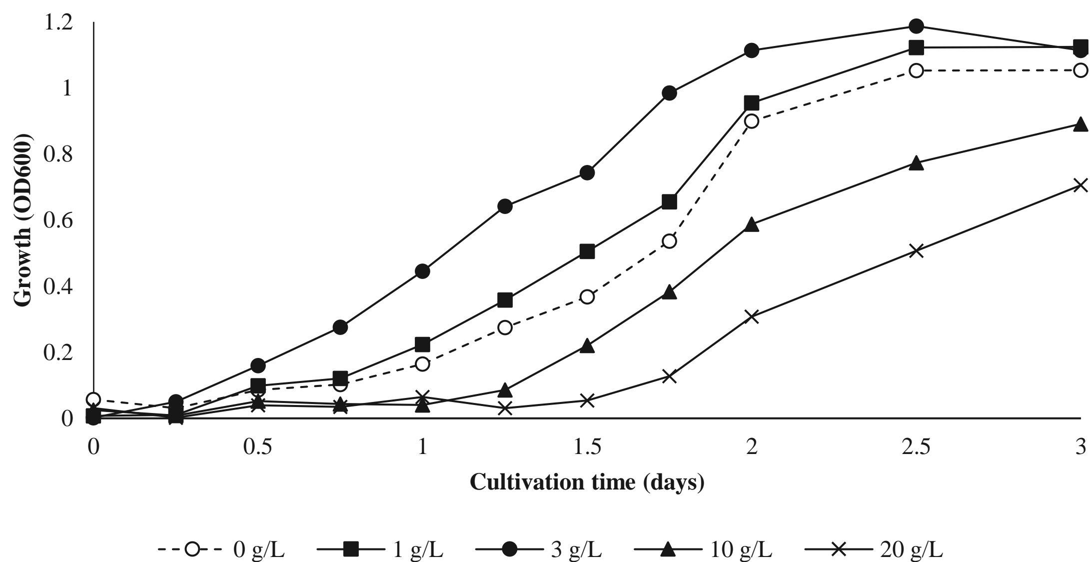


Figure 4.3. Growth curve of PD630 on varying concentrations of [Emim][Cl] in R2A media

4.3.3 Generation of a hyper-tolerant *R. opacus* PD630 strain by adaptive evolution

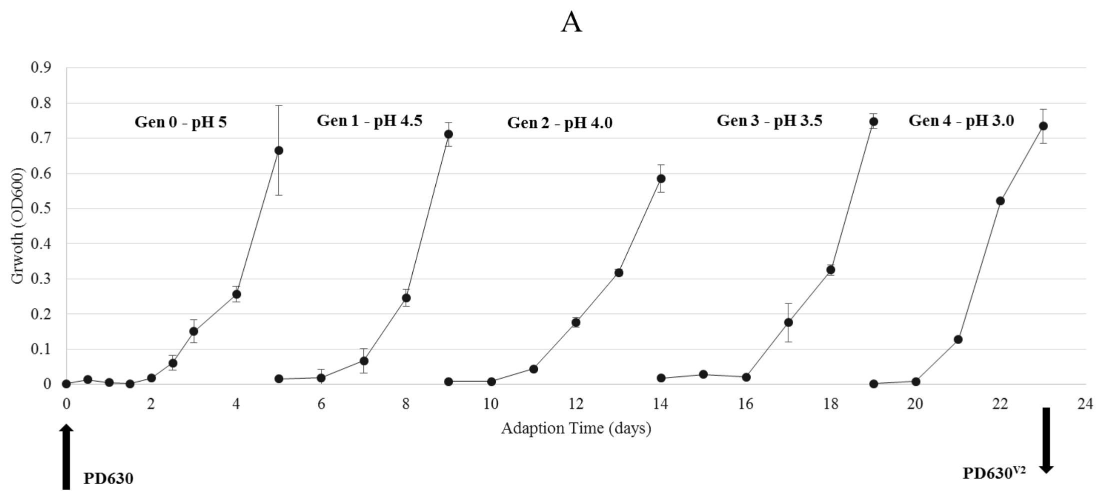
PD630 was subjected to adaption to low pH, [Emim][Cl], and HMF. Sub-lethal levels of each inhibitory factor found in the previous experiments were used as references for the adaptive/directed evolution strategy. For pH adaption, wild-type

PD630 was subjected electroporation and plated on R2A agar medium adjusted to an initial inhibitory pH of 5. Cells were observed routinely for 7 days, and six isolate colonies that indicated rapid and robust growth were transferred and re-streaked and purified on the new R2A agar adjusted to pH 5. The isolate strains were then used for serial transfer cultivation. The isolates were inoculated in separated flasks with R2A liquid medium flask adjusted to pH 5. After reaching approximately mid-exponential phase, the culture was transferred to a fresh R2A liquid medium with a pH of 4.5. This serial transfer was repeated for a total of four serial transfers at pH 4, 3.5, and 3. Figure 4.4 illustrates the serial transfer adaption method for low pH tolerance. At the final generation, cells were plated for single colonies on R2A agar medium adjusted to pH of 5, and re-streaked and purified on LB agar medium. The cells were identified as strain PD630^{V2} and tested for growth against the wild-type PD630 in R2A media adjusted to a pH of 3 (Figure 4.4B). Although PD630^{V2} did show signs of inhibition initially, the growth of PD630^{V2} was noticeability more rapid than WT PD630.

For IL tolerance, acid-tolerant strain PD630^{V2} was subjected to increased tolerance to [Emim][Cl] for double tolerance. Once again, PD630^{V2} was subjected to electroporation and plated on R2A agar supplemented with 20 g/L of [Emim][Cl]. Two isolate that indicated robust growth were purified on new media. The adaptive evolution procedure was repeated similarly for [Emim][Cl] tolerance with a stepwise increase in [Emim][Cl] concentration starting from 20 g/L, to 30, 40, 45, and 50 g/L during the serial transfer. Figure 4.5 illustrates the serial transfer adaption method for [Emim][Cl] tolerance. After the final generation, the strain was plated for single colonies on R2A

agar supplemented with 20 g/L [Emim][Cl] and purified on LB agar. This isolate strain was identified as strain $PD630^{V3}$. This mutant strain was tested for growth against the wild-type PD630 in R2A media supplemented with 50 g/L of [Emim][Cl] (Figure 4.5B). $PD630^{V3}$ was shown to grow at a much faster rate than WT PD630 in the IL rich media. The double tolerant strain $PD630^{V3}$ was once again subjected to electroporation and the adaptive evolution procedure was repeated similarly for HMF tolerance for a triple tolerant strain. The serial transfer concentration started at 0.2 g/L, and increased to 0.5, 1.0, 2.0, and 2.0 mg/L for four generations (Figure 4.6). The final isolate strain was therein identified as strain $PD630^{V4}$ and tested for growth against the WT PD630. Similarly, $PD630^{V4}$ was observed to grow more rapidly than WT PD630 in R2A media supplemented with 2.0 mg/L of HMF (Figure 4.6B).

A



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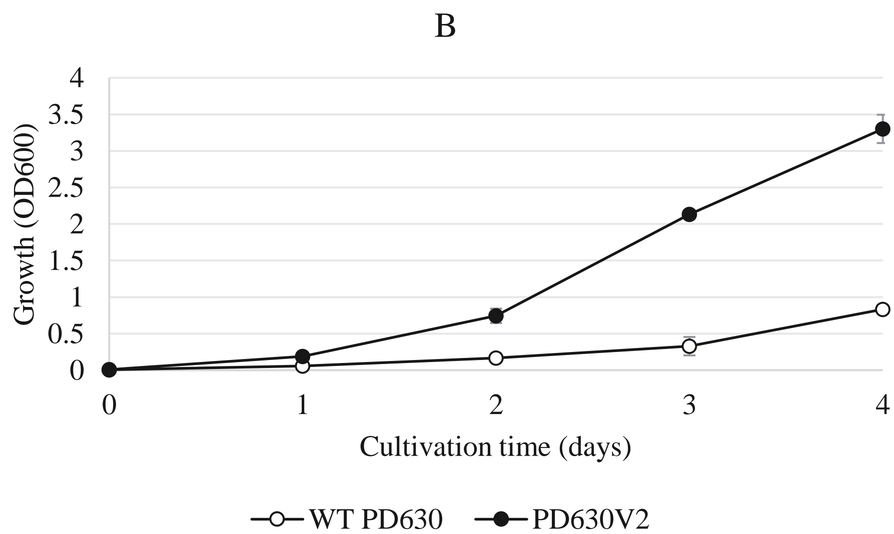
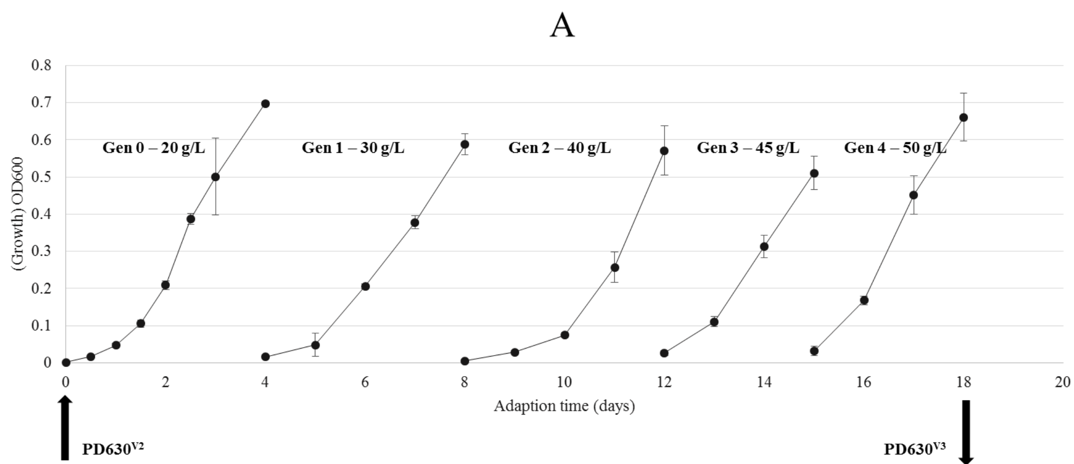


Figure 4.4. A) Adaptive evolution for pH tolerance through serial transfer cultivation, B) Growth of $PD630^{V2}$ and WT PD630 on R2A media adjusted to pH value of 3

A



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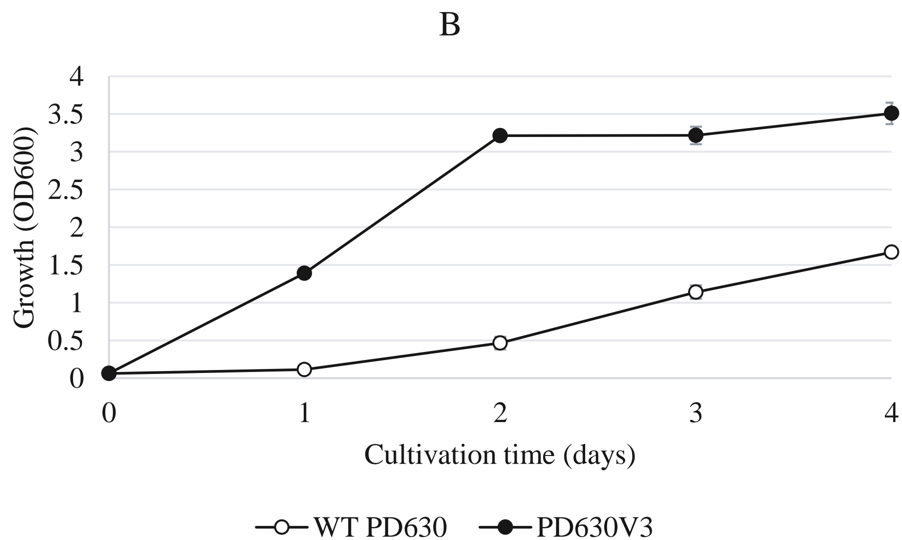

Figure 4.5. A) Adaptive evolution for [Emim][Cl] tolerance through serial transfer cultivation, B) Growth of PD630^{V3} and WT PD630 on R2A media supplemented with 50 g/L [Emim][Cl]

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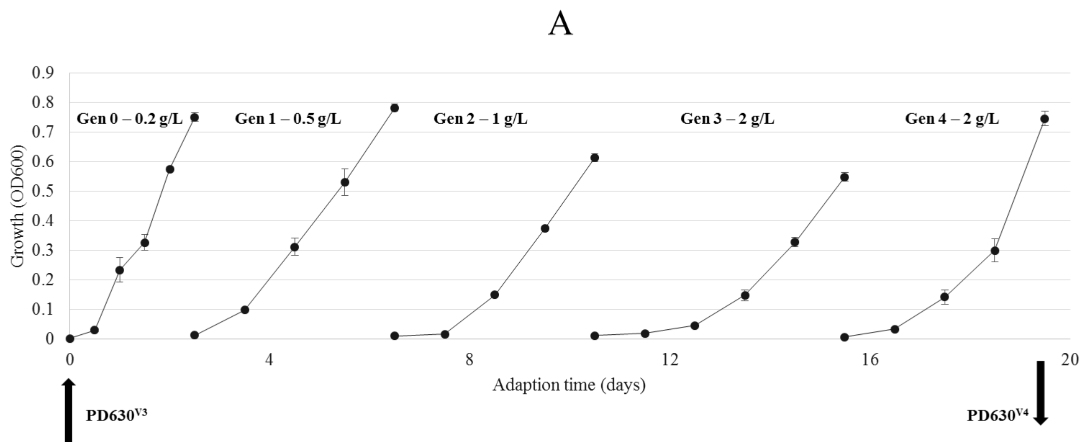

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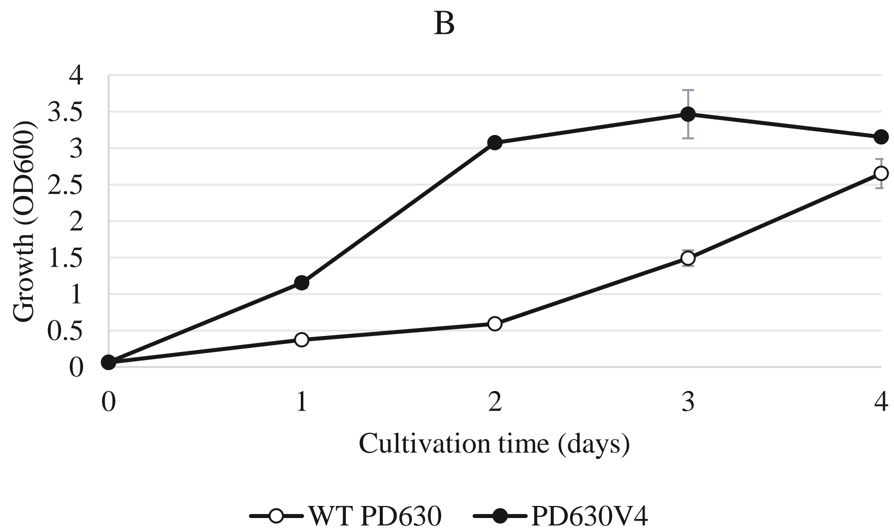


Figure 4.6. A) Adaptive evolution for HMF tolerance through serial transfer cultivation, B) Growth of PD630^{V4} and WT PD630 on R2A media supplemented with 2 g/L HMF

To test against the synergistic effects of the inhibitory factors and the ability of $PD630^{V4}$ to maintain the triple tolerance, $PD630^{V4}$ growth was tested again the two mutant strains and the WT strain on mock hydrolysate, that is R2A media supplemented with 40 g/L of [Emim][Cl], 0.6 mg/L of HMF, and pH adjusted to 3 (Figure 4.7). $PD630^{V4}$ was considered to have maintained the triple tolerance, as it shows less inhibition in the mock hydrolysate than the other strains.

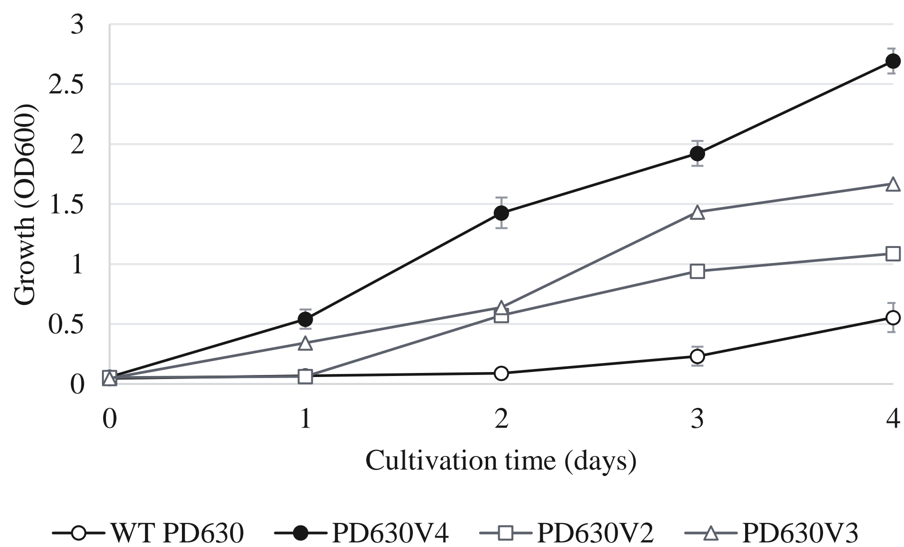
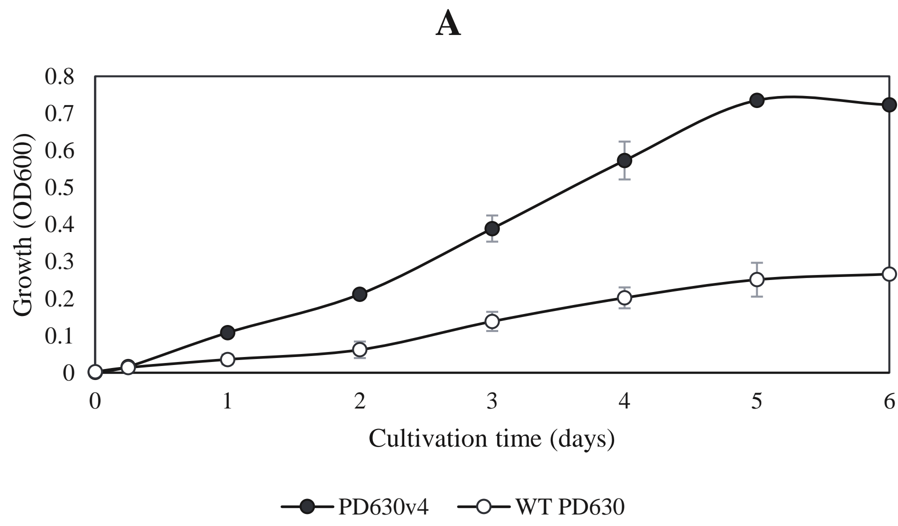


Figure 4.7. Growth curve of four strains on mock hydrolysate (R2A media supplemented with 40 g/L of [Emim][Cl], 0.6 mg/L of HMF, and pH adjusted to 3)

4.3.4 Comparison of evolved *R. opacus* PD630 strain and wild-type *R. opacus* PD630 on biomass hydrolysates

The newly adapted $PD630^{V4}$ was tested for growth with all crude biomass hydrolysates (Figure 4.8). It was found that $PD630^{V4}$ was significantly less inhibited in each crude hydrolysate compared to PD630. $PD630^{V4}$ required at least 5 days of cultivation before reaching a stationary phase on SCG hydrolysate. For both corn husk and BG hydrolysate, $PD630^{V4}$ required 4 days of cultivation before reaching stationary phase.

TAG accumulation was also compared in $PD630^{V4}$ and WT PD630 in each hydrolysate after 6 days of cultivation (Figure 4.9). Interestingly, WT PD630 accumulated more TAG than $PD630^{V4}$ in R2A media and corn husk, even though PD630 was not observed to reach stationary phase in corn husk after 6 days. TAG quantity was similar in BG for both $PD630^{V4}$ and PD630, even though PD630 did not reach as high of OD. $PD630^{V4}$ accumulated higher amounts of TAG in SCG than WT PD630, likely because PD630 only reached an OD of approximately 0.2 after 6 days of cultivation.



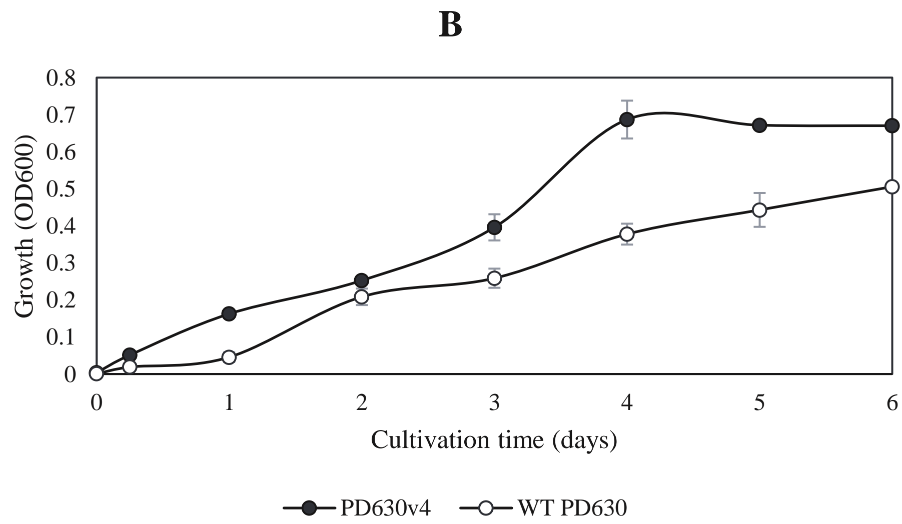


Figure 4.8. Growth curve of $PD630^{V4}$ and wild-type PD630 on crude (unadjusted) hydrolysate, A) SCG, B) corn husk, and C) BG

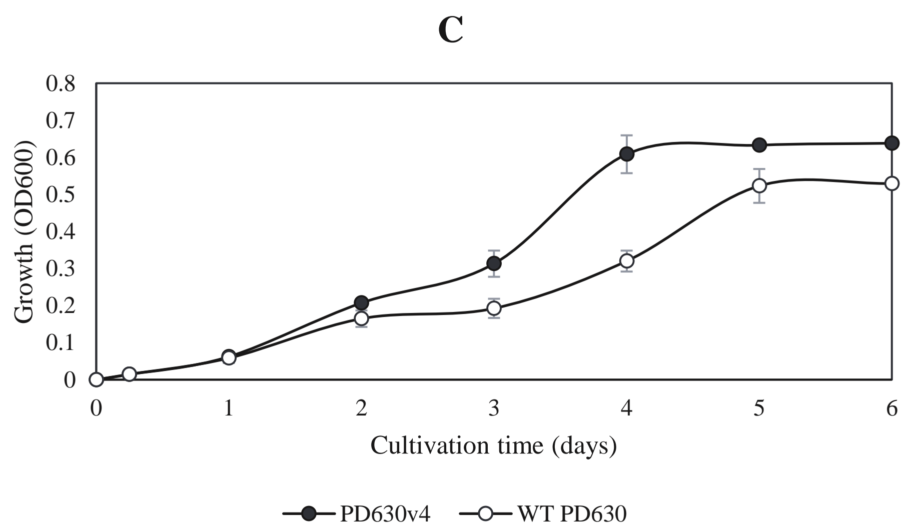


Figure 4.8. Continued

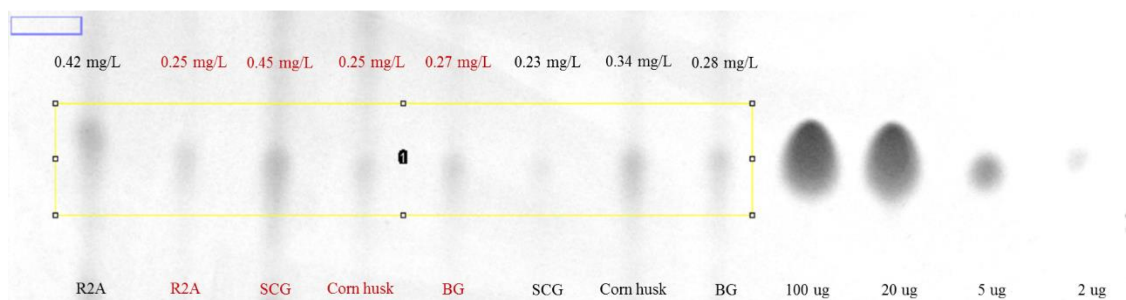


Figure 4.9. TAG accumulations by $PD630^{V4}$ (red) and PD630 (black) on each hydrolysate after 6 days of cultivation

5. SUMMARY, CONCLUSIONS AND FUTURE STUDIES

5.1 Summary and conclusions

This study has investigated the potential of using chemical hydrolysis aided by ionic liquid pretreatment to yield sufficient fermentable sugars for three different types of biomass. This is the first study to utilize a chemical hydrolysis using an IL, with a downstream compatible tolerant PD630 for TAG production. The hypothesis of this study situates that an ionic liquid pretreatment will aid in dilute acid hydrolysis of biomass for fermentable sugars, which can be used in tandem with a downstream hyper-tolerant bacterial strain for lipid production. The hypothesis is confirmed was supported and highlighted by the results below:

1) Pretreatment of MCC, SCG, corn husk, and BG using [Emim][Cl] as a solvent prior to dilute acid treatment, yields reducing sugars and glucose in the final hydrolysate.

2) The alternative chemical hydrolysis of biomass does not yield as much reducing sugars as established treatments utilizing cellulolytic enzymes, however it yields sufficient sugars in a relatively shorter time than enzymatic treatment.

3) The ionic liquid pretreatment is more successful at lignin removal than dilute acid for biomass SCG, corn husk, and BG subjected to the same treatment conditions.

4) The alternative chemical hydrolysis yields sufficient reducing sugars and glucose for usage as growth substrate for PD630 when the hydrolysate is detoxified and neutralized by removing residual [Emim][Cl] and adjusting the pH to 7.

5) However, original conditions in the crude hydrolysate of the chemical treatment will inhibit PD630 from growing on the biomass hydrolysate. The original levels of inhibition were quantified in terms of pH, residual [Emim][Cl], and HMF concentration.

6) The minimum inhibitory concentration for wild-type PD630 was elucidated for each of these inhibitory factors. PD630 can experiences inhibition at 2 g/L of HMF, at acidic conditions with pH of approximately 4, and at 20 g/L of [Emim][Cl].

7) Based on quantifying the inhibitory factors in the hydrolysates, a triple-tolerant mutant of PD630, $PD630^{V4}$ was successfully developed using the adaptive/directed evolution strategy. This strain is able to tolerate and grow more rapidly in higher inhibitory levels of pH, IL, and HMF than the wild-type PD630.

8) $PD630^{V4}$ demonstrated less inhibition in SCG, corn husk, and crude hydrolysate, than the wild-type and was able to successfully grow on each hydrolysate and accumulate TAG.

5.2 Future studies

This study has implications in other bio-refinery process research utilizing chemical treatment. It also has implications on the utilization of microbial systems as an effective catalyst for bio-fuel production. The following are suggestions for future studies that aim to continue studies in these related fields.

1) Further investigation into the mechanisms of tolerance in $PD630^{V4}$ is needed. DNA sequencing and difference in gene expression or gene identifications are needed to further understand the ability for $PD630^{V4}$ to tolerate these conditions.

2) Optimization of the chemical treatment conditions to yield more reducing sugars, increasing removal of lignin, and reduce inhibitory by-product formation such as HMF.

2) Implications of using various different IL on the chemical process and the adapted strain.

3) The ability to recycle ILs is a major advantage in using IL pretreatment. More research is needed into an efficient system for IL separation to fully exploit these advantages. There will inevitably be sugar loss in the IL liquid portion in forms of hemicellulose and some cellulose; effective IL separation strategies would allow for the complete usage of the biomass. In addition, lignin valorization and reuse is a popular option if effective IL separation strategies are found.

4) The hormetic effect of [Emim][Cl] on PD630 can be further investigated. Mechanisms of hormesis can be further explored. In addition, implications of different ILs and their effects on other oleaginous microorganisms can be further investigated.

5) The successful implementation of the adaptive/directed evolution strategy prompts more studies on different bio-fuel producing microorganisms. Preferably, this tolerance adaption strategy can be used in tandem with recombinant microorganisms that can utilize a wide variety of substrate in addition to glucose.

6) For a more effective system, PD630^{V4} can be genetically modified to express cellulolytic enzymes and utilize other hemicellulose sugars to fully exploit the advantages of microbial systems.

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