Exploring substrate specificities of novel *Burkholderia sp.* isolates towards sustainable energy: benzoate degradation by new *B. xenovorans* strains

by

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Abstract

Sustainable energy technologies demand the use of renewable sources of energy and materials, such as biomass. Remediation of toxic wastes is a related problem. The recalcitrance of the aromatic heteropolymer lignin, commonly found in plant biomass, is a major challenge for its sustainable transformation to energy and biomaterials. Biological approaches for degrading lignin and xenobiotic aromatic pollutants show great promise and are effective in some recent applications. This work aims at investigating the biodegradation activity of the newly discovered *Burkholderia sp.* isolates in alkali lignin, aromatic hydrocarbons, and *n*- or *iso*-alkanes under aerobic condition. Results show that no degradation was observed for alkali lignin inoculated with *B. xenovorans*. However, the novel *B. xenovorans* strains were able to grow and utilize benzoate (1 mg mL^{-1}) as the sole carbon source in a minimal medium (M9) at relatively fast rate. HPLC analysis showed the presence of catabolic intermediates that were further used by the bacteria for growth. A ^{1}H NMR analysis confirmed the presence of catechol as the central intermediate having a yield of 31.21 mg after hours of incubation. Furthermore, the new endophytic *B. phytofirmans* isolates also showed growth on M9 plates containing isooctane as the sole carbon source. More work is needed to determine the mechanism of degradation in isooctane, a recalcitrant branched alkane, by the novel *B. phytofirmans*. Overall, this study reveals the degradation potential of the new *Burkholderia sp.* isolates, in which the provided information can be harnessed for advanced applications and biotransformation strategies.

Keywords: renewable energy; biomass; lignin; biodegradation; *Burkholderia sp*.; aromatic hydrocarbons; biotransformation;

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List of Abbreviations

ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
APPL	Acid-precipitable polymeric lignin (APPL)
box	benzoate oxidation
ben-cat	benzoate-catechol
CLEA	Cross-linked enzyme aggregate
CoA	Coenzyme A
DMP	Dimethoxy phenol
DyPs	Dye-decolorizing peroxidases
ESI	Electrospray ionization
HPLC	High performance liquid chromatography
LB	Luria-Bertani
LiP	Lignin peroxidase
M9	Minimal media
MnP	Manganese peroxidase
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
OD_{600}	Optical density at 600 nm
PAH	Polyaromatic hydrocarbon
PCB	Polychlorobiphenyl
PORL	Pre-treatment to overcome recalcitrance of lignocellulose
RCF	Reductive catalytic fractionation
rRNA	Ribosomal RNA
SSF	Simultaneous saccharification and fermentation
TCA cycle	Tricarboxylic acid cycle

Chapter 1.

Introduction

1.1. General introduction

The rate of global energy consumption has been on the rise for over a century. Fossil fuels remain the dominant source of energy and chemicals, of scales ranging from that for a global commodity (e.g., benzene-toluene-xylene) to fine chemicals (e.g., starting materials for pharmaceuticals). It also is now known that many environmental problems, such as air and water pollution, and climate change, are the result of fossil fuel consumption. To minimize the release of environmentally detrimental gases (e.g., CO_2, NO_x), several governments have legislated the use of renewable resources, especially biomass, to produce energy and value-added chemicals. The Canadian Government has implemented measures to support the production of renewable fuels. Part of these measures is to support next-generation technologies for biofuel production from non-conventional feedstocks.[1] Likewise, the U.S. Department of Agriculture and U.S. Department of Energy have set ambitious goals to derive 25% of U.S. commodity chemicals and 20% of transportation fuels from biomass by 2030.[2]

Combustion of fossil fuels is not the only process that gives rise to detrimental environmental effects. Common pollution sources, including oil spills and waste water streams, are hazardous to the environment and to human health. Remediation approaches are needed to address these problems. Remediation takes many forms, including approaches that are based on physical, chemical, thermal, and biological agents. Governments of different countries include such strategies in proper contingency plans for handling pollutions.[3, 4] Of special significance is biological remediation, or bioremediation, which has the potential to be the best approach in terms of low cost, environmental friendliness, and the success rate for remediation.[3]

The field of biotechnology is diverse and shares complementary facets for use in energy and environmental remediation. Consequently, there is a long-standing and often overlapping interest in the development of technology to valorize biomass and to mitigate environmental pollution. A great deal of current technological advancement is directed at

improving biorefineries. These facilities are used for processing biomass and waste streams to obtain value-added products, energy, or to produce non-toxic by-products. Much like conventional petroleum refineries, which produce fuels and chemicals from crude oil, a biorefinery uses renewable biomass sources to produce multiple products, including biofuels, commodity chemicals, and fine chemicals. It is important to note that there is an economic motivation for a biorefinery to produce chemicals (fine, commodity, specialty) in addition to biofuels, and this has been strongly emphasized elsewhere.[5,6]

1.2. Bioprocessing

The term “bioprocessing” evokes images of large, modern factories. However, humans have long been using biological processes to obtain useful products. One important historical example is the use of yeasts to ferment sugars from grapes or grains, to produce wine or beer, respectively. These simple examples provide a broad definition of bioprocessing wherein the natural biological processes of an organism use the molecules found in a low value starting material to generate a product of greater value.[7]

In modern facilities (including breweries and wineries), bioprocess technology is quite advanced. These modern processes still use living cells to convert simple or inexpensive starting material to valuable products, but the scales are usually very large. The large-scale processes are called “biomanufacturing” and are used in the manufacture of drugs and foods, and in the treatment of industrial waste. In many ways, biomanufacturing and bioprocesses could surpass other industrial technology if appropriate organisms or systems can be identified. The advantages of the bioprocess over common industrial process are many and include: (1) mild reaction conditions (i.e., those amenable to propagating living organisms); (2) the use of inexpensive and often sustainable starting materials, and (3) the potential production of less hazardous wastes.[8]

The overall procedure for a given bioprocessing scheme presents some challenges that are worth noting. Many of these challenges can be traced to the use of enzymes or components from whole organisms to catalyze biochemical reactions. Many challenges are associated with the cost (capital investment and operational cost) of bioprocessing. There are several contributing factors to these costs. First, reaction conditions (such temperature, pressure, pH, oxygen content and other variables) must be controlled. Second, reactions often lead to formation of many unwanted by-products,

presenting hurdles to the isolation of the desired product. However, advances in technology are changing the economics of bioprocessing. The advent of automated and computerized equipment makes reaction control and monitoring straightforward, thereby increasing the efficiency in production. Likewise, new genetics tools also are making it easier to understand the origin of by-product formation, and organismal viability in bioprocess conditions. In many cases, modified organisms or enzymes are being used.[9]

Modern bioprocessing has evolved into a multidisciplinary field, requiring knowledge from scientific disciplines including biology, molecular biology, chemistry, biotechnology, physics, engineering, and computer science. As members of these fields continue to learn from each other and develop technology, industrial scale bioprocesses are expected to advance. This is especially true for separation and purification technology. The impact of more advances in biotechnology will be felt in food processing, agriculture, pharmaceutical development, waste management, medical research, and other fields of science and industry.[7]

1.3. The bioprocess method development

The workflow of bioprocessing is can be broadly classified into two parts: the upstream and downstream processing. The following sections define these two parts and put them into context of an overall process.

1.3.1. Upstream processing

In this stage of the bioprocess, cells (prokaryotes or eukaryotes) are grown in bioreactors. “Bioreactor” is a broad term that is used for the environmentally controlled reaction vessel needed for a given bioprocess. Designs and implementation can vary from a small, laboratory-scale culture to industrial-scale operation, as shown in Figure 1.1. The upstream stages involve media preparation, cell culture, and fermentation. When the cells have reached the required density (defined as number of cells per unit volume), they are collected for the next stage of bioprocessing. Furthermore, part of the upstream process is to ensure that particulate and/or inhibitory compounds are removed from the process, including the medium, instruments, and air. It is essential that unwanted biological contaminants, such as fungi, virus, and bacteria, are removed, such that only the desired cells will have access to nutrients.[7] A cell strain known to synthesize the target value-

added product is selected and can be optimized to maximize its ability to produce desired amounts of the product.[7,10]

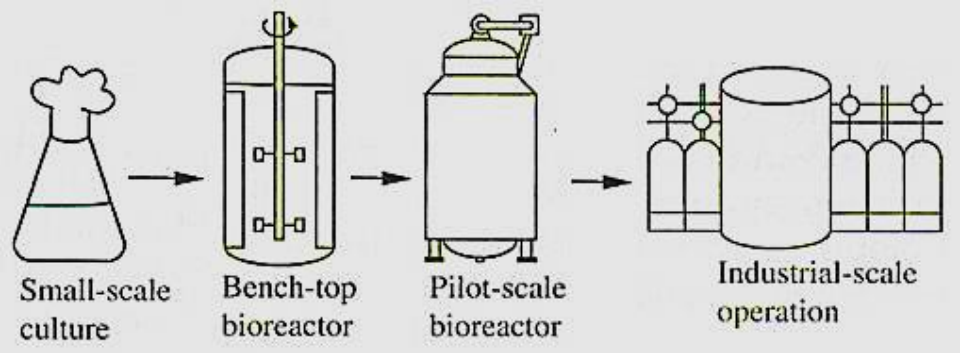

Figure 1.1 Different operational types of bioreactors, from small-scale culture to industrial-scale operation. Adapted with permission from Elsevier.[11]

1.3.1.1. Media preparation

This step involves the development and implementation of the recipes for each bioreactor stage, from inoculum to harvest. The vessel containing the media can be a tank, bottles, flask, carboy, or bag. All cells have specific nutrient requirements in a given process. The composition of these nutrients is essential to produce the desired product (enzyme or metabolite) of the bioprocess. The typical composition of microbial media includes: nitrogen (usually ammonium), fats/lipids, carbohydrates (glucose or other sugars), vitamins, phosphorus (usually as inorganic phosphate), and trace amounts of transition metal salts. Mixtures of amino acids also are routine additives.[10]

1.3.1.2. Cell culture

This step involves the growing of cells in a controlled, aseptic environment. The growth of all living things can be characterized by their cell division[12] when given a suitable environment. The minimal environment usually consists of a growth medium as a nutrient source, the proper atmosphere (i.e., aerobic or anaerobic), and proper temperature. The growth rate and overall health of the cells can be correlated with solution turbidity. Measurements of turbidity are carried out by collecting optical densities using a spectrophotometer. Sometimes, the cells are physically counted by taking serial dilutions and counting under magnification (e.g., using a hemocytometer).[13]

1.3.1.3. Fermentation

This step (specifically for bacteria and yeast) involves the conversion of substrates to the desired product(s). The specific product from the fermentation is determined by the type of microorganism carrying out the process and the substrate present in the fermentation media. For example, yeast fermentation in fruit juice produces ethanol,[14] bacterial fermentation of cereals and milk produces vinegar and some cheese products, respectively.[15] Fermentation approaches are commonly associated with the food and beverage industry.

The fermenter, or bioreactor, provides the appropriate conditions for successful production. The process conditions, which carry over from above, include: temperature control, agitation, aeration control and monitoring oxygen levels, pH control, containment, and so on. Containment is necessary for potentially hazardous organisms. The release of viable cells from the process is undesirable because of the possibility of detrimental effects to humans, animals, or the environment.[7]

The five major groups of commercially important fermentation processes are:

- **Group I:** Those processes that produce cells or biomass as the product. The cells or their extracts can be used as an ingredient or a substitute for foods, and is suitable for human or animal consumption.[16]

- **Group II:** Those processes that produce enzymes. Here, useful enzymes are produced in large quantities, which is due (in part) from the advent of modern recombinant DNA techniques.[17]

- **Group III:** Those processes that yield target metabolites via natural processes. In this case, cells use components in the growth media (e.g., nitrogen, sugars) for survival and produce the desired products. The metabolic properties are organism-specific and determine the environmental condition(s) that are suitable for growth. This can be used to tailor the right microbe to a matching industrial process. Microbial metabolites are of two types: primary and secondary metabolites. Many products derived from primary metabolism are of economic importance and generated by fermentation. Secondary metabolites often possess pharmacological properties such as antimicrobial activity, enzyme inhibitors, promoters and drug or drug-like molecules.[18,19]

- **Group IV:** Those processes that produce recombinant protein products. The development of molecular biology and recombinant DNA technology gives access to a new range of products. One exciting example is the production of recombinant human proteins. This includes transfecting non-human cells with genes from higher organisms, where the hosts are capable of synthesizing the desired foreign proteins. *Escherichia coli* and *Saccharomyces cerevisiae* are common examples.[20]

- **Group V:** Those processes that transform a starting material into a structurally related value-added analog. Biotransformation usually involves structural modification of a chemical compound by organisms or enzyme systems, that leads to the formation of molecules with different properties.[21,22] Microbial biotransformation is widely used in transformation of pharmaceutical substances[22] and in the remediation of pollutants e.g., hydrocarbons, metals.[23]

Fermentation technology has been used to obtain commercial products that can be classified using the five groups mentioned above. Examples of the diverse set of bioproducts include: biofuels and biochemicals, biopharmaceuticals and nutraceuticals (nutritional supplements), foods and beverages, and enzymes. Given the breadth of products, there is a good economic driving force for application of fermentation processes that could support a bio-based economy.[7] Biofuels and biochemicals, which are products of the transformation of lignocellulosic biomass, will be discussed later in this Chapter.

1.3.2. Downstream Processing

"Downstream processing" refers to the steps involved with handling of products derived from the fermentation process to meet purity and quality requirements. In general, downstream processing refers to the isolation and purification of a biotechnological product to yield a product that is suitable for its desired use. In practice, this can be a straightforward process (e.g., precipitation), or one that requires many steps (e.g., chromatographic separations). The many different biotechnological products mentioned above require similarly diverse techniques for recovery, purification, and characterization. An example flowchart of the entire downstream process is shown in Figure 1.2.[24] The key divisions of downstream processing are explained below.

1.3.2.1. Cell harvest

This is the first step and it involves the removal of insoluble material from the fermentation medium. Usually this is whole cells and cell debris, but other solids can be removed, such as particulate matter or insoluble products. The physical operations used to perform this are centrifugation, filtration, extraction, precipitation, sedimentation, flocculation, electro-precipitation, and/or gravity settling. Centrifugation is commonly used for separating solid particles from the liquid phase and is based on density differences between particles to be separated in the medium.

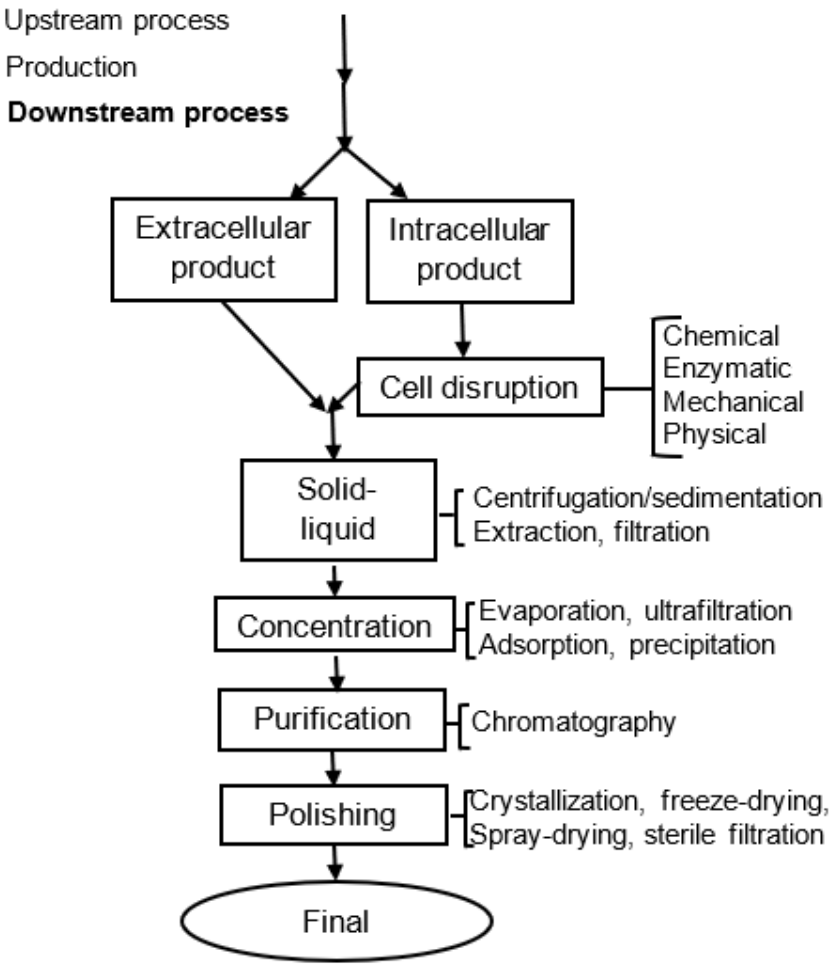

Figure 1.2 Flowchart of the downstream processing.[24]

1.3.2.2. Concentration

This is a pre-treatment step in downstream processing to prepare the product for the purification process. Concentration is usually necessary because chromatographic columns operate under specific concentration or pH conditions. In this step, the desired

product is partially purified by removal of water, solvents, buffer salts, and any other compounds that would interfere with separations. Operations involved in achieving this include solvent extraction, adsorption, precipitation, ultrafiltration and diafiltrating. Extraction of compound(s) of interest either by liquid-liquid extraction or solid-liquid extraction are examples of commonly used procedures.

1.3.2.3 Purification

This process is carried out to remove contaminants that have similar physical or chemical properties to those of the desired product. This is an important difference with respect to the concentration step, which generally separates compounds with features different from the desired product. Purification usually requires the use of sensitive and sophisticated equipment and therefore tends to be the most expensive step. Chromatography is the major tool in obtaining high purity products in downstream processing. The common chromatographic techniques used include: ion exchange, reverse phase, affinity, gel permeation, hydrophilic interaction, and size exclusion. Other purification methods also can be applied, such as crystallization and fractional precipitation.

1.3.2.4. Polishing

This is the final processing step. Here, the pure product is prepared and packaged in a convenient and easily transported form. This final product is now at its highest value. Failures at this stage are not acceptable and can result in products with contamination. Therefore, quality control is emphasized, especially for biopharmaceuticals. Associated operations in polishing are crystallization, lyophilization, desiccation, and spray drying.[24,25,26]

Bioprocesses show great economic potential. According to research from the Business Communication Company (BCC), the demand for microbes and microbial products pushed the market value to over $140 billion in 2014.[27] Markets continue to grow rapidly (to an estimated $300+ billion by 2020), or in other words, and a growth rate of *ca*. 15% per year from 2015 to 2020.[27] In an increasing number of areas, bioprocesses are replacing synthetic production processes due to emergent technical and economic advantages. At present, a major focus is on using sustainable raw materials for bioprocessing. One example of a renewable raw material is biomass. In particular, there

is interest in using biomass waste and side streams in the agricultural and forestry sectors. However, biocatalytic conversion of biomass is challenging, owing to its complex composition, variable origins, and the presence of chemically or physically recalcitrant and contaminating compounds in waste streams. Work on understanding and improving the robustness of microbial strains to make them more suited to ferment plant biomass materials is being done.[8]

1.4. Lignocellulosic biomass

Lignocellulosic biomass (more conveniently referred to simply as “biomass”) is thought to be among the most promising alternative sources of organic molecules that are traditionally derived from petroleum.[28] Biomass-derived materials (or renewable feedstock) can have some similar chemical structures to those in fossil-based chemicals (e.g., aromatics).[29] Biomass is considered highly sustainable because it is generated through photosynthesis using water, CO_2, and sunlight. As such, it is considered to be the only sustainable source of organic carbon on earth and an ideal alternative to petroleum for the production of fine chemicals and fuels with net zero carbon emission.[30,31] Equally attractive is that biomass is globally abundant and can be utilized to produce biofuels, biomolecules, and biomaterials.[32,33]

There are several important concerns about the use of biomass as an alternative source of fuel and feedstock. These concerns involve issues centered on the use of food sources and water resources in the production of biomass. To address the former issue, the use of second-generation (i.e., non-edible) biomass is proposed. This ameliorates competition with the use of land and resources used for the production of food. Lignocellulosic feedstocks are advantageous second generation sources because they derive from non-edible portions of plants.[34] Furthermore, waste biomass generated from agriculture, forestry, and food industry are generated yearly in large quantities and can be utilized to produce value-added products.[35,36]

The development of the conversion of lignocellulosic biomass to value-added chemicals remains a big challenge despite its great promise in many applications. Due to the chemical and physical recalcitrance and the complex heterogeneity of the lignocellulosic matrix,[32] pre-treatment methods are usually applied to change the physical and chemical properties such that the material is appropriate for conversion to desirable

products. However, pre-treatment of biomass is an expensive procedure, especially with respect to energy.[37] The overarching challenge in utilizing lignocellulosic biomass is to economically obtain high-value chemicals at high yields and purity.[38] Extensive research is being undertaken to solve this problem.[39] Importantly, there have been developments in biorefinery and biofuel technologies to refine biomass in analogy to petroleum refinery for producing renewable oil and green materials. Companies such as Comet, Logen, Enerkem, and Lignol have described promising results in the development of biorefining technologies to produce advanced biofuels, biochemicals, and biomaterials from second generation biomass feedstock.[40]

The topics covered in the next sections will provide context for the above challenges by describing the structure and sources of lignocellulosic biomass, highlighting the different pre- and post- treatment methods for the degradation of lignocellulosic biomass into its components, and relating those topics to biotechnological importance of lignocellulosic biomass.

1.4.1. Structural characteristics of lignocellulosic biomass

Lignocellulosic biomass comes from plant sources and is mostly composed of cellulose, hemicellulose, and lignin polymers (Figure 1.3).[41] Biomass varies in composition depending on the source. Because lignocellulose is associated with plant structures, it is naturally resistant to degradation. This resistance is tied to the crystalline properties of cellulose, the hydrophobic nature of lignin (including crosslinking), and protection of cellulose matrix by lignin and hemicellulose.[32,42,43]

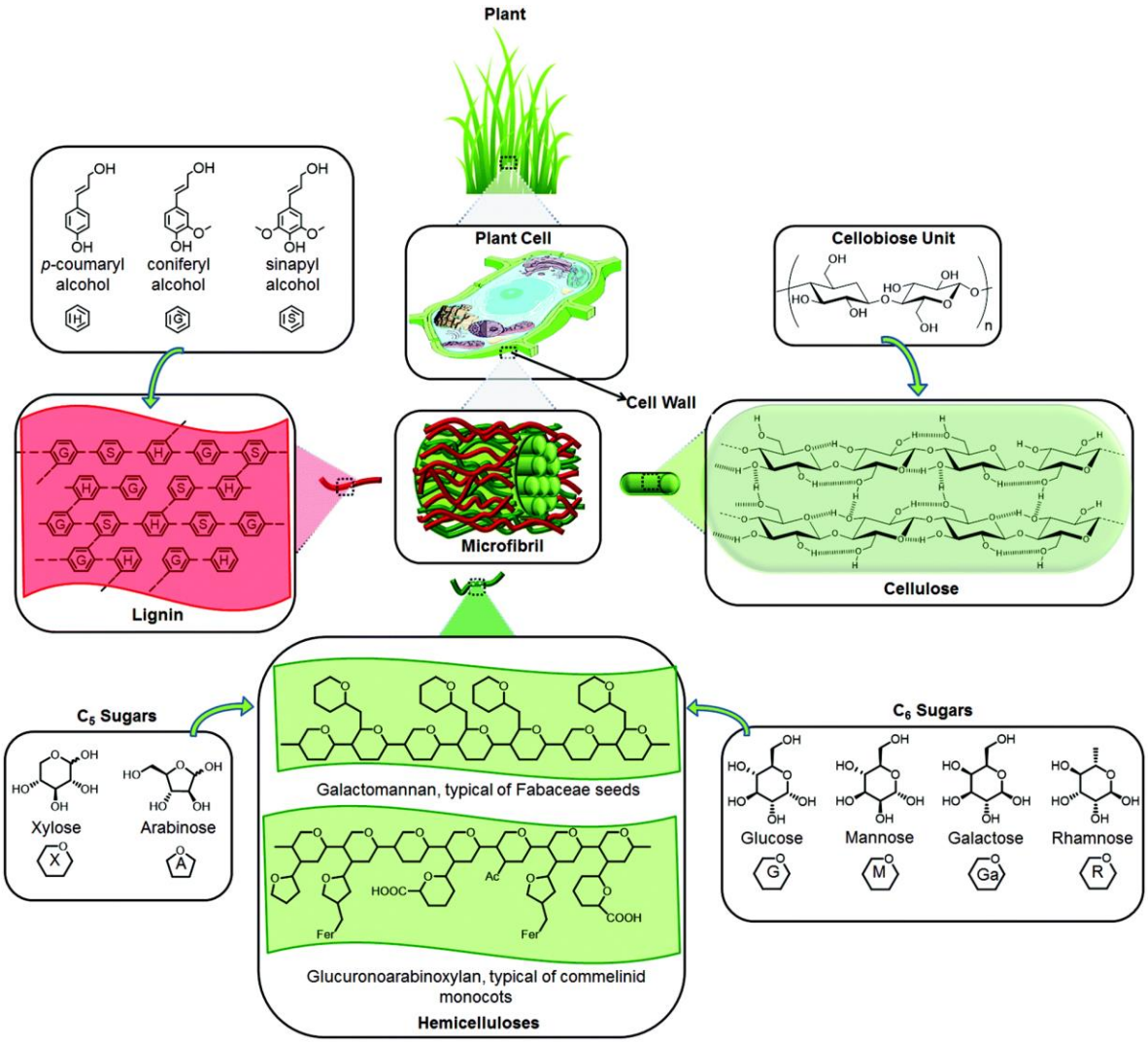

Figure 1.3 The main components and structure of lignocellulose. Adapted with permission from The Royal Society of Chemistry.[41]

1.4.1.1. Cellulose

Cellulose is usually the main component of lignocellulose (30-60% by mass). The cellulose biopolymer is very stable and has the general formula of $(C_6H_{10}O_5)_n$. It can be comprised of up to 12,000 cross-linked glucose monomers, giving it an average molecular weight of over 100,000. The (1,4*)-D*-glucopyranose (cellobiose) subunit is a major component in cellulose and is attached via β-1,4 linkages.[44] Cellulose's 3D structure is supported by extensive intramolecular and intermolecular hydrogen bonding networks involving the glucose units (Figure 1.3). Individual cellulose chains typically aggregate into bundles known as microfibrils. This gives rise to cellulose's crystalline and amorphous macrostructures.[36]

1.4.1.2. Hemicellulose

Hemicellulose is the second most abundant biopolymer in lignocellulose (10-40% by mass). In contrast to cellulose, hemicellulose is structurally amorphous. The hemicellulose network involves the random linkage of xylans, galactomannans, glucomannans and xyloglucans. The hemicellulose heteropolymers are classified based on the number of carbons of the monosaccharide units. They include the 5-carbon monosaccharides (pentoses, such as xylose, arabinose), 6-carbon monosaccharides (hexoses, such as mannose, glucose, galactose), and acetylated sugars (Figure 1.3).[41] Hemicellulose and cellulose form non-covalent bonds between the surface of each cellulose microfibril, and cross-links with lignin that provide structural strength. Finally, the hemicellulose heteropolymers differ in composition from hardwood to softwood.[42]

1.4.1.3. Lignin

Lignin is the most complex and, on average, smallest biomass fraction (6-30% by mass). It is a complex, heterogeneous polymer composed largely of phenylpropanoid units linked predominantly through ether bonds.[45] The function of lignin is to provide physical strength to plant structures and rigidity to cell walls. In addition to those structural roles, lignin helps to protect against insects and pathogens.[46] The lignin structure is formed naturally by oxidative coupling of the three phenylpropane building blocks: coniferyl alcohol, *p*-coumaryl alcohol, and sinapyl alcohol. Coupling of those monomers gives rise to the phenylpropanoid monomeric units of the lignin polymer: guaiacyl (G), *p*-hydroxyphenyl (H), and syringyl (S) (Figure 1.3).[47] Chapter 2 of this thesis describes a study involving lignin, focusing on valorization of the recalcitrant lignin polymer by use of microbial degradation.

1.4.2. Biotechnological significance of lignocellulosic biomass

Lignocellulosic biomass has a different elemental content than compounds derived from petroleum; biomass has higher percent composition of oxygen and a correspondingly lower percent of hydrogen and carbon. In principle, this allows for more classes of products to be obtained from lignocellulosic biorefineries than petroleum refineries. However, most processing technologies are still at the pre-commercial stage and a relatively large range of techniques are being explored.[40,48] The only commercially available biomass processing

technologies are for bio-ethanol/biofuel production and fermentation of glucose to lactic acid.[40,49]

Industrial biotechnology and biocatalysis have been used in both above technologies to convert conventional biomass and renewable feedstock into chemicals. For example, enzymatic techniques have been applied to transformations of fats/oils, polysaccharides, and lignocellulosic biomass.[50] Table 1.1 presents an overview of transformations applied to carbohydrates obtained from lignocellulosic biomass. In addition to transformation of biomass through more efficient routes, research efforts are currently directed toward the identification of new and non-conventional feedstock streams as opportunities. These sources include lignin. Valorization and exploitation of lignin is still in the development stage, and further optimization of the recent technological advances will provide a market for lignin-derived products.[51]

Table 1.1 Biocatalyzed transformations of polysaccharides and sugars.

Carbohydrates	Biocatalyst	Biotransformation	Product
1. Cellulose			
Microcrystalline cellulose	CLEAs of *Trichoderma reesei* cellulase	Hydrolysis	Glucose[52]
Cellulose from corn cob (1st step)	Cellulase from *Trichoderma reesei* (immobilized) 1st step	Hydrolysis	Glucose[53,54]
Cellulosic hydrolysate (2nd step)	*Lactobacillus delbrueckii* (immobilized cells) 2nd step		Lactic acid[50]
Cellulose from sugar beet pulp	Cellulase	Hydrolysis	Cellobiose[50]
Cellulosic biomass sugars	Glucose isomerase	Isomerization	D-Xylulose[55]
Peanut-shell hydrolysate	Xylose isomerase	Isomerization	convert D-xylose to D-xylulose in ethanol production[56]
Cellulosic biomass	Cellulases and xylanases	Hydrolysis	Ethanol[57,58]
Sugar cane biomass			
Waste woody cellulosic materials			
Switch grass (*Panicum virgatum L.*)			
2. Grain products and cane sugar juice or molasse	Xylose isomerase	Isomerization	convert D-xylose to D-xylulose in ethanol production[58,59,60,61,62]
3. Starch	Amylases,	Hydrolysis	Maltose, glucose[63]

	Glucoamylase and Saccharomyces cerevisiae	Saccharification and fermentation (SSF)	Ethanol[64]
4. Lignocellulosic biomass	Cellulales; hemicellulases	Hydrolysis	Biofuels[65,66]
5. Glucose	Glucose isomerase	Isomerization	Fructose[67]
6. Galactose	*Aspergillus oryzae* β-galactosidase	Oligomerization	Galactooligosaccharides (GOS)[68,69]
7. Lactose	β-galactosidase	Transgalactosylation	Galactooligosaccharides (GOS)[70,71]

Multiple processing steps are required to convert lignocellulosic biomass into value-added products. These steps include: pre-treatment, enzymatic hydrolysis, and fermentation.[72] Pre-treatment steps can include mechanical, chemical, or enzymatic approaches, or combinations thereof. Biorefineries can be fully integrated to carry out this series of processes. These steps can be grouped under upstream processing, which was explained above. Figure 1.4 illustrates the thermo-chemical and biochemical processing of lignocellulosic biomass into different products.[73] There are several recent reviews on the conversion of biomass derived sugars to commodity chemicals.[74,75,76,77] The focus of this work is on biocatalytic routes involving fermentation.

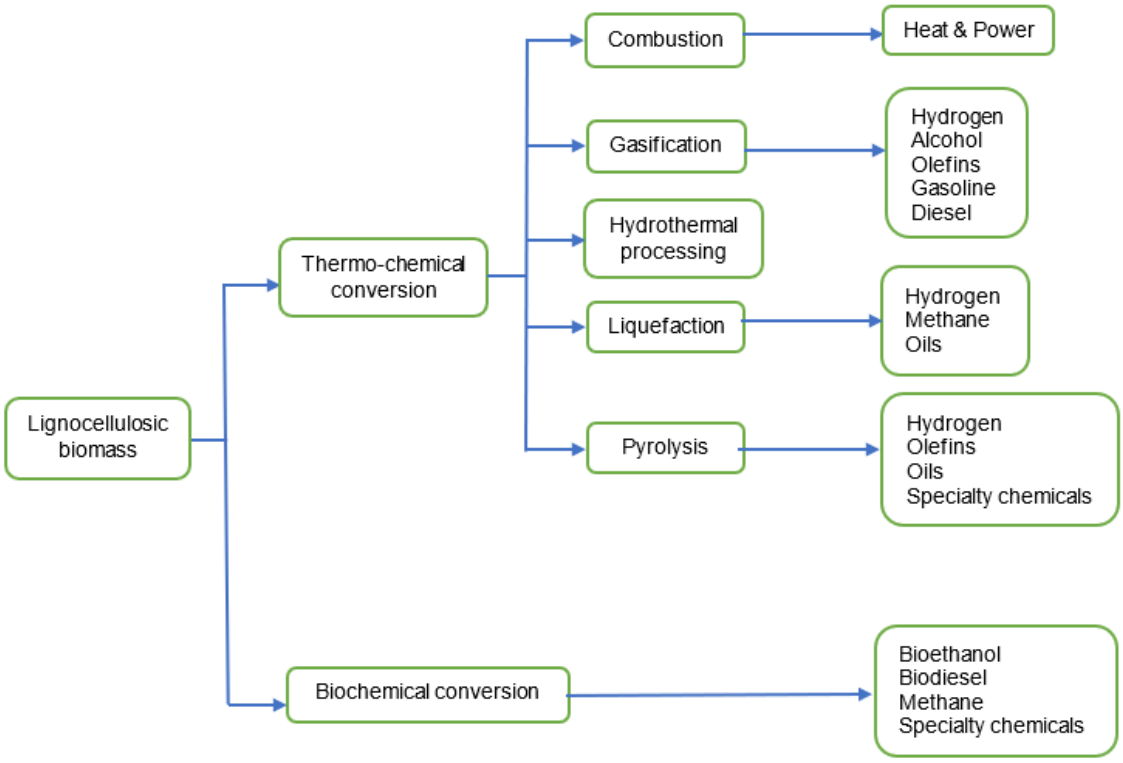

Figure 1.4 **Thermo-mechanical and biochemical processing of lignocellulosic biomass into various values added biotechnological products. Adapted with permission from Elsevier.[73]**

1.4.3. Lignocellulosic biomass treatment methods

Pre-treatment is an important step for the recovery of the three major lignocellulose components. Pre-treatment also is required for more efficient downstream processing. All of these steps can introduce added costs. When lignocellulosic biomass is pre-treated, the tight binding natural structure of the cellulose-hemicellulose-lignin matrix will become less compact. This is crucial because it enhances the access for enzymes or chemicals used for degrading cellulose, hemicellulose and lignin.[32,40,34,37] Historically, a single-step and cost-effective pyrolysis treatment method was applied, but that approach is not efficient. During pyrolysis, biomass is deconstructed resulting in “bio-oil” which has a complex mixture of hundreds of compounds that are difficult to separate. The compounds also have an increased oxygen content, which is not necessarily desirable.[41]

Other strategies for pre-treatment can be categorized under mechanical (or physical), chemical, physicochemical, and biological methods (Figure 1.4). Often, combinations of strategies from those categories are used.[45] In general, all of these

techniques involve the fractionation, solubilization, hydrolysis and separation of cellulose, hemicellulose, and lignin components.[37] Some specific examples of pre-treatment approaches are microwave irradiation, milling, steam explosion, ammonia fiber explosion, supercritical CO_2 treatment, alkaline hydrolysis, liquid hot-water pre-treatment, organosolv processes, wet oxidation, dilute- and concentrated-acid hydrolysis, and biological agents.[36,37] A few additional details about these processes are set out in Appendix C. An effective pre-treatment is characterized by several criteria: (a) avoiding excessive reduction of biomass particle size, (b) preservation of the hemicellulose fraction, (c) minimization of degradation products, (d) overall energetic efficiency, (e) maximization of the yield of fermentable sugars, and, (f) use of cost effective catalysts and regenerating high-value lignin co-product.[78] Each the above-mentioned criteria should be considered when evaluating pre-treatment options with maximum utility to produce a desired product.

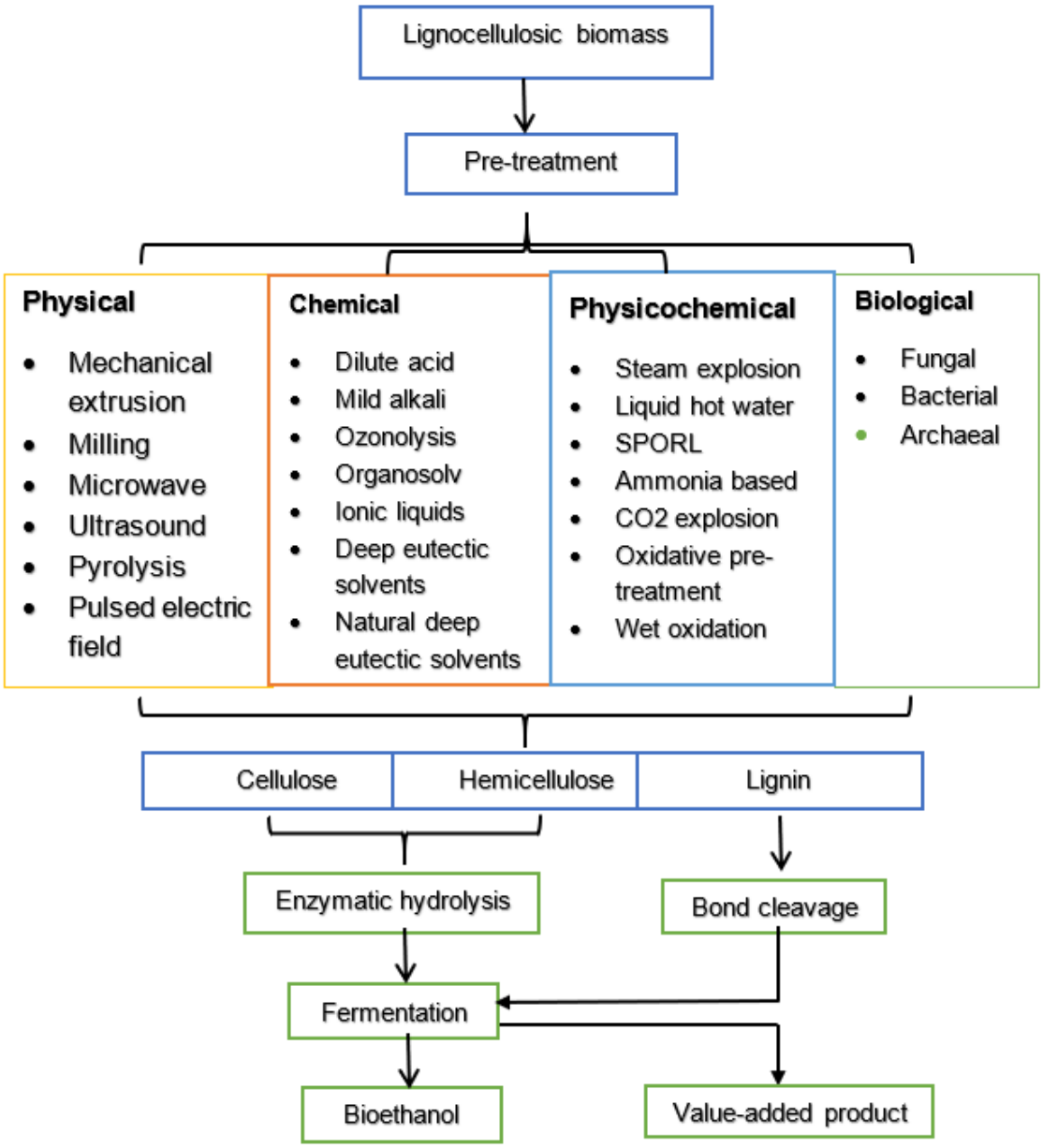

Figure 1.5 Overview of different pre-treatment processes. Adapted with permission from the Creative Commons.[79]

The above section highlights cost as an important consideration, as it is in many industrial applications. One strategy to minimize cost associated with handling and treating lignocellulose is to fully integrate biorefineries by combining biomass pre-treatment methods with the other processes, like enzymatic hydrolysis (e.g., enzymatic saccharification), fermentation, and recovery of products (Figure 1.5).[37] Overall, any future developments of lignocellulosic transformation on a commercial scale will depend on improvements to pre-treatment technologies, especially cellulolytic or ligninolytic enzyme

producing microorganisms. Developments in these areas will enable the fullest exploitation of biomass components and process integration.[80]

1.5. Bioremediation

One of the other important applications of bioprocessing is the cleanup of toxic wastes. Soil, ground water, and surface water contamination are caused by many urban activities, industrial operations, intensive agricultural practices, and improper transport and disposal practices. Common pollutants include petroleum hydrocarbons, polycyclic aromatic hydrocarbons, solvents, pesticides, herbicides, fertilizers and metals. Pollution is a threat to the ecosystem as it affects its functions, decrease biodiversity, and causes potential health risks to humans and animals.[81]

A number of environmental pollution concerns have motivated the search for new, eco-friendly, non-invasive, inexpensive, and efficient environmental clean-up techniques.[82] Bioremediation processes can satisfy all of those criteria. In this context, bioremediation is the use of entire organisms, or sometimes microbial enzymes, to remove foreign compounds from contaminated soil or water.[83] The bioremediation process transforms those contaminating, toxic substances, into benign products. Ideally, the toxic substance is transformed into innocuous products, such as carbon dioxide and water.[83]

The origin of bioremediation strategies, specifically those employing microorganisms, can be traced to 1930.[84] That early work involve petroleum clean-up, but today, bioremediation is a common technique used to restore many different types of polluted sites.[85] We know now that microorganisms degrade organic pollutants by using them as a source of carbon and energy or in other metabolic processes. In addition, microorganisms can detoxify heavy metals by changing their oxidation state or trapping them in a coordination complex. Either of those outcomes can dramatically change water solubility, bioavailability, and therefore toxicity.[85,82] Continuing research efforts are focused on understanding the mechanisms by which microorganisms act upon pollutants.

1.5.1. Bioremediation methods

Bioremediation can be carried out in two modes: *in situ*, where the contamination occurred or *ex situ*, in an artificially created environment.

1.5.1.1. In situ bioremediation

The *in situ* approach is used when contamination affects a large area and it is impossible to excavate and transfer all of the polluted material.[86] One drawback here is that the outcome is difficult to predict. For example, controlling the propagation of any given microorganism may be challenging under different circumstances.[94] When the scale is smaller, the *ex situ* approach can be more appropriate (see below).

- There are three basic *in situ* bioremediation methods, as outlined below. The focus here is on soils, but the same genera; principles apply to contaminated water.

- **Natural attenuation.** This process involves the use of microorganisms that are naturally found in a contaminated location to carry out degradation. The method takes advantage of natural pathways and the ecosystem reverts to its original condition over time. Natural attenuation minimizes hazards to a natural habitat that may be introduced in the methods described below. The natural processes can be slow or ineffective which is an important drawback.[87,88]

- **Bioaugmentation.** The population of native microorganisms capable of degrading contaminants is not always large enough to restore an environment in a reasonable timeframe (i.e., faster than many years, as for U.S. “Superfund” sites) or the contaminant is present in a high enough concentration that it impedes natural processes. Bioaugmentation, which involves the introduction of specific degraders, increases the bioremediation efficiency in these situations.[88,89] Organisms used in bioaugmentation are degraders of the specific pollutant(s) present and can survive in a foreign and unfriendly habitat. Isolation and propagation of organisms is done before their introduction onto polluted site, and recent developments in DNA manipulation and directed evolution allow, to some degree, their functional enhancement in the laboratory. Likewise, genetically modified microorganisms can also be incorporated into the polluted site.[88,90] Competition between the exogenous and natural populations of microorganisms determines the result of the bioaugmentation process.[88]

- Biostimulation. This method is used to accelerate in situ bioremediation processes by modifying the content of the contaminated soil or water. It involves the introduction of nutrients (e.g., manure, rice straw, biogas slurry, spent mushroom compost, and

corncob) or electron acceptors (nitrogen, oxygen, carbon, phosphorus).[89,91,92] 1.5.1.2. Ex situ bioremediation

In *ex situ* treatment appraoches, the polluted medium is removed and transferred to artificial treatment sites. After treatment, the clean soil can be returned to the original site.[93] *Ex situ* methods tend to be efficient for removing pollutants, since careful control of the physico-chemical parameters is possible. This level of control also is associated with shorter remediation times. The potentials disadvantages of the *ex situ* approach include additional cost and risk of dispersing contaminant during transport, which can be addressed with proper transportation procedures. Examples of liquid cleanup sites are artificial wetlands. On the other hand, semi-solid or solid wastes require special reactors. Alternatively, treatment of contaminated solids can be carried out using compost techniques.[86,94,95]

- **Constructed wetlands.** This refers to an artificial wetland that is used to treat waste water with origins in agriculture or industry, and sometimes from domestic activities.[96] The system is engineered to utilize organisms and plants (phytoremediation). The plants remove about seven to ten percent of pollutants through metabolism, accumulation, and immobilization at the interface of roots and soil. Microorganisms decay organic compounds present in the water undergoing treatment and they also utilize decayed plants as a carbon source.[95]

- **Slurry bioreactors.** This approach uses controlled reactions and can be carried out under a controlled atmosphere.[94] They make use of naturally occurring or modified organisms that have specific metabolic capabilities.[97] The ability to control the operating conditions of slurry bioreactors make this approach one of the most applied technologies used in bioremediation. The method can be applied to problematic sites, such as soils with high contents of organic and clay matter, sites where recalcitrant pollutants are present, and sites with highly toxic contaminants.[98] The desired organismal metabolic activity toward degradation can be enhanced in this method.[97,99]

- **Landfarming.** In this widely used soil bioremediation technology, polluted material (usually solids) is spread out in a thin layer on the ground surface. The contaminated soil is periodically turned to provide aeration. Nutrients, minerals, and water are also added to stimulate growth.[100,101] Landfarming is inexpensive and effective when

applied to easily biodegradable low molecular weight, low concentration contaminants.[94,100,101] The approach is slower for high molecular weight compounds (PAHs), chlorinated, or nitrated contaminants.[102]

- **Composting and biopiles.** Composting involves the use of organisms, heat, and air to treat solid waste and sewage.[94] Thermophiles are often used and these are specific bacteria that survive at higher temperatures (40-70 °C). The risk of contamination by pathogens is typically low, because those undesired organisms are mostly inactive at 70 °C. Composting is simple, eco-friendly, can be applied to large amounts of waste, and results in the total decomposition of pollutants.[86,103,104] Composting is suitable to remediate soils contaminated with petroleum hydrocarbons, chlorophenols, solvents, pesticides/herbicides, polycyclic aromatic hydrocarbons, and nitro-aromatic derivatives.[104,105] Biopiles, on the other hand, refer specifically to an advanced form of composting that requires more components, including a treatment bed, an aeration system, an irrigation/nutrient system, and a run-off collection system. Critical reaction variables such as moisture, heat, nutrients, oxygen, and pH are controlled to enhance biodegradation. Petroleum-contaminated soils have been treated with this remediation technology.[94,105]

1.5.2. Factors affecting bioremediation of pollutants in the environment

The choice of method involves many factors, including the contaminant, the site (i.e., marine or soil), and the costs that can be borne. Different factors influencing degradation of petroleum hydrocarbon pollutants have been reported by many authors as shown on Table 1.2.

Table 1.2 Factors influencing bioremediation of environmental contaminant

Factor	Effect
1. Temperature	Pollutants persist longer at lower temperature.[106]
	At low temperatures, the viscosity of oil increases, while the volatility of the toxic low molecular weight hydrocarbons reduces, thus, delaying the onset of biodegradation.[107]
	Temperature also affects the solubility of hydrocarbons. Although hydrocarbon biodegradation can occur over a wide range of temperatures, the rate of biodegradation generally decreases with the decreasing temperature.[108]

	Elevated temperature increases solubility of hydrocarbon pollutants, decreases viscosity and transfers long chain n-alkanes from the solid phase to the water phase.[109,110]
	Highest degradation rates generally occur in the range 30–40 °C.[111,112]
	High temperatures and salinity hamper microbial growth and their products.[113]
2. pH	Microorganisms and enzymes exhibit pH - dependent activity.[106]
	pH is between 6 and 9.[114]
3. Oxygen availability	Aerobic condition or anaerobic condition.[115]
4. Water content	Transport of pollutants and the degraded products; degradation of pollutants.[106]
5. Reduction / oxidation potential	Concentrations and ratios of electron donors/acceptors determine pathways and efficiency of degradation.[63]
6. Nutrients	The acceleration of microbial turnover of chemical pollutants generally depends on the supply of carbon nutrients such as N and P.[116,117]
	To stimulate microbial degradation, nutrients in the form of fertilizers (water soluble e.g., KNO_3, $NaNO_3$, NH_3NO_3, K_2HPO_4 and $MgNH_4PO_4$), slow release and oleophilic are added.[118]
7. Type of pollutant or hydrocarbon	The pollutants as substrates must be available and accessible either to microorganisms or to their extra cellular enzymes for metabolism to occur.[119]
	Biodegradability of hydrocarbons can be ranked as: linear alkanes > branched alkanes > low-molecular-weight alkyl aromatics > monoaromatics > cyclic alkanes > polyaromatics – asphaltenes.[120,121]
	Some compounds, such as the high molecular weight polycyclic aromatic hydrocarbons (PAHs), may not be degraded at all.[122]
8. Site condition	The soil or water properties and the indigenous microbial population affect the degree of biodegradation.[123]
9. Microbial communities	A successful strategy for *in situ* bioremediation can be the combination, in a single bacterial strain or in a syntrophic bacterial consortium, of different degrading abilities with genetic traits that provide selective advantages in a given environment.[124]
10. Organic matter	Influence degradation and sorption/entrapment.[106]
11. Co-contaminant/the presence of possible inhibitors	To know whether bioremediation itself is appropriate or not.[125]

The following section covers bacterial approaches in bioprocessing, providing the knowledge gap on how bacteria degrade organic pollutants and biomass. Understanding bacteria catabolic pathways, mechanisms and responsible enzymes is an effective means to define important factors for efficient remediation, and to produce value-added chemicals from plant biomass.

1.6. The role of bacteria in biodegradation and bio-product formation

Microorganisms have developed physiological mechanisms to adapt to a wide variety of environmental factors in order to survive. Acquiring nutrients is a challenge facing all living organisms, but especially microorganisms. A means of survival by microorganism is to feed on decaying matter, mostly from plant biomass. In so doing, they developed mechanisms that allow energy uptake from plant biomass.[126] One of the mechanisms they use involves enzymes that degrade the plant cell wall or organic matter. These enzymes degrade the lignocellulosic biomass or other organic compounds, which can include pollutants, and the readily metabolizable carbohydrate monomers or small molecules produced are taken up by the microorganism. The use of plant biomass by microorganisms is essential for life on Earth, because it is a primary contributor to carbon flux in the biosphere.[126] The following sections provide a brief overview of microbial degradation of lignocellulose and petroleum hydrocarbons with the focus on bacteria, highlighting some of the known bacteria, their metabolic pathways and mechanisms of degradation.

1.6.1. Microbial biodegradation of lignocellulosic biomass

The capacity to degrade lignocellulose is mainly distributed among fungi and bacteria. Most of the known fungi and bacteria are selective in their mode of action and are classified based on the lignocellulosic component they degrade, as described by the associated terms cellulolytic, hemicellulolytic, and ligninolytic. There is an intense interest in understanding the degradation of each of the three lignocellulose components; cellulose and hemicellulose produce fermentable sugars upon hydrolysis, while lignin is a rich source of aromatic compounds. A complicating factor is that lignin is resistant to hydrolysis, so it is typically removed via pre-treatment before the cellulose and hemicellulose can be accessed. Therefore, understanding the molecular mechanism of lignin deconstruction will be beneficial.[126,127]

1.6.1.1. Cellulose biodegradation

Degradation of cellulose to fermentable glucose is performed by cellulases. This class of enzymes catalyzes the hydrolysis of β-1,4-glycosidic bonds of cellulose. Fungi, bacteria, and actinomycetes produce three types of cellulase: β-1,4-endoglucanases,

exoglucanases/cellobiohydrolases, and β-glucosidase. The endoglucanases cleave cellulose chains internally to disrupt the crystalline structure of cellulose. That process releases polysaccharide units that are degraded by cellobiohydrolases and/or β-glucosidase. The cellobiohydrolases cleave the ends of exposed polysaccharide chains to form cellobiose (a disaccharide). Finally, β-glucosidases hydrolyze cellobiose to give two glucose monomers. These three types of enzymes act synergistically and sequentially to break down the complex crystalline cellulose structure. A schematic of cellulose degradation is shown in Figure 1.6. Glucose represents about 60% of the total sugars available in cellulosic biomass (see above). Once glucose is made available, the yeast *Saccharomices cerevisiae* is the most important microorganism able to ferment glucose (hexose) to generate ethanol.[126,127]

Most cellulose is degraded aerobically, but 5-10% is degraded anaerobically. The mechanism of aerobic and anaerobic degradation differs significantly.[128] The best characterized cellulase systems are the aerobic fungi which include: *Fusarium solani, Penicillium funiculosum/pinophilum, Talaromyces emersonii, Sporotrichum pulverulentum, Trichoderma koningii, and T. reesei*. Cellulolytic bacteria can be found in different genus such as *Ruminococcus, Erwinia, Thermobifida, Fibrobacter, Cytophaga, Clostridium,* and *Sporocytophaga*.[126,128] Most of the cellulolytic bacteria produce mainly endoglucanases. The most extensively studied bacterial cellulase system is that of *Clostridium thermocellum*, an anaerobic bacterium. *C. thermocellum* produces a very active cellulase in the form of complex enzymes termed cellulosome. The cellulosome degrades crystalline cellulose extensively in the presence of Ca^{2+} or Mg^{2+} and a reducing agent, preferably dithiothreitol.[128]

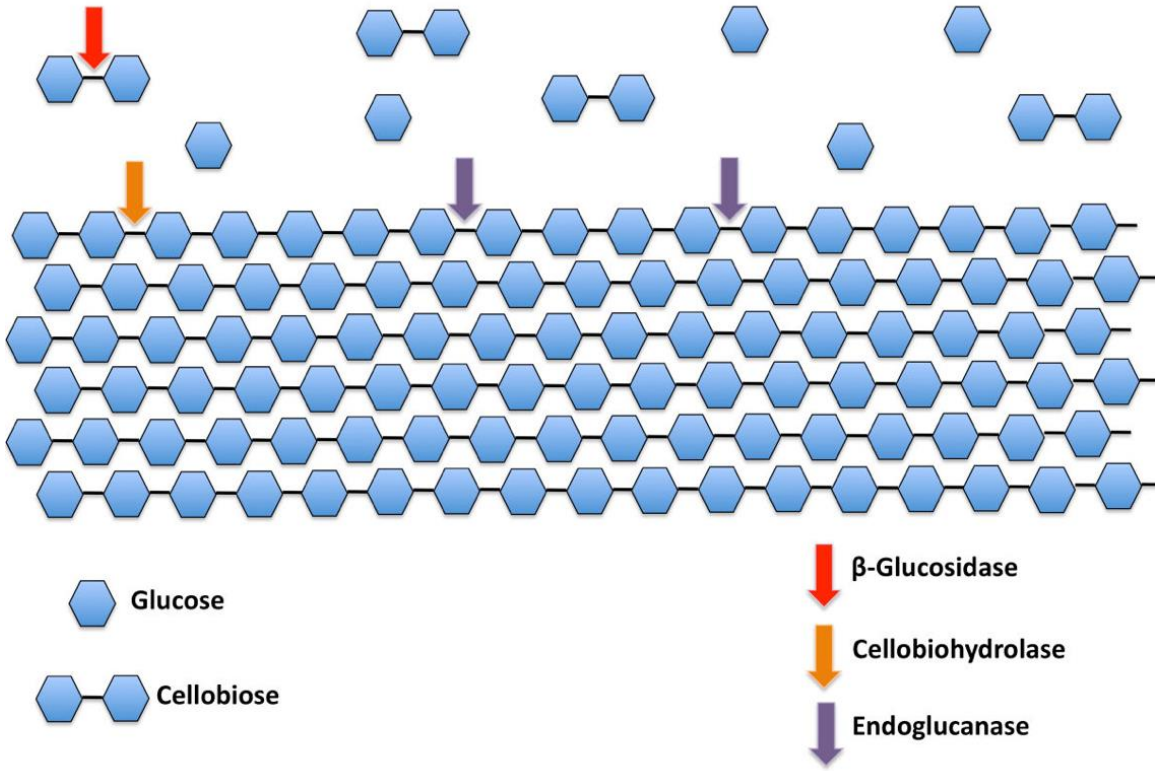

Figure 1.6 **Schematic view of cellulose degradation. Endoglucanases hydrolyze cellulose bonds internally, while cellobiohydrolases cleave cellobiose units from the ends of the polysaccharide chains. The released cellobiose units (disaccharide) are finally hydrolyzed by β-glucosidases, releasing glucose, the main carbon source readily metabolizable by microorganisms. Adapted with permission from the Creative Commons.[126]**

1.6.1.2. Hemicellulose biodegradation

Fungi, bacteria, and actinomycetes can degrade hemicelluloses either aerobically and anaerobically. Hemicellulose decomposition is similar to that of cellulose where extracellular enzymes depolymerize the polymeric structure to produce sugars that are transported into the cell for catabolism or anabolism. Hemicellulose degradation is faster than cellulose degradation because it is more readily cleaved by enzymes due to the greater accessibility of its amorphous structure. Degradation of different types of hemicellulose (e.g., xylans and mannans) by bacteria that produce hemicellulases have been reported.[128] Examples include: *Aeromonas sp., Bacteroides sp., Butyrivibrio sp., Ruminococcus sp., Clostridium sp., Pseudomonas sp., Xanthomonas alfalfae, Cellulomonas sp., Streptomyces olivochromogenes, Polyporous versicolor,* and *Trichoderma harzianum*.[128]

Aerobic degradation of hemicellulose involves the hydrolysis of hemicellulose by secreted hemicellulolytic enzymes known as hemicellulases. The action of those enzymes ultimately produces sugars (e.g., xylose, arabinose, galactose, and mannose). The sugars then undergo fermentation to produce ethanol, CO_2 and H_2O. Hemicellulases are comprised of three enzymes: exoxylanase, endoxylanase, and β-xylosidase (which cleaves xylose chains and other xylobioses). In contrast, anaerobic degradation process of hemicellulose involves three steps: (a) hydrolysis of polymeric substrates to monomers; (b) fermentation of those monomers to organic acids, hydrogen, and CO_2; and (c) transformation of organic acids, H_2, and CO_2 into methane.[128]

1.6.1.3. Lignin biodegradation

Lignin is a potential source of aromatic chemicals and is generated in large amounts as a waste product in the cellulosic biofuel and pulp/paper industries. The degradation of lignin to improve the release of fermentable sugars from lignocellulosic biomass presents a challenge to produce biofuels or chemicals from lignocellulose. Degradation of lignin has been most intensively studied in white-rot and brown-rot fungi. Different genera of bacteria have also been reported to break down lignin.[129] The well known lignolytic enzymes from fungi include: multi-copper oxidases (laccases) and heme-containing peroxidases (lignin peroxidase or LiP, versatile peroxidase or VP, and manganese peroxidase or MnP).[127,130] The bacterial lignin-degrading enzymes include dye-decolorizing peroxidases (DyPs) and small laccases (sLacs).[127] The discovery of novel lignin-degrading enzymes from bacteria provides advantages over fungal enzymes in terms of relative ease of protein engineering and expression. Fungal mechanisms of lignin degradation tend to be complex and involve post-translationally modified enzymes that complicate their study and application using recombinant methods. This has hindered the commercial application of fungi delignifying systems since their discovery over 50 years ago.[131]

Research effort has recently focused on characterizing delignifying bacteria. However, to date our understanding of mechanism(s) of bacterial delignification remains modest. There are several examples of delignifying bacteria,[127] but the best characterized ones are: *Amycolatopsis sp. 75iv3* (formerly called *Streptomyces setonii* and *S. griseus* 75vi2),[132] *Streptomyces viridosporus* T7A,[133] *Rhodococcus jostii* RHA1,[134] and *Pseudomonas putida*.[135] Most of these bacteria are isolated from tropical regions, except

Amycolatopsis sp. 75iv3, which was found in soil from Idaho, USA.[132] Two strains of the proteobacteria, *Burkholderia sp.*, were reported to show delignifying activity. Strain LIG30 was isolated from the tropics,[136] while the strain CCA53 was isolated in Japan.[137] New strains of the *Burkholderia sp.,* have recently been discovered in non-tropical locales, such as British Columbia, Canada (Appendix A). Some of these organisms could show potential to degrade lignin or organic pollutants. The goal of this research is to investigate the biodegradation activity of the newly discovered *Burkholderia* strains. Further details on lignin structure and the mechanism of fungi and bacteria degradation are presented in Chapter Two.

1.6.2. Microbial biodegradation of Petroleum Hydrocarbons

The diversity and quantity of hydrocarbons present in contaminated sites often complicates bioremediation efforts. The four classes of petroleum hydrocarbons are: the saturates (*n*-alkanes, cycloalkanes), the aromatics, the asphaltenes (phenols, fatty acids, ketones, esters, and porphyrins), and the resins (carbazoles, sulfoxides, pyridines, quinolines, and amides).[138] In natural sites, bacteria, yeast, and fungi can participate in biodegradation. The reported efficiencies for degradation vary widely, with values ranging from less that 1% to 100% for some marine bacteria.[140] Similar ranges are reported for other bacteria and fungi. In many cases, complex mixtures of hydrocarbons from crude oil can be degraded by consortia of microorganisms having broad enzymatic capacities.[140] For example, Jones *et al.*, reported the biodegradation of *n*-alkanes and alkyl aromatics in marine sediments by the microorganisms *Arthrobacter, Burkholderia, Mycobacterium, Pseudomonas, Sphingomonas,* and *Rhodococcus*.[139]

Bacteria are the most active agents in petroleum degradation, as several of them are known to feed exclusively on hydrocarbons. *Acinetobacter sp*. was found to be capable of utilizing *n*-alkanes (C_{10} - C_{40}) as the sole carbon source. In references 140-141, bacteria from *Gordonia, Brevibacterium, Aeromicrobium, Dietzia, Burkholderia, Sphingomonas*, and *Mycobacterium* genera were isolated from crude oil polluted soil. These organisms were proposed as key contributors for remediation of hydrocarbon contaminated sites.[140-141]

1.6.2.1. Mechanisms

The three known mechanisms involved in the degradation of petroleum hydrocarbons are: (1) specific enzyme mediated reactions; (2) attachment of microbial cells to substrates; and (3) production of biosurfactants. The mechanism of (1) and (3) have been well studied while that of (2) is unknown.[140]

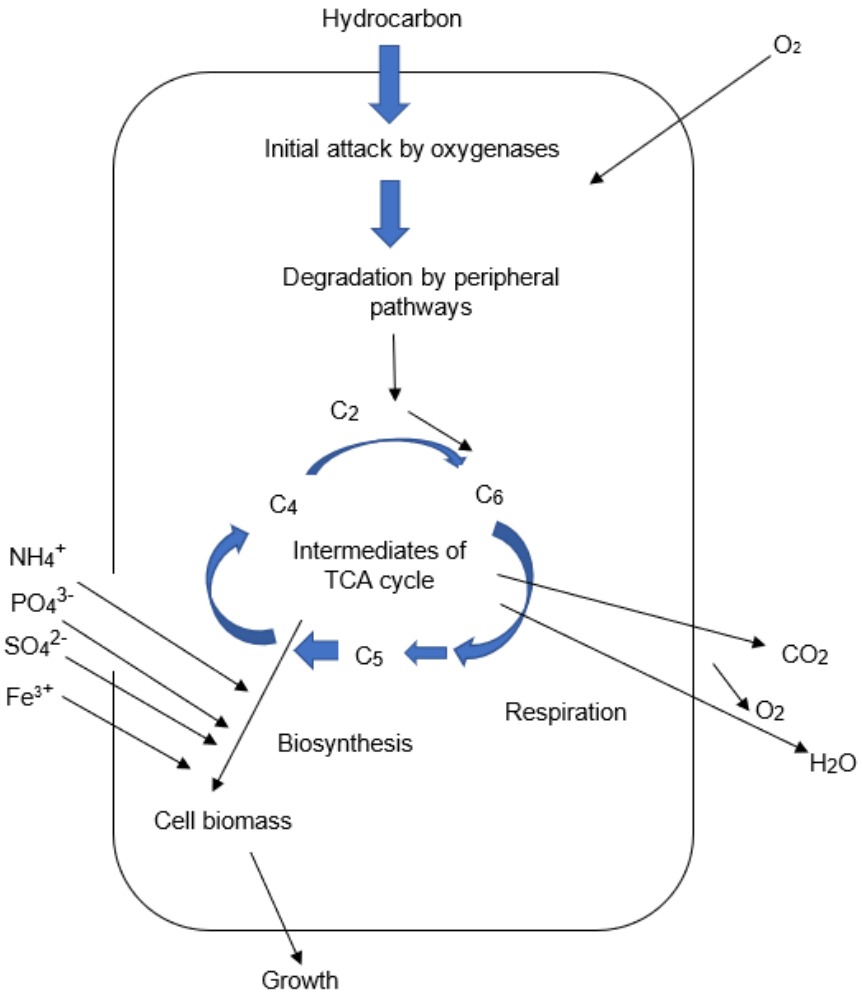

Figure 1.7 Major principle of aerobic degradation of hydrocarbons by microorganisms. Adapted with permission from the Creative Commons.[140]

Many organic pollutants can be degraded rapidly and completely under aerobic conditions, as shown in Figure 1.7. In this scheme, the organic pollutant enters the microorganism cell following an oxidative process where oxygen is incorporated enzymatically. The enzymes usually include oxygenases, dioxygenases, hydroxylases, and peroxidases.[142] The organic pollutant is converted, step by step, in subsequent

pathways into compounds that can be used in metabolism, e.g., the tricarboxylic acid cycle (TCA). The TCA cycle generates precursor metabolites such as acetyl-CoA, pyruvate, succinate which are used up by the organism for growth. The mechanism of enzymatic oxidation of aromatic pollutant is shown in Figure 1.8.[140]

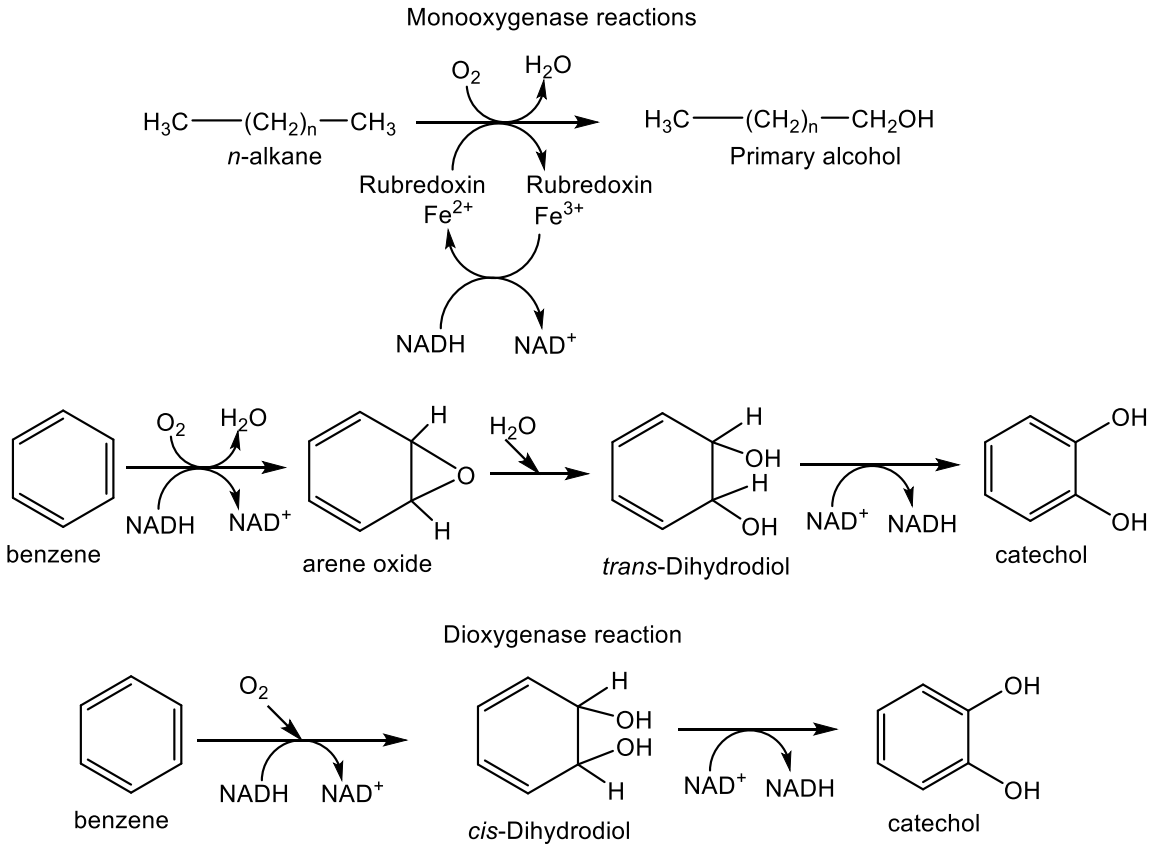

Figure 1.8 Enzymatic reactions involved in the processes of hydrocarbons degradation. Adapted with permission from the Creative Commons.[140]

Table 1.3 sets out some enzymatic systems that microorganisms use to incorporate oxygen in different organic compounds to initiate biodegradation. The application of recent genomic or proteomic research to several polycyclic aromatic hydrocarbon (PAH) degrading bacteria (e.g., *Burkholderia spp.* and *Mycobacterium spp.)* has revealed the existence of multiple dioxygenases.[142]

Table 1.3 Enzymes involved in biodegradation of petroleum hydrocarbons.[140]

Enzymes	Substrates	Microorganisms
Soluble Methane Monooxygenases	C_1–C_8 alkanes alkenes and cycloalkanes	*Methylococcus, Methylosinus, Methylocystis, Methylomonas Methylocella*
Particulate Methane Monooxygenases	C_1–C_5 (halogenated) alkanes and cycloalkanes	*Methylobacter, Methylococcus, Methylocystis*
AlkB related Alkane Hydroxylases	C_5–C_{16} alkanes, fatty acids, alkyl benzenes, cycloalkanes and so forth	*Pseudomonas, Burkholderia, Rhodococcus, Mycobacterium*
Eukaryotic P450	C_{10}–C_{16} alkanes, fatty acids	*Candida maltosa, Candida tropicalis, Yarrowia lipolytica*
Bacterial P450 oxygenase system	C_5–C_{16} alkanes, cycloalkanes	*Acinetobacter, Caulobacter, Mycobacterium*
Dioxygenases	C_{10}–C_{30} alkanes	*Acinetobacter sp.*

As an alternative to the oxidative processes described above, some organisms initiate metabolism of hydrocarbons using biosurfactants. Biosurfactants aid in the formation of emulsions by forming micelles, thereby, lowering the oil surface tension. The hydrophobic region of the microbial cell surface takes in the micelles and the encapsulated compound is subsequently degraded.[143] Biosurfactants come in different classes that depend on the organism. For example, biosurfactants may be rhamnolipid or glycolipid derivative, as reported in *P. putida, P. chlororaphis, P. aeruginosa,* and *R. erythopolis*. A microbial consortium (*Pseudomonas aeruginos*a and *Rhodococcus erythopolis*), where biosurfactants were present was reported to degrade 90% of the hydrocarbons in the soil contaminated with oil sludge.[140]

1.7. *Burkholderia* species

The genus *Burkholderia* comprises diverse species of Gram-negative bacteria. *Burkholderia* species are ubiquitous in many habitats, including in humans.[144] To date, more than 80 *Burkholderia* species have been reported. They have been classified based on phylogenetic analyses, including those of the 16S rRNA and genome sequences.[144,137] These classification groups are also referred to as Clades. *Burkholderia* Clade I contain clinically relevant and phytopathogenic *Burkholderia* species. Clade I may be referred to as the *Burkholderia cepacia* complex (BCC), which are the causative agents of major infections in both plants and animals. Clade II is composed of the environmental *Burkholderia* species, which are newly named as the genus *Paraburkholderia gen. nov*.[144] For example, conversion of nitrogen in legumes is assisted by *B. mimosarum, B. nodosa, B. sabiae, B. tuberum, and B. phymatum*.[137] Furthermore, growth rates of some plants are modulated by *B. phytofirmans and B. unamae*.[137]

Several *Burkholderia* species are potential biocatalysts for biodegradation/bioremediation or for the manufacture of small molecules. This is because they possess wide array of secreted extracellular products or robust metabolic capabilities. For example, *B. cepacia* and *B. fungorum* were shown to degrade diesel-, PAH-, and carbofuran-contaminated soils and water.[137,145] Some *Burkholderia* species showed lignin degrading capabilities,[131] which could be harnessed to generate lignin-derived biochemicals and second-generation biofuels from lignocellulosic biomass. Thus, *Burkholderia* species show great potential in their application as biochemical agents in bioprocessing. Furthermore, several antibiotics are produced by *Burkholderia* species.[137]

The *Burkholderia* isolates used in this work were collected from shallow plant roots in Southern British Columbia by members of the Linington Lab at Simon Fraser University. The *Burkholderia* species were identified to be from *xenovorans* and *phytofirmans* species based on their 16S rRNA sequence. Detailed information on these isolates are presented in Appendix A. The new *B. xenovorans* isolates share some genetic history with the known polychlorinated biphenyl (PCB) degrading *B. xenovorans* LB400. Therefore, based on the shared history we assumed they may be capable of cleaving biphenyl linkage in lignin and also degrade a number of aromatic pollutants.

1.8. Contribution of genomics, proteomics and metabolomics in understanding bacterial degradation

The advent of next generation sequencing methods has led to a rapid increase in the number of genomic sequences or expressed sequence tags of bacterial species. This include several biomass and petroleum hydrocarbon degrading bacteria from *Mycobacterium, Acinetobacter, Arthrobacter,* and *Burkholderia* genera. Proteomics and metabolomics have been recently employed in the studies of environmental microbiology and have shown great impact in biodegradation and bioremediation.[142] Proteomics is useful in identifying proteins and their biological roles in biodegradation, while metabolomics can be used to profile degradation products (in biomass or hydrocarbons), as well as primary and secondary metabolites.[142]

Specifically, for this thesis, a few examples involving bioremediation shows the power of "omics" techniques. Detailed transcriptomes in LB400 during the catabolism of polychlorinated biphenyl (PCB) were analyzed by microarray techniques.[142,146] The results showed large changes in the genomic and proteomic levels during the PCB catabolism. Importantly, the expression of transporter proteins was up-regulated. Proteomics studies have also shown that several *Mycobacterium* species have multiple dioxygenases and related enzymes, which may be involved in different substrates.[142] Comprehensive profiling of metabolites during the degradation of phenanthrene by *Sinorhizobium sp.* C4 showed a large metabolome differences in comparison to cultures containing natural carbon sources. Genomics, proteomics and metabolomics are becoming indispensable in elucidation of mechanisms of biodegradation and biotransformation of biomass and organic pollutants in the environment, especially when complex mixtures of chemicals and microbial consortia are involved.[142] These omics studies have also enhanced the field of metabolic engineering and synthetic biology. For example, valuable products that are natural intermediates of catabolic processes can be produced by eliminating enzymes responsible for their further metabolism.[147]

1.9. Aims and Objectives

The work described in this thesis aims to investigate and characterize the biodegradation activity of the newly isolated *Burkholderia* strains. The biodegradation activity was assessed in alkali lignin (a commercial lignin source), aromatic hydrocarbons

and *n*- and *iso*-alkanes. Furthermore, the objective is to identify metabolites or intermediates produced and to propose the mechanism of degradation.

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Chapter 2.

Lignin: Structure, chemistry and biocatalytic degradation

2.1. Introduction

The pulp and paper industries are a major source of pollution in terms of the generation of large amounts of lignocellulosic waste. Lignin is a primary component of that waste, due in large part to its overall recalcitrance toward fragmentation to monomers or smaller subunits that could be transformed into useful products (see Chapter 1). The goal of the manufacturing process is to break down the structure of the lignocellulosic biomass (wood and plant materials) into constituent fibres (i.e., pre-treatment, see section 1.4.3). Cellulose fibre is the desired product for paper making, while lignin is eliminated as waste in forms that vary depending on the pre-treatment method. Harsh pre-treatment conditions add another complication that makes waste lignin difficult to degrade.[1] The backdrop for the challenge of lignin valorization is the complex, heterogeneous polymeric structure, which is often modified during pre-treatment. The exact structure of lignin also varies, depending on its source and the method of pre-treatment.[2,3] Ultimately, this means that biorefineries must deal with complex mixtures of lignin oligomers and related low molecular weight products.

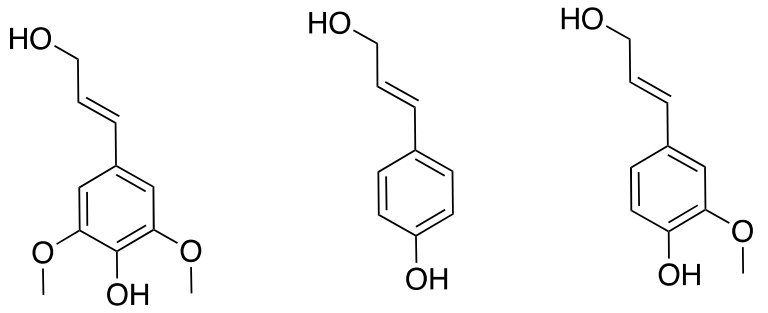
Reviews on biomass valorization via biocatalytic routes focus mainly on cellulose and hemicellulose.[4,5] However, there are interests in biological valorization of lignin to more useful products.[6] A wide range of organisms such as fungi, bacteria, and actinomycetes are reported to degrade cellulose and hemicellulose into valuable products. However, only a select few fungi and bacteria are presently known to break down lignin.[7] In this Chapter, the lignin macromolecule and its main structural differences, linkages and isolation methods are first introduced. Next, known lignin degraders, degradation mechanism(s), and current trends in bacteria lignin biodegradation are described. Finally, the bacterial route for converting lignin is described. This involves the use of the bioprocessing method development (section 1.3) on a laboratory scale. Some of the downstream bioprocessing methods for isolating metabolic products from lignin

biodegradation are outlined. Experiments exploring the ability of *Burkholderia* to degrade lignin are described.

2.2. Lignin biosynthesis, structure and linkages

Lignin is a three-dimensional amorphous polymer consisting of methoxylated phenylpropane structures that are linked randomly through ester, ether, and C-C bonds.[8] In plant cell walls, lignin fills the spaces between cellulose and hemicellulose and holds the lignocellulose matrix together (cf. Figure 1.3).[9] Considerable effort has been dedicated to fully characterize the structure of the lignin polymer and an understanding of its structure-activity relationship is gradually developing. Many of the principal structural features, constituents, and linkages of lignin have been elucidated by spectroscopic analysis,[8] thermogravimetry,[10] oxidation/reduction,[11,12] photochemical degradation,[13] ozonation,[14] and computational studies,[15] which complement wet chemical methods. These studies resulted in improvements in the structural characterization of lignins, although the exact structure of untreated lignins found in plants (native lignin) is still not well defined.

The biosynthesis of lignin is complex process that involve radical polymerization of three primary monomers (monolignols): *p*-coumaryl, coniferyl, and sinapyl alcohols (Figure 2.1).[8] The monolignols are oxidized to phenoxy radicals by redox enzymes (e.g., peroxidases, phenol oxidases, and laccases). The phenoxy radicals include the guaiacyl (G), syringyl (S) and the p-hydroxyphenyl (H) units. Phenoxy radicals rapidly undergo dehydrodimerization reactions that result in random phenol coupling. Ultimately, these coupling reactions lead to the formation of a complex crosslinked lignin in plants.[7] Lignin abundance in plants decreases in the order of softwoods > hardwoods > grasses, and the lignin composition, molecular weight, and quantity differ from plant to plant.[16]



sinapyl alcohol *p*-coumaryl alcohol coniferyl alcohol

Figure 2.1 The three monolignols, the building blocks of lignin.

During lignin formation several different chemical bonds are formed between the monolignol building blocks. Further crosslinking is possible in the aforementioned pre-treatment processes. The most common lignin linkages include β-aryl (β-O-4), biphenyl (5-5), phenyl coumaran (β-5 or α-O-4), biphenyl ether (4-O-5), and resinol (β-β or γ-O-α) (Figure 2.2).[17] The β-aryl linkage is the most abundant, comprising more than half of the inter-subunit bonds in lignin.[18] The β-O-4 ether bond can be cleaved during the pre-treatment step, and it serves as a principal pathway in which the lignin is depolymerized.[8] The breaking of these linkages tends to lead to the formation of phenolic compounds with water solubility.[8] However, the carbon-carbon bonds in lignin are amongst the most difficult to break, and most of these survive the pre-treatment processes.[8] The development of biocatalysts or chemical catalysts capable of cleaving these more recalcitrant linkages (particularly the biphenyl or 5-5 linkages) is enormously challenging and has not yet been adequately addressed. The 5-5 linkages are highly prevalent in softwood conifers such as spruce, pine, and hemlock. Furthermore, additional carbon-carbon bonds can be formed during lignin pre-treatment, such as in alkali-promoted condensation reactions during Kraft pre-treatment.[8] Ultimately, this complicates structural characterization and impedes the development of new catalysts for bioprocessing.

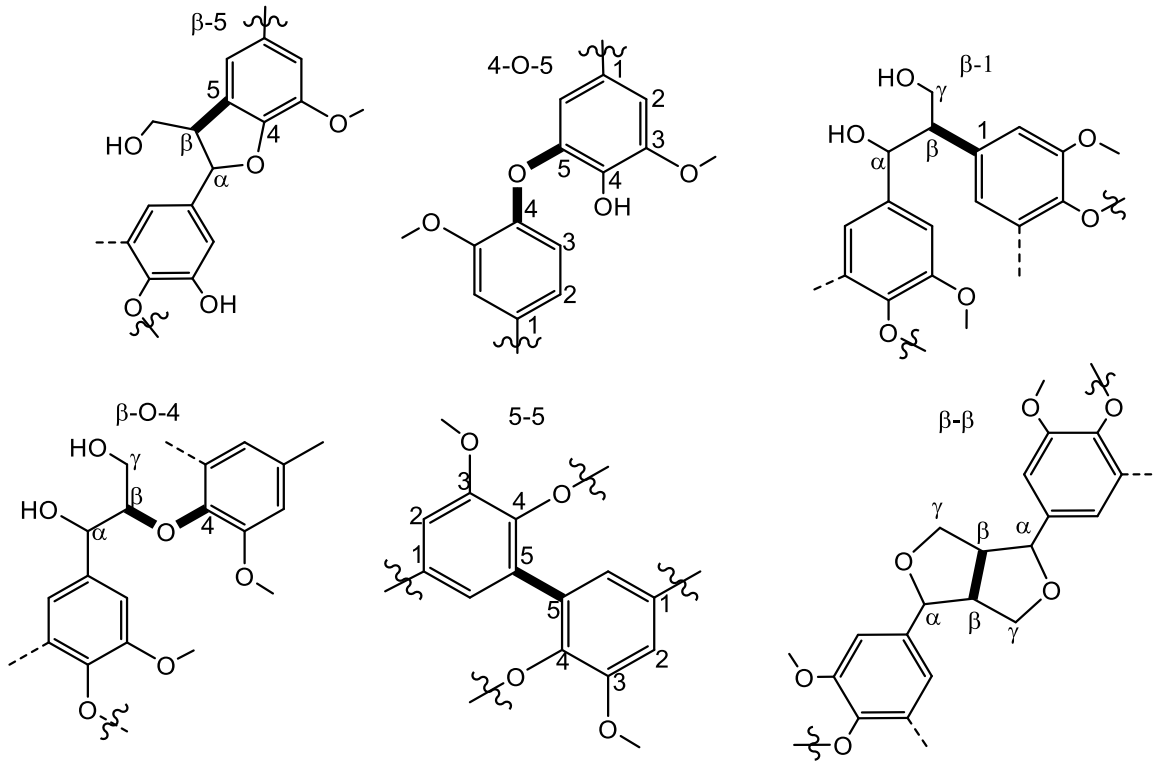


Figure 2.2 Common lignin linkages and their numbering schemes. Adapted with permission from Springer Nature.[19]

The identification and quantification of the structures and linkages in lignin is challenging, even with advanced NMR techniques, owing to complexity of the variability in lignin's structure and its sensitivity to isolation techniques.[18,20] The relative abundance of *p*-coumaryl, coniferyl, and sinapyl alcohol monolignols determines the composition of lignin from different sources. Softwood lignin contains about 90% coniferyl alcohols, while hardwood lignin has roughly equal proportions of coniferyl and sinapyl alcohol, although there are many exceptions.[21] The composition of grassy lignins are comparatively heterogeneous and contain a variable mixture of all three monolignols. Because the sinapyl aromatic rings possess more methoxy groups (Figure 2.1), they readily form linear linkages with little or no biphenyl linkages, in contrast to softwood.[22]

2.3. Lignin isolation for microbial utilization

The pre-treatment of lignin is an important step to consider in any biorefinery. Currently, a diverse range of options are available for biomass pre-treatment, which aim at cleanly separating all fractions of lignocellulosic material (Section 1.4.3). Also, pre-treated lignin in the pulp and paper industry (i.e., Kraft lignin or lignosulfonate) are likely

candidates for further valorization. At the industrial scale, the commonly used pre-treatment methods are steam explosion, dilute-acid, and ammonia-based treatments (brief definitions of the commonly used pre-treatment methods are presented in Appendix C). Other methods are being tested in pilot facilities.[6] On the bench scale, more selective approaches are being used, such as treatment/extraction using ionic liquids or other solvent-based fractionations (Organosolv), which are able to selectively isolate lignin from whole biomass.[23,24]

One important new approach is called Reductive Catalytic Fractionation (RCF). In this method, reducing conditions and a redox catalyst are used to cleave the β-O-4 linkages in lignin.[6,25,26] The RCF method generates monomers and dimers at high yields (~50%) on hardwoods (e.g., birch). The RCF concept is yet to be deployed on industrial scale, though a recent report shows a successful demonstration of RCF on *Miscanthus* (a member of the grass family) with a yield of nearly 70% aromatic products from lignin.[27] The high yields in these examples have generated much interest.

Finally, high pH pre-treatment, such as the use of NaOH, can partially fractionate lignin from biomass; this is called “alkali pre-treatment”. The lignin stream from alkali pre-treatment is highly condensed and characterized by the presence of minimal β-O-4 linkages. In addition, thiol groups are present, arising from the use of sodium sulfide.[3] Alkali (a.k.a. Kraft) lignin exhibits a very broad molecular weight distribution from the monomer range to oligomers.[28] From the biorefinery point of view, it is highly essential to develop pre-treatment technologies that will generate well-characterized and reproducible lignin streams that are suitable for efficient conversion.[6]

In the context of this Chapter, the most desirable lignin stream for microbial conversion is comprised of water-soluble monomers at high concentration. For context, sugars obtained from cellulose and hemicellulose are typically generated at 100 to 200 g L^{-1} concentrations.[6] However, the emerging pre-treatment depolymerization approaches barely produce any lignin subunits, let alone at such high concentrations. Rather they generate complex, heterogeneous mixtures of oligomeric fragments and low molecular weight products that are often in an organic oil phase.[6] Approaches that use oxidation, either chemical or biological could be promising in terms of producing water-soluble, low molecular weight compounds. At present, the state-of-the-art, only successfully gives low yields of aldehydes (e.g., vanillin) or ring-opened dicarboxylic acid mixtures (e.g., adipic

acid).[9] A recent review by Beckham *et al*. covers how lignin streams from pre-treatment approaches can be improved to make them suitable for biological conversion to value-added chemicals.[6]

2.4. Biocatalytic degradation of lignin: mechanism and enzymes

Following pre-treatment, lignin can be susceptible to a wide range of biochemical transformations that can give rise to valuable chemicals. In nature, microbial lignin degradation has been studied in white-rot and brown-rot fungi; in these cases the lignin is mineralized.[29,30] The white-rot fungi produce a range of extracellular lignolytic enzymes, such as heme-dependent lignin peroxidase (LiP), manganese peroxidases (MnP), and versatile peroxidases (VP), as well as the multi-copper oxidases (laccases), that degrade lignin via several mechanisms.[17]

2.4.1. Mechanism

The current model for microbial lignin degradation is described[3,17] as "oxidative combustion" or "enzymatic combustion." This involves the production of “mediators,” which are a broad range of small molecule redox reagents that work in concert with metalloenzymes. Example of these mediators include: veratryl alcohol radical,[31] Mn(III) coordination complexes,[32] reactive oxygen species, or those produced in secondary radical cascades,[30] as shown in Figure 2.3(a). The mediators, rather than the enzymes themselves, are thought to react directly with the lignin substrate to generate radical sites. A radical is formed within the lignin polymer, resulting in a cascade of bond scission reactions and that ultimately lead to production of smaller aromatic compounds, CO_2, and water[17] (Figure 2.3(b)).

Fungal lignin degradation has been studied for over three decades. These fundamental studies are broadly concerned with fungal enzymes and there is not yet a commercial biocatalytic process for lignin depolymerization. This is due to the challenges associated with fungal protein expression and fungal genetic manipulation. Recent advances in recombinant DNA technology may soon change this, but at present there are crucial hurdles that need to be addressed prior to widespread application. In contrast, lignin metabolism by bacteria shows different reactivity and may provide a more tractable

path toward large applications. Therefore, in recent years, there has been renewed interest in bacterial lignin depolymerization.[17] Bacterial lignin degradation activity has been best characterised in actinobacteria, proteobacteria, as well as in the gut of wood-infesting termites and beetles.[33,34]

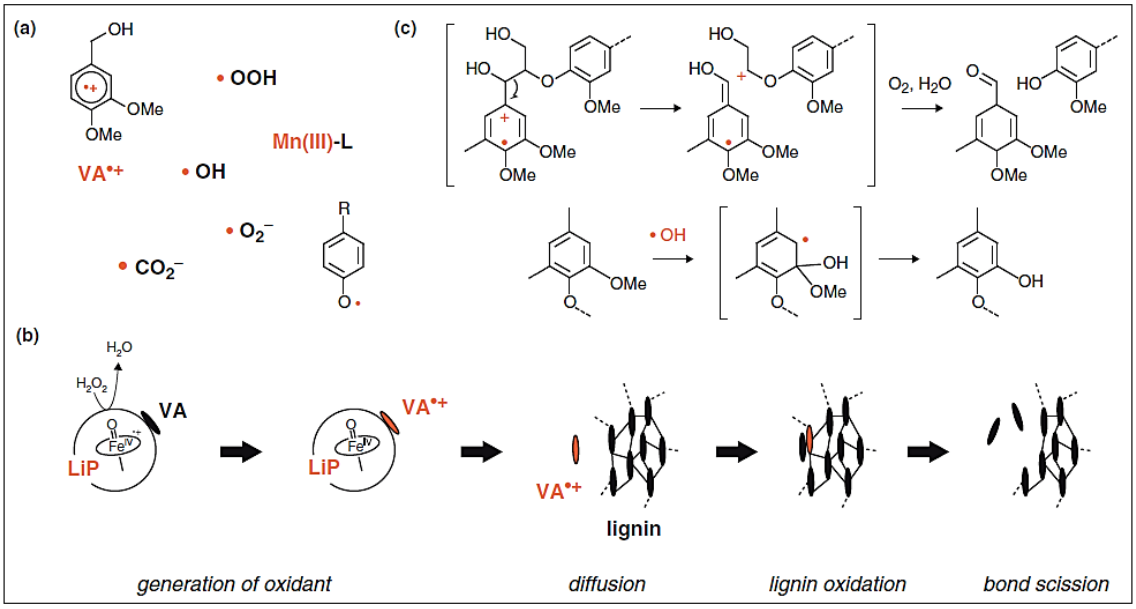


Figure 2.3 **Microbial degradation of lignin. (a) Various mediators (small molecule oxidants) produced by metalloenzymes such as LiP, MnP, and laccases, as well as secondary oxidants produced by radical cascades. Oxidizing species are represented in red. (b) The current model for lignin degradation involves enzymatic generation of the radical mediator, which can then diffuse to the lignin substrate and transfer the oxidizing equivalent to the polymer. Upon formation of a lignin based radical, bond scission reactions will ensue that lead to depolymerization. (c) Proposed reactions of model dimers leading to formation of observed products in fungal ligninases. Adapted with permission, Copyright (2001) American Chemical Society.[30]**

2.4.2. Lignolytic Enzymes

There are several literature reports of bacteria that can break down lignin,[35] although the degree of lignin metabolism is not as complete as fungi.[36] For bacteria, the main products are high molecular weight metabolites known as acid-precipitable polymeric lignin (APPL). [17] The gram-positive bacterium, *Streptomyces viridosporus* T7A, was the first reported to show APPL production. This bacterium also shows the presence of extracellular oxidative enzymes during lignin metabolism.[37] Advances in DNA sequencing and spectroscopy enabled further characterization of secreted oxidative systems of other

lignin-reactive bacteria. Genome sequencing of *Amycolatopsis sp.* 75iv2 (formerly *Streptomyces setonii* and *Streptomyces griseus* 75vi2) revealed the presence of several genes that code for heme peroxidases, laccases, and cytochrome P450s. Of importance with respect to the heme enzymes was the observation of gene products associated with the production of extracellular peroxide.[38,39] Peroxide is the oxidant that activates the heme enzymes, which subsequently activate mediators that go on to oxidize lignin (see above).

Using a spectroscopic assay, Ahmad *et al*. identified extracellular enzymes in *Rhodococcus jostii* RHA1 and *Pseudomonas putida*, both of which can degrade lignin.[40] This led to the discovery of dye-decolorizing peroxidases (DyPs) in *R. jostii* RHA1. DyPs are unique heme peroxidases because they feature a distal aspartate instead of the more commonly found histidine. DyPs are so named because of their ability to carry out extracellular oxidation reactions of dyes and other aromatic compounds.[17,42,43] In addition, an *in vitro* study shows that the recombinant DypB catalyzes oxidative C_α-C_β cleavage of a β-O-4 bond in lignin model compounds and can oxidize Mn^{2+}, in analogy to fungal, lignin-degrading manganese peroxidase.[44]

Copper-containing bacterial laccases extract electrons from organic compounds accompanied with the reduction of dioxygen to water. Laccases are prevalent in actinobacteria,[45] and can be found in α-, β- and γ-proteobacteria.[34] Laccases from *Streptomyces coelicolor* A3(2*), Streptomyces lividans* TK24, *Streptomyces viridosporus* T7A, and *Amycolatopsis sp.* 75iv2 have received the most attention. These enzymes were found to catalyse C_α oxidation of lignin model compounds.[46] Emerging genomic data reveal that laccases are widespread among bacteria, and can play roles in pigmentation, sporulation, and metal tolerance.[47] Some laccases from the bacterium *Thermus thermophilus* offer advantages such as long half-lives at high temperatures (over 14 h at 80 °C[48]) and a halotolerant laccase from *Streptomyces psammoticus* displays activity under alkaline conditions.[49]

β-etherases are newly discovered enzymes from bacteria that can cleave the β-aryl bonds in the presence of glutathione. Studies were carried out on a selection of lignin model compounds and fluorescently labelled polymeric lignin substrates, specifically using *Sphingobium* SYK-6 and *Novosphingobium* strains.[3] A summary of the currently known

bacterial enzymes that degrades lignin is presented in Table 2.1, with the majority reported to be active with lignin model compounds rather than polymeric lignin.

Table 2.1 Summary of bacterial enzymes for lignin breakdown.

Enzyme	Bacteria	Cofactor	Co-substrate	Low MW substrates	Lignin model compounds		Polymeric lignin		Ref
					Substrates	Reaction	Substrate	Products	
DypB	*Rhodococcus jostii RHA1*	Heme Fe	H_2O_2	ABTS, Mn(II)	β-O-4	Cα - Cβ cleavage	Kraft lignin	33	
	Pseudomonas fluorescens	Heme Fe	H_2O_2	ABTS, Mn(II)	NR		Lignocellulose	Lignin dimer 50	
sDyp2	*Amycolaptosis sp 75iv2*	Heme Fe	H_2O_2	ABTS, Mn(II)	β-O-4	NR	NR		51
Laccase	*Streptomyces coelicolor*	Cu	O_2	ABTS, DMP	β-O-4	Cα oxidation	Ethanosolv	Higher MW 46	
CopA	*Pseudomonas stutzeri*	Cu	O_2	ABTS, DMP	β-O-4	NR	HP lignin	Aromatic 52 monomer	
Etherase	*Sphingobium SYK6*		Glutathione		β-O-4	β-Ether cleavage	NR		53
	Novosphingobium sp.		Glutathione		β-O-4	β-Ether cleavage	Fluorescent lignin		54

NR, not reported
MW, molecular weight

2.4.3. Biodegradation of recalcitrant lignin biphenyl fragments

Degradation of aromatic compounds by bacteria via oxidative pathways is well studied. The primary reactions are *ortho*-cleavage and *meta*-cleavage of catecholic intermediates. For the lignin fragments, catabolic pathways produce vanillin (and related compounds) in *Sphingobium sp*. SYK-6 (Figure 2.4).[33,6] Of special importance is the lignin biphenyl component, which can account for more than 10% of the structure depending on the lignin source. Bacterial biphenyl degradation by genera such as *Sphingomonas, Burkholderia, Rhodococcus, Pseudomonas, Achromobacter, Comamonas, Ralstonia, Acinetobacter*, and *Bacillus* is well documented.[55] Compounds related to biphenyl and polychlorinated biphenyl (PCB) can be degraded by bacteria such as *R. jostii* RHA1 and *Burkholderia xenovorans* LB400. The products of degradation from those bacteria are chloro-benzoate and 2-hydroxypenta-2,4-dienoate.[56] The lignin model biphenyl compound

5,5'-dehydrodivanillate is also transformed to vanillate by *Sphingobium sp*. SYK-6 using a set of at least 5 catabolic genes (Figure 2.4).[33,57]

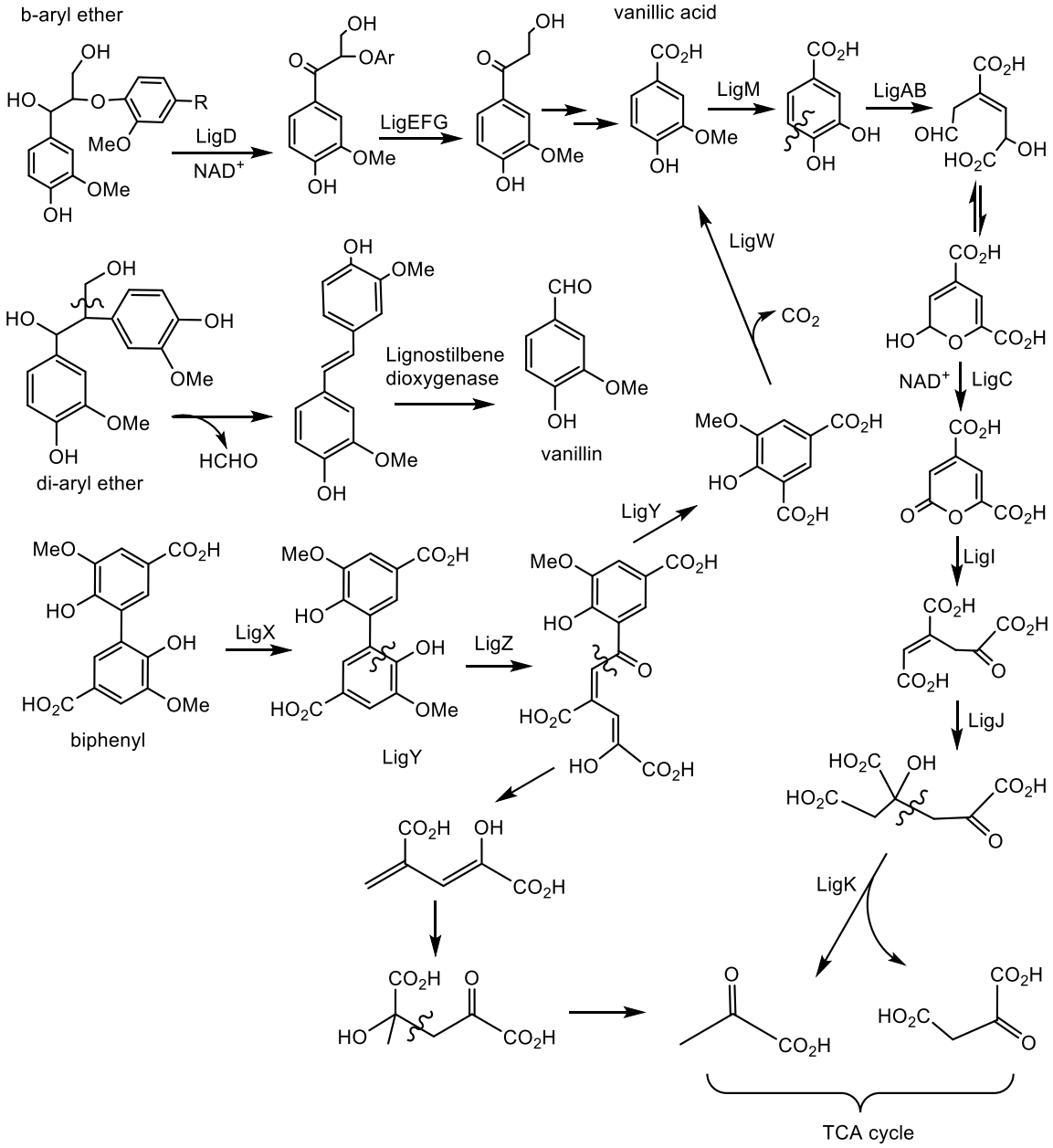


Figure 2.4 Pathways and gene products identified in *Sphingobium sp*. SYK-6 for degradation of lignin components. Adapted with permission from Elsevier.[33]

As noted above, lignin structures are complex. This hinders studies of natural or processed lignin and many researchers have used low molecular weight lignin model compounds for detailed study. The use of lignin model compounds is justified by several

features. First, they contain linkages that are analogous to those found in lignin. As such, it is thought that study of reactions of models can yield insight into the degradation and reaction of the polymer structure as a whole. Second, structurally related compounds (either as monomers or dimers) are often found in lignin streams after pre-treatment, so methods for their valorization to high value chemicals are therefore relevant for the bioprocess integration.[58] This will be of great help in evaluating the concentration and toxicity of lignin-derived aromatic molecules present in the product stream during the fermentation process.[6,59] Finally, the use of model compounds present far fewer analytical challenges in comparison to the complex lignin polymer. Because only one type of linkage is often present in the model compound, systematic analysis of the reaction paths, and thus catalytic efficiency, is greatly simplified.[58]

2.5. Recovery of metabolic products of lignin degradation

Downstream bioprocessing of lignin product streams requires consideration of three groups of metabolic products: (1) intracellular products, (2) extracellular water-soluble products, and (3) extracellular phase-separating products.[6]

The intracellular metabolites are high molecular weight compounds produced and stored in the microbial matrix. Examples include fatty acids, carotenoids, and triacylglycerides. Recovery of these internal metabolites is carried out by collecting cells and removing from the growth media. Cells are disrupted using chemical, enzymatic or mechanical means and the products are collected and separated.[60,61] With respect to lignin utilization, monomeric and oligomeric lignin residue may be problematic if they are carried over during product recovery, requiring costly cleanup steps (see Chapter 1 for additional discussion in the context of the overall bioprocess).[6]

Extracellular metabolic products can be soluble or insoluble. The soluble fraction is typically derived from lignin via oxidative pathways. Products include catechol, muconate, protocatechuate, vanillin, and metabolites from the tricarboxylic acid cycle. These compounds can be separated from the supernatant (after pelleting cells) by overcoming the target molecule's hydrophilicity.[6] Techniques such as chromatography, membranes, reactive distillation, crystallization, or solvent extraction are applied to recover the desired compound.[61] This step is challenging due to interference from other

metabolites or media components that have similar physicochemical properties (e.g., solubility, pK_a, partition coefficient) as the compound of interest.[6]

Finally, extracellular phase-separating or insoluble products, such as fatty acids and derivatives (e.g., fatty alcohols, fatty esters, long-chain hydrocarbons), and isoprenoids derivatives (e.g., isoprene, farnesene, bisabolene), can be recovered based on the hydrophobicity of the molecule of interest.[6] In analogy to the soluble fraction, interaction between the desired products and broth components (salts, lipids, proteins) remain a challenge for effective separations.[62] In general, separation strategies for lignin-derived chemicals must account for the energy, operational, and capital costs in an economically and environmentally favourable manner to justify its further development and scale-up beyond the bench.[42]

This work described in the Chapter investigates the capability of the newly discovered *Burkholderia xenovorans* strains to degrade polymeric lignin, using commercial alkali lignin on a laboratory scale. The strains were also tested on the aromatic compounds anthracene, biphenyl, and benzoate.

2.6. Materials and methods

2.6.1. Chemicals and media

Alkali lignin, as a representative of Kraft process lignin, was purchased from Sigma-Aldrich (St. Louis, MO) with a purity of 99%. All solvents and chemicals used were obtained commercially and were of high purity grade.

Luria-Bertani (LB) medium (pH 7.0) contained 10.0 g/L tryptone, 5.0 g/L yeast extract, and 5.0 g/L NaCl (from Bioshop Canada Inc. Burlington, Ontario). The M9 salt or minimal medium contained 6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 1 M $MgSO_4$, 1 M $CaCl_2$, 100 mM thiamine, 0.03 % glucose and 10 mL of trace element solution (5 g/L Na_2EDTA, 0.5 g/L $FeCl_3.6H_2O$, 0.05 g/L $ZnCl_2$, 0.01 g/L $CuCl_2$, 0.01 g/L $CoCl_2.6H_2O$, 0.01 g/L H_3BO_3, 1.6 g/L $MnCl_2.4H_2O$). All media were prepared under sterile conditions by autoclaving and using sterile syringe filters.

2.6.2. Bacteria strains, culture conditions and measurement of growth

Burkholderia xenovorans isolates suspected to show biopolymer degrading activity were provided by the Linington lab. The *Burkholderia* isolates were obtained from sites in southern British Columbia and were screened based on phylogenetic analysis of the 16S ribosomal RNA (more information is presented in Appendix A). Phylogenetic analyses reveal that these new strains share similar history with the known polychlorinated biphenyl (PCB) degrader *B. xenovorans* LB400.[63] The *Burkholderia* species used in this work are the *B. xenovorans* (U86373) and *B. phytofirmans* (AY497470). *B. xenovorans* strains include: RL17-329-BIC-A, RL17-337-BIC-C, RL16-009-B52D-A, RL16-009-BSJ-nal-C, RL16-009-BSH-A, in which their lignin and aromatic compound degrading ability were examined.

To test if the bacteria strains can grow on, and degrade, lignin alkali and a number of aromatic compounds, the *B. xenovorans* strains were grown in LB medium under overnight incubation at 30 °C and 180 rpm using Stuart orbital incubator SI500. Cultures were collected in the mid-log phase and centrifuged at 3000 rpm for 5 min using Thermo Scientific Sorvall Biofuge Primo. Cell pellets were resuspended in M9 media and used to inoculate the substrate solutions. Stock solutions of lignin and each aromatic compound were prepared in dimethyl sulphoxide (DMSO) at a concentration of 100 mg mL^{-1}. M9 media was supplemented with the lignin and individual aromatic compounds to achieve a final concentration of 1 mg mL^{-1}. A total of 5% of the M9 culture of each bacteria strain was used to inoculate the M9 media containing 1 mg mL^{-1} lignin, or individual aromatic compounds as the sole carbon source. Control cultures included: (1) M9 media with 1 mg mL^{-1} of lignin and aromatic compounds without bacteria inoculum and (2) M9 media without lignin and aromatic compounds, but with bacteria inoculum. Solutions were left to shake (180 rpm) at 30 ^{o}C for 6 days, and samples were removed daily from 0 to 6 days. All bacteria were grown under aerobic conditions. Bacterial growth was monitored with measurement of optical density of the culture at 600 nm (OD_{600}) using the Cary100Bio UV-Visible spectrophotometer.

2.6.3. Sample preparation for HPLC analysis

Samples (1 mL) were collected and centrifuged at 12,000 × g for 5 minutes to obtain cell-free culture supernatants. The supernatant was subjected to two phase organic solvent extraction. The supernatant was thoroughly mixed with an equal volume of ethyl acetate (at pH 7) by vortex mixing. The organic layer was collected, and the remaining aqueous layer was acidified to pH 2-3 with 1 M HCl (30 µL) and then thoroughly mixed with two volumes of ethyl acetate. Again, the organic and aqueous layers were separated. The organic layers (collected from acidic and neutral phase extractions) were combined and evaporated to dryness with rotary evaporator (Buchi Rotavapor R 110, Switzerland). Dried samples were concentrated by dissolving in 200 µL of 30 % acetonitrile in water for HPLC analysis.

2.6.4. High performance liquid chromatography (HPLC)

HPLC analysis was performed on an Agilent 1200 Series system (Agilent Technologies, Cheshire, UK), equipped with a photodiode array detector and a multimode source for 6100 Series single quad LC/MS (G1978B) electrospray ionization mass spectrometer (ESI/MS) system. Both detectors are coupled to an Agilent Chemstation (version B.04.03) for data processing**.** The organic residue was re-dissolved in 200 µL of 30 % acetonitrile/70 % water, and 20 µL of sample was injected for each sample onto a reversed-phase column Synergy 10u Fusion-RP 80A (250 mm x 4.6; 5 µm particle). Solvent B was 99.6% water/0.4% formic acid (v/v) and solvent C was 100% methanol. The flow rate was 1 mL min^{-1}. The linear gradient for solvent C was as follows: 0 min, 25%; 10 min, 95%; 15 min, 98%; 20 min, 98%; 25 min, 25%. The identity of each compound in the chromatogram was established based on their masses shown in the mass spectral window.

2.7. Results

2.7.1. Growth of the *B. xenovorans* strains on the lignin substrate

The growth of *B. xenovorans* strains RL17-329-BIC-A, RL17-337-BIC-C, RL16-009-BSH-A and RL16-009-B52D-A on lignin substrate was evaluated for six days based on OD_{600} measurement (Figure 2.5). All the strains displayed almost similar growth pattern

and their maximum growth was attained after around 24 hours incubation. The *B. xenovorans* growth on M9 control culture without lignin and cultures with lignin were similar.

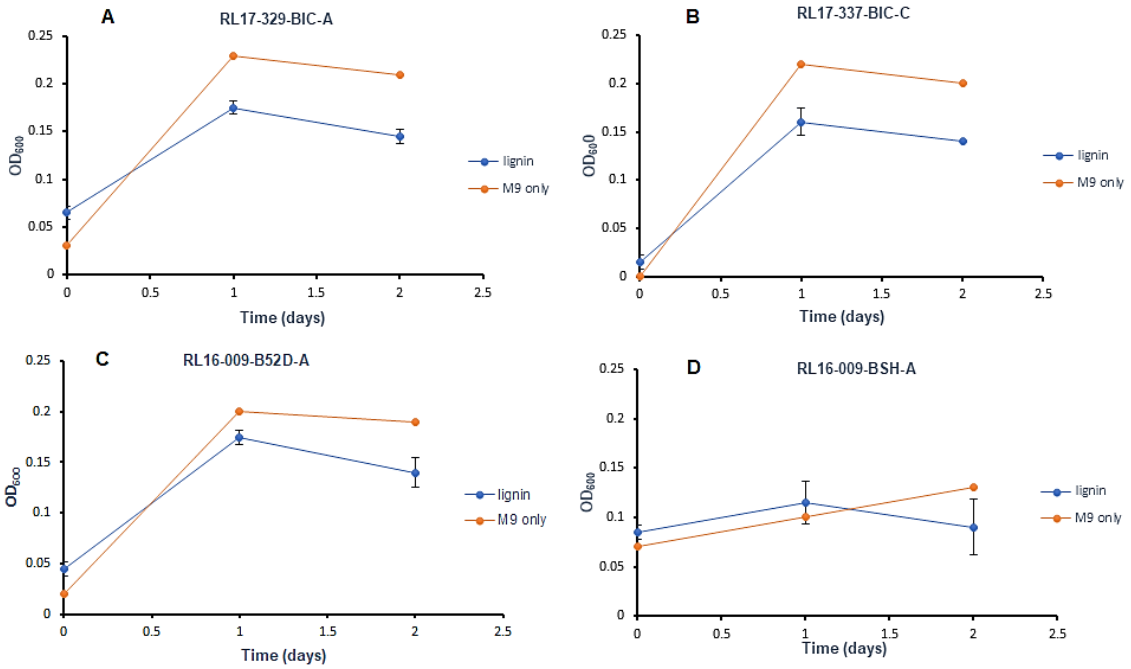


Figure 2.5 Growth curves of the *B. xenovorans* strains on M9 medium containing 1 mg mL^{-1} of lignin monitored by OD_{600}. Error bars indicate the standard deviation for 3 number of experiments.

2.7.2. Small scale lignin degradation trials

To verify whether the *B. xenovorans* strains were able to degrade lignin, their cultures were subjected to HPLC analyses. Aliquots were removed at regular intervals and analyzed by reverse phase HPLC. A number of peaks were observed presumably corresponding to lignin fragments. It was found out that the HPLC chromatograms obtained from the lignin control culture and the bacteria cultures remain essentially unchanged with time (Figure 2.6). This observation, together with the growth curve (Figure 2.5), imply that the *B. xenovorans* strains were unable to degrade and grow on the alkali lignin. The only exception is organism RL-17-337-BIC-C, which showed some activity on lignin (note changes to the LC peak at retention time 7.67 minutes). These efforts were abandoned due to the very poor growth with lignin as the carbon source.

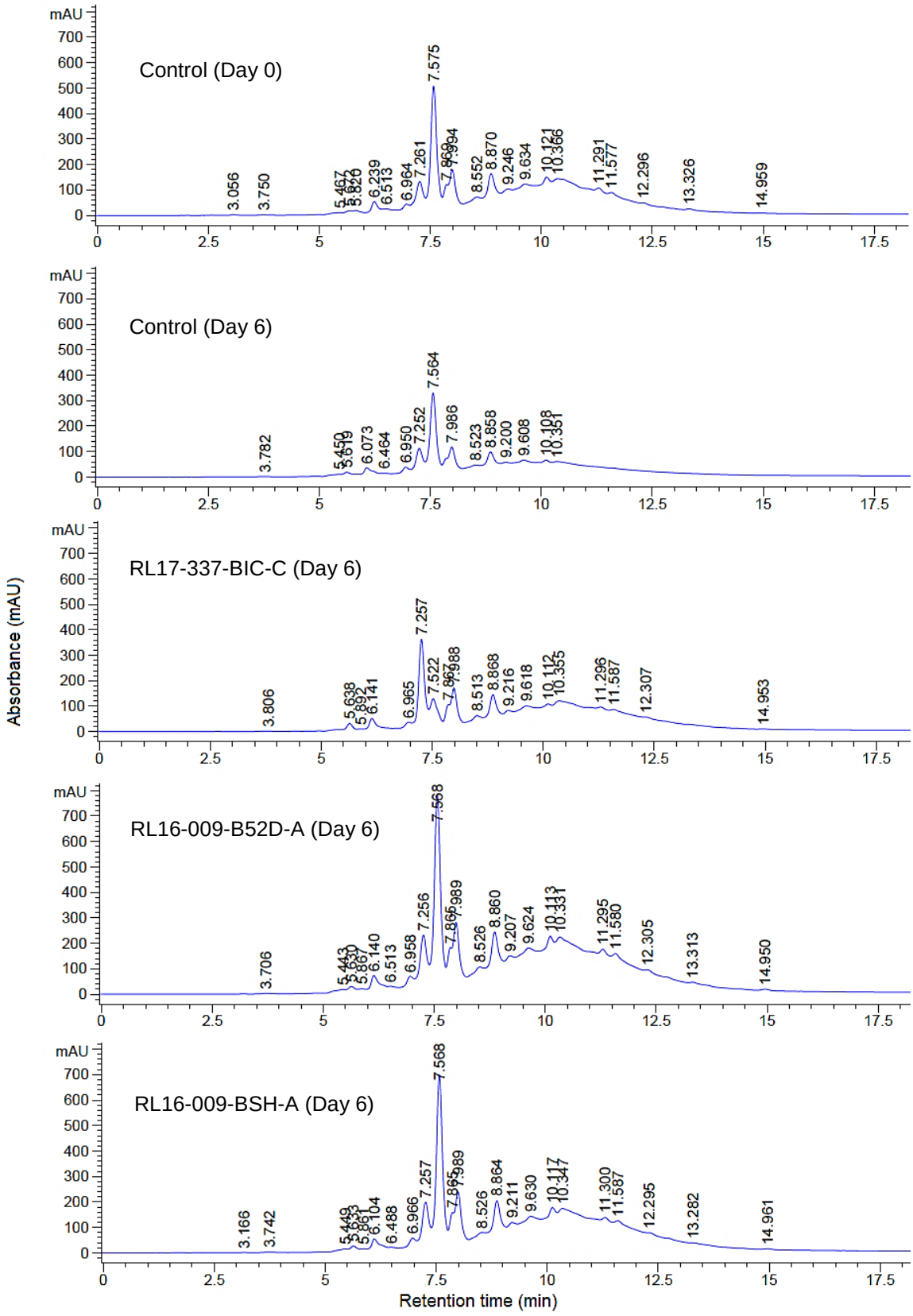

Control (Day 0)
Control (Day 6)
RL17-337-BIC-C (Day 6)
RL16-009-B52D-A (Day 6)
RL16-009-BSH-A (Day 6)
mAU
Absorbance (mAU)
Retention time (min)

Figure 2.6 HPLC chromatograms showing lignin (1 mg/mL) fragments for control (a) and in *B. xenovorans* strains (b) samples at day 6. UV detection at 280 nm.

2.7.3. Growth of the *B xenovorans* strains on aromatic compounds trials

To verify whether the *B. xenovorans* strains were able to grow on the selected aromatic compounds such as anthracene (a common environmental pollutant), biphenyl, and benzoic acid. These compounds serve as lignin model compounds and are also known environmental pollutants. The growth experiments were conducted in same manner as with the alkali lignin using liquid cultures in M9 media. The results show that the *B. xenovorans* strains grew on the benzoic acid cultures, but no growth was observed for the anthracene and biphenyl cultures as shown in Table 2.2. The degradation analysis of the *B. xenovorans* on benzoic acid is detailed in Chapter 3.

Table 2.2 Growth of B. xenovorans on M9 Medium containing aromatic compounds (1 mg mL^{-1}) monitored by OD_{600}

Strain		Substrate	
	Anthracene	Biphenyl	Benzoate
RL17-329-BIC-A	–	–	+
RL17-337-BIC-C	–	–	++
RL16-009-B52D-A	–	–	++
RL16-009-BSH-A	–	–	+
RL16-009-BSJ-nal-C	–	–	+

\+ bacteria growth
– no bacteria growth
++ high level of growth at 20 -24 hours

2.8. Discussion

Inspection of the HPLC chromatogram shown in Figure 2.6, shows some fragment peaks in the control sample without bacteria inoculum. This demonstrates that the alkali lignin used contains some fragments whose masses are in the monomeric and dimeric range. The observed chromatograms were the same for both the control cultures and the *B. xenovorans* cultures. There were no noteworthy new peaks after 6 days of incubation in cultures containing the *B. xenovorans,* so we conclude that new fragments were not

produced via bond cleavage by the bacteria. Also, since no changes were observed in the fragment peaks with time, it shows the monomeric or oligomeric units are not substrates for the *B. xenovorans* strains. It has been shown that Kraft lignin often contains greatly reduced β-O-4 content and high presence of biphenyl (5-5) linkages. The 5-5 content are highly refractory as they typically survive, and are even formed, during the Kraft pulping process.[3,28] Thus, the alkali lignin is thought to be structurally modified during the pre-treatment process giving rise to highly crosslinked, heterogeneous structure that may prevent easy access of microbial enzymes.[64] Alkali lignin is not considered as representative of the native lignin structure and chemistry.

Our *B. xenovorans* strains are known to share genetic history based on 16s rRNA sequence with the PCB degrader *B. xenovorans* LB400. However, phylogenomic analysis may not perfectly represent the evolutionary history.[65] The phylogenomic results can be brought into question by further study (e.g., gathering of additional data, analyzing the existing data with improved methods).[65,66] The mechanism of microbial lignin breakdown is similar to those of multiple aromatic compounds (Figure 1.8). Other bacteria are thought to breakdown lignin by releasing lignolytic enzymes that cleave the lignin bonds to produce lower molecular weight compounds. The resulting lower molecular weight compounds or fragments can be further modified, and then are taken up to produce metabolic processes such as triacylglycerides or polyhydroxyalkanoates depending on the microbe, which are used for growth under favourable conditions.[6,36] The growth curves in Figure 2.5 indicate that the *B. xenovorans* strains only utilize the nutrients present in the M9 media, thereby, showing minimal growth with OD_{600} around 0.2. The results show that no lignin carbon was utilized, because no further growth was observed in the cultures containing lignin and the *B. xenovorans*.

The *B. xenovorans* strains were unable to grow in cultures of anthracene and biphenyl but were able to grow in cultures containing benzoate within 24 hours, as shown in Table 2.2. This suggests that the *B. xenovorans* strains are not specific degraders of biphenyl bonds, either as a model compound or as biphenyl linkage present in the polymeric lignin. To be sure if our *B. xenovorans* strains can degrade PCBs, an experimental verification is required. Follow-up studies to characterize the benzoate degradation of the *B. xenovorans* strains are presented in Chapter 3.

2.9. Conclusions

Although our new *B. xenovorans* strains may be genetically related to *B. xenovorans* LB400, but they did not show delignification activity on alkali lignin. Furthermore, the alkali lignin contains fragments which are not potential substrates for the *B. xenovorans* strains. Additional growth tests carried out on aromatic compounds such as anthracene, biphenyl, and benzoic acid, show that these strains were only able to grow on benzoic acid, as discussed later in this thesis.

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Chapter 3.

Biodegradation of benzoate by newly isolated *Burkholderia xenovorans* strains

3.1. Introduction

Approximately 25% of the Earth's biomass is comprised of aromatic compounds. The only organic compounds found in higher abundance are carbohydrates. Owing to the inherent kinetic stability (toward bond activation/oxidation at ambient temperature) of aromatic rings, their rate of non-biological depletion is relatively slow. However, many organisms have evolved bio-degradative capabilities to utilize wide variety of aromatic compounds in biological processes. Plant-derived aromatic compounds, such as lignin, are widely distributed in natural systems and bacteria and fungi have evolved ways of utilizing such substrates.[1] Man-made aromatic pollutants, however, pose major challenges to the environment. The compounds are, in many cases, not easily degraded naturally owing to their chemical complexity and thermostability.[2] Examples of petroleum-derived pollutants released in the environment include biphenyl and polychlorinated biphenyls (PCB), polycyclic aromatic hydrocarbons (PAHs), and simpler aromatics such as benzene, toluene, ethylbenzene and xylenes (BTEX). Man-made pollutants include pesticides, herbicides, and flame retardants.[3,4] The presence of such compounds in the environment often poses a serious risk to both ecosystem function and human health. Although anthropogenic activity is a main source of these contaminants, they also can form during natural events.[5]

Bacteria are commonly implicated in the degradation of chemical pollutants. Many of the principal pathways and associated enzymes involved in the degradation processes of common pollutants have been described.[6,7] The ecological importance of characterizing such bacterial metabolic pathways is increasingly evident. One great example is the potent PCB-degrading organism *B. xenovorans* LB400 (abbreviated as LB400).[2] To fully understand the process of PCB degradation in LB400, a detailed description of its benzoate degradative pathways was performed.[2,8] Not only were key intermediates identified, but the production of those compounds was correlated with the activity of specific enzymes. The intermediates found during degradation of many aromatic

compounds (e.g., PCBs) include benzoate,[9] which motivated us to explore benzoic acid as a model of aromatic degradation in newly described *B. xenovorans* strains.

3.2. Benzoic acid and its derivatives

Benzoic acid is the simplest aromatic carboxylic acid. It can be found in plant and animal tissues and is produced by microbial fermentation of certain food products. Benzoic acid is synthesized industrially and commonly included as a preservative in food, cosmetics, hygiene products, and other pharmaceuticals. Furthermore, It is used as an additive, nucleating agent, intermediate, stabilizer and/or catalyst in the coolant, solvent, photography, plastic, textiles, pesticide, paper, and dye industries.[10] Derivatives of benzoic acid, such as the sodium, potassium, and calcium salts, alkyl benzoate esters, and hydroxybenzoate esters (parabens) can be naturally found and/or chemically synthesized, and are widely used in different industrial sectors.[10]

Benzoic acid is a colorless aromatic compound with a melting point of 122.4 °C, a boiling point of 249.2 °C. The pK_a of its carboxylic group in water is 4.19 at 25 °C. In its neutral form, it has meager solubility in acidic water and good solubility in polar organic solvents. Upon deprotonation, the aqueous solubility greatly increases.[11] Benzoic acid was originally obtained by sublimation of benzoin resin, but currently it is industrially produced by aerobic oxidation of toluene catalyzed by cobalt- or manganese-containing catalysts.[10]

Benzoic acid and its derivatives are released into the environment due to their widespread production and use, often resulting in pollution of water, soil, and the atmosphere. In one example contaminated site, the concentration of benzoic acid ranged from 0.01 to 27.5 mg L^{-1} in the groundwater at up to 12 m in depth.[12] The depth at which pollution is found highlights the problem of high mobility of water-soluble pollutants. The toxicology and adverse effects of benzoic acid are debated. Some toxic and adverse effects have been reported and the topic has been extensively reviewed.[10, 13,14,15] Due to daily exposure to benzoic acids, the Food and Agriculture Organizations and World Health Organizations (FAO/WHO) established an acceptable daily intake (ADI) of equal to or less than 5 mg kg^{-1} body weight per day (expressed as benzoic acid equivalents) for benzoic acid, benzoate salts, benzaldehyde, benzyl acetate, benzyl alcohol, and benzyl benzoate.

This value was confirmed in 2001[16] and is still in effect. Anything beyond the established ADI may be detrimental to human health.

Bioremediation (section 1.5) can be effective in addressing pollution problems in aquatic and soil surroundings caused by aromatic compounds. Many aerobic benzoate-degrading bacteria have been isolated and the pathways for the aerobic degradation of benzoate are well established.[7,11] The focus of this Chapter is the investigation of the benzoate degradation pathway by the newly isolated *B. xenovorans* strains.

3.3. Benzoate catabolic pathway in *B. xenovorans* LB400

Genomic, transcriptomic, and proteomic studies have been influential in defining the metabolic processes in the benzoate degradation chemistry of *Burkholderia xenovorans* LB400.[8] Based on genomic analyses, LB400 encodes two benzoate catabolizing pathways: a benzoate-catechol (*ben-cat*) pathway and the more recently discovered benzoate oxidation (*box*) pathway, which is present as two paralogues. The two *box* pathways are encoded by gene clusters in a plasmid and in a chromosome.[2,17] The mechanism of the *box* pathway is different from the *ben-cat* pathway, though they both result in common succinyl and acetyl coenzyme A (CoA) products. The products are used as metabolic fuels in the tricarboxylic acid cycle[1] (cf. section 1.6.2.1).

The *ben-cat* and *box* pathways exhibit some differences that are worth noting. Each intermediate in the *box* pathway exists as a CoA thioester[17,18,9] and only the *box* pathway is intimately involved in PCB degradation in LB400.[2,17] The number of dioxygenases encoded also is different, with the *ben-cat* pathway encoding two dioxygenases, and the *box* pathway encodes only one.[2] The two pathways also have different oxygen requirements, where the aromatic bond cleavage reactions in the *box* pathways is oxygen independent. Finally, the organism-level mechanism of regulation and substrate specificity are different.[2] The overall reaction process involved in the *ben-cat* and in the *box* pathways in LB400 is presented in Figure 3.1.[9]

The *box* pathway is not unique to LB400; the bacterium *Azoarcus evansii* also metabolizes benzoate using *box* enzymes and a mechanism has been proposed.[19,20] Analogous investigation of the *box* pathway from *B. xenovorans* LB400 have also been carried out.[2] Comparing the *box* pathway reports on *A. evansii* and LB400, respectively,

shows that the presence of the two *box* pathways in LB400 is unique.[2,17,18,19,21] This is crucial because *A. evansii*, for example, is not capable of degrading PCBs and thus the *box* pathway is likely to have evolved differently than the *box* pathway in LB400. The purpose of the research described in this Chapter is to investigate the intermediates of benzoate-degradation by the new *B. xenovorans* strains, thus providing insights into the pathway of benzoate metabolism. Investigation of different aromatic substrates is used to distinguish the possibility of different *box* pathways, in analogy to the above studies.

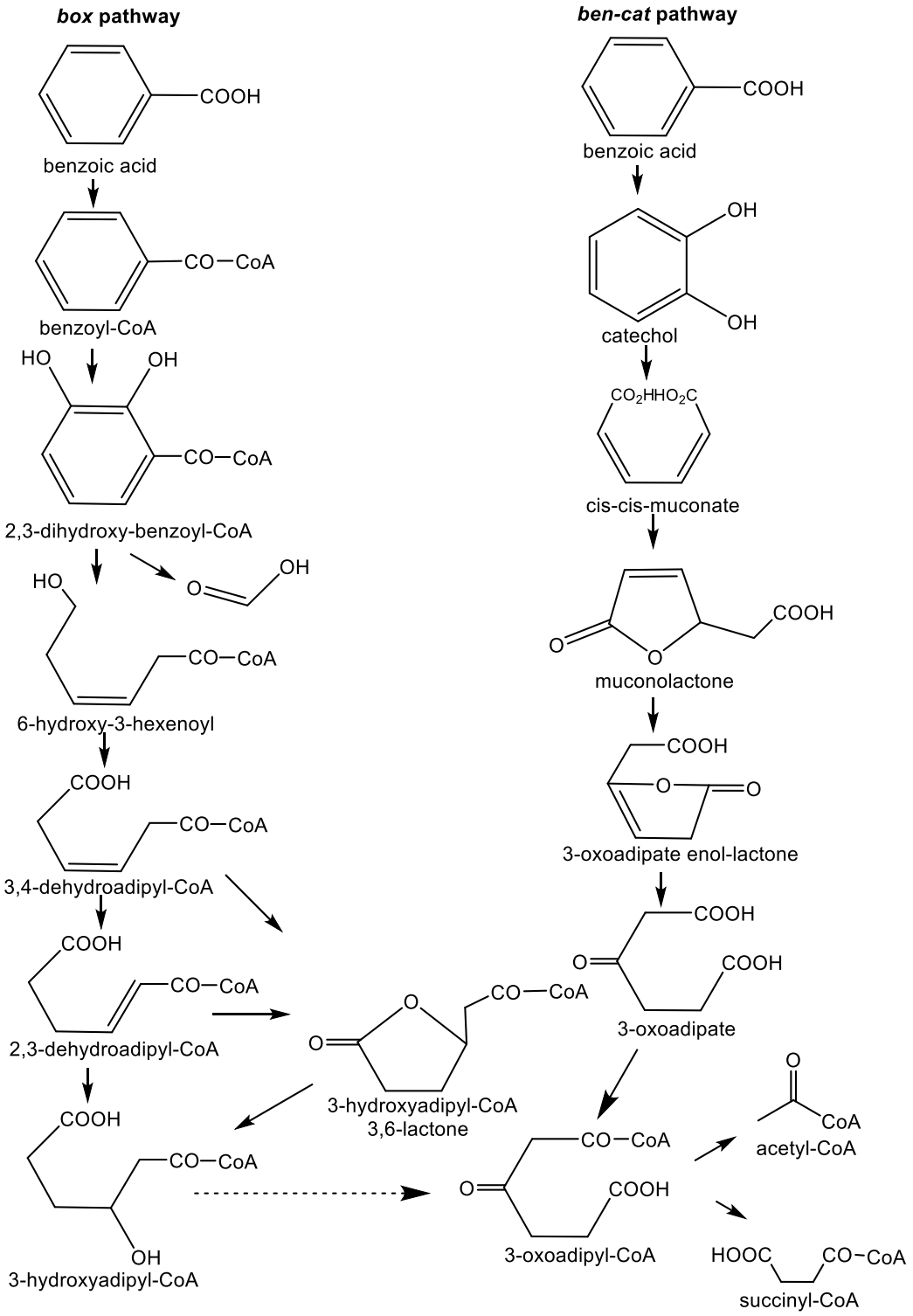

Figure 3.1 **The two different benzoate pathways in B. xenovorans LB400. Adapted with permission from the American Society for Microbiology.[9]**

3.4. Techniques for studying the aerobic degradation of benzoate

Techniques for the study of degradative pathways are well established.[22] Several technological developments have been made with the help of more sophisticated specialized analytical equipment, as outlined below. Standard experimental methods are largely involved with the identification of metabolic intermediates, by analyzing the soluble compounds secreted into the growth medium.[23] Labelling experiments, involving the use of heavy isotopes (^{2}H, ^{13}C) or radioisotopes, have been used to determine the fate of particular substrates,[24] and in quantitative and qualitative determinations of aromatic metabolites.[25] Spectrophotometry and gas chromatography (GC) are useful in the identification of intermediates, and chromatographic techniques such as size exclusion and HPLC have been used widely to identify, quantify, and isolate metabolites.[26,27,28] Ideally, compounds identified using HPLC or GC should be conclusively assigned by mass spectrometry (MS)[27] or by comparison to authentic standards. Nuclear magnetic resonance (NMR) is also used in direct analysis of the crude product mixture formed in the reduction of benzoyl-CoA.[25] That approach indicates that NMR is advantageous in analyzing samples directly without the risk of disruption during sample work up. In fact, metabolic analysis can, in some cases, be carried out *in vivo* using intact cells.[23,29] NMR is a powerful tool that will continue to be of importance in identifying the structures of metabolic intermediates.[30]

Degradative pathways observed by both biochemical and chemical analysis provides the basis for proposing mechanisms for bacterial metabolism of a given compound. For example, in previous studies, potential intermediates in benzoate metabolism were tested chemically based on known chemical reactions.[22,33] Likewise, known reactions can provide a means for proposing enzymatic transformations.[23] For example, carboxylation of phenol at the 4 position is analogous to the Kolbe-Schmidt reaction.[34]

3.5. Materials and Methods

All materials and methods including the: chemicals and media, bacteria strains, culture conditions, growth measurement, sample preparation and HPLC analysis for the benzoate degradation experiment were carried out as described in section 2.6. The *B.*

xenovorans strains used for the benzoate degradation are RL17-329-BIC-A, RL17-337-BIC-C, RL16-009-B52D-A, and RL16-009-BSH-A.

3.5.1. Purification of the crude degradation product by HPLC

The purification experiment was carried out with the assistance of Jake Haeckl from the Linington Lab. A scale-up bacteria culture of 1 mg mL^{-1} of benzoate in 1 L M9 media (i.e. 1 g/L) was conducted for the purification experiment. The dried crude benzoate degradation products were reconstituted in acetonitrile and centrifuged. The crude products were fractionated using a 250 × 4.6 mm Kinetex 00G-4605-EO C_{18} column (100 Å, 5 µ; Phenomenex, Torrance, California). The gradient used was: 5% MeOH for 5 min, 50% MeOH for 10 min, and 98% MeOH over 10 min. Fractions collected from the HPLC were dried under nitrogen and weighed by mass difference to obtain their recoveries before NMR analysis.

3.5.2. ^{1}H NMR spectroscopic analysis

Samples for NMR analysis were prepared by dissolving the dried collected fractions in deuterated chloroform ($CDCl_3$). The samples were transferred to an NMR tube. ^{1}H NMR spectra were recorded for the metabolic products of benzoate. ^{1}H NMR measurements were performed using the Bruker AVANCE 500 MHz NMR spectrometer (with 8 number of scans) as well as the Bruker AVIII HD 600 with QCI cryoprobe (with number 64 scans). Chemical shifts are reported in ppm relative to the signal of $CDCl_3$ at 7.29 ppm.[35]

3.6. Results

3.6.1. Growth of the *B. xenovorans* strains on benzoate

The growth rate of the *B. xenovorans* strains was monitored spectrophotometrically at OD_{600} for 6 days (Figure 3.2). The control cultures contain bacteria without benzoate (in blue), while the cultures containing bacteria and benzoate are shown in orange. The results show that all the bacteria strains were able to utilize and grow with benzoate as the sole carbon source. As evidenced by differences in growth patterns between the control culture and benzoate culture. Strains RL17-337-BIC-C and

RL16-009-B52D-A showed a maximum growth at 24 hours while strain RL17-329-BIC-A at 48 hours and strain RL16-009-BSH-A after 4 days. Since strains RL17-337-BIC-C and RL16-009-B52D-A displayed a fast and high growth on benzoate, a time-course analysis was performed to determine the maximum growth rate as well as rate of benzoate utilization.

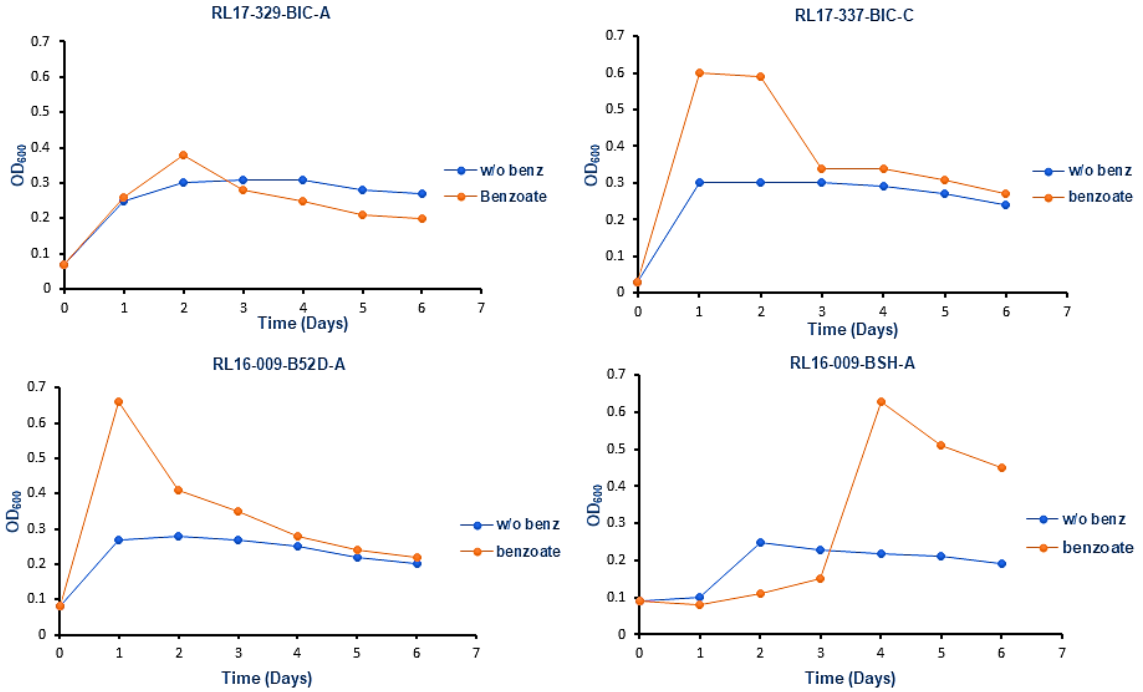

Figure 3.2 **Growth curves of the B. xenovorans strains on M9 medium containing 1 mg mL^{-1} of benzoate (orange) monitored by OD_{600}. Growth in M9 only without benzoate is blue.**

3.6.2. Time-course growth characterization of strains RL17-337-BIC-C and RL16-009-B52D-A on benzoate

The time-course growth analysis of strains RL17-337-BIC-C and RL16-009-B52D-A was carried out as described above except that OD_{600} measurement was taken more frequently (i.e., at 0, 2, 4, 6, 8, 24, 48 and 72 hours). The result is presented in Figure 3.3 (*top*). These data show that both strains display a similar growth pattern. For strain RL16-009-B52D-A, another time-course experiment at 12, 14, 16, 18, 20,22, 24, 48 hours (Figure 3.3 - *bottom*) was carried out in order to ascertain the beginning of the exponential phase. This is a complementary experiment to the previous time-course experiment

(Figure 3.3 - *top*). The results show that the exponential phase for both strains began at 12 hours until 24 hours where the stationary phase was reached.

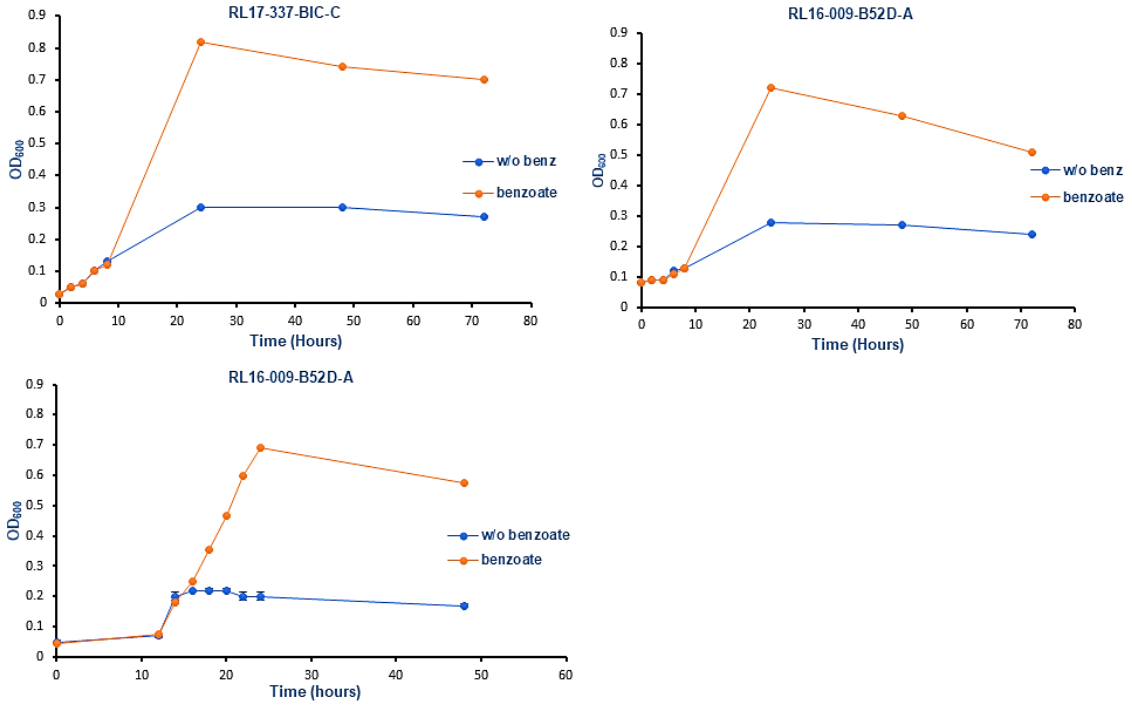

Figure 3.3 **Time-course growth curves at 0, 2, 4, 6, 8, 24, 48 and 72 hours of strains RL17-337-BIC-C and RL16-009-B52D-A (top) on 1 mg mL^{-1} benzoate (orange), and time-course growth curve at 12, 14, 16, 18, 20,22, 24, 48 hours of strain RL16-009-B52D-A (bottom) monitored by OD_{600}. Growth in M9 only without benzoate is blue. The error bars indicate standard deviation of 3 biological replicates.**

3.6.3. HPLC analysis of benzoate degradation by the *B. xenovorans* strains

As a means to test if the bacteria strains were able to utilize and degrade benzoate, their cultures were extracted and analyzed by HPLC, as described above. Aliquots were removed at the 24 h interval and analyzed by reversed-phase HPLC coupled to UV (254 and 280 nm) and MS detectors. The results obtained with chromatographic study show loss of benzoate starting material, which is consistent with the observations from OD_{600} analyses which show greater cell densities when benzoate is added. A decrease in the benzoate peak was observed in the LC chromatograms for all cultures containing benzoate and the bacteria, while the peak for the control culture (with benzoate and no bacteria) remains the same (Figures 3.4 – 3.7). Strains RL17-329-BIC-A and RL16-009-

BSH-A degraded benzoate at a slower rate, within two and four days, respectively. In contrast, strains RL17-337-BIC-C and RL16-009-B52D-A show high level of degradation within 24 h, again mirroring the OD_{600} data. It was also observed that when the benzoate peak in chromatograms began to decrease, new peaks that were tentatively assigned as extracellular metabolites appeared. Representative data are shown in the Figures below. This behaviour was observed for all the *B. xenovorans* strains investigated. These metabolite peaks further decrease over time once the cells reach the stationary phase (based on OD measurements). These observations demonstrate that benzoate was used up by the *B. xenovorans* strains. The chromatographic peaks for the metabolites at the MS detector window were barely detected, making it difficult to directly identify the metabolites by their masses. Poor metabolite detection is a result of poor ionization of these products which may be caused by factors such as pH, sensitivity, or matrix effect (ion suppression).[36]

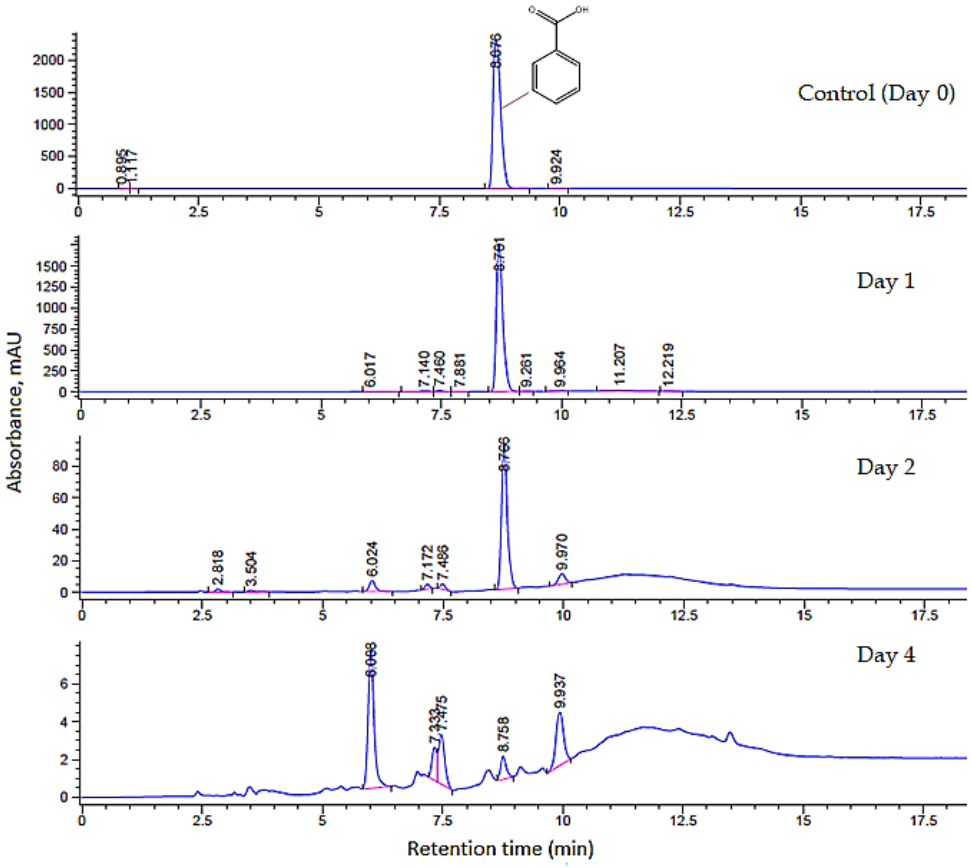

Figure 3.4 **HPLC chromatograms showing degradation of benzoate in strain RL17-329-BIC-A. UV detection is at 280 nm. Benzoate peak is shown at time 8.7 min. Note the differences in y-axis range for each chromatogram.**

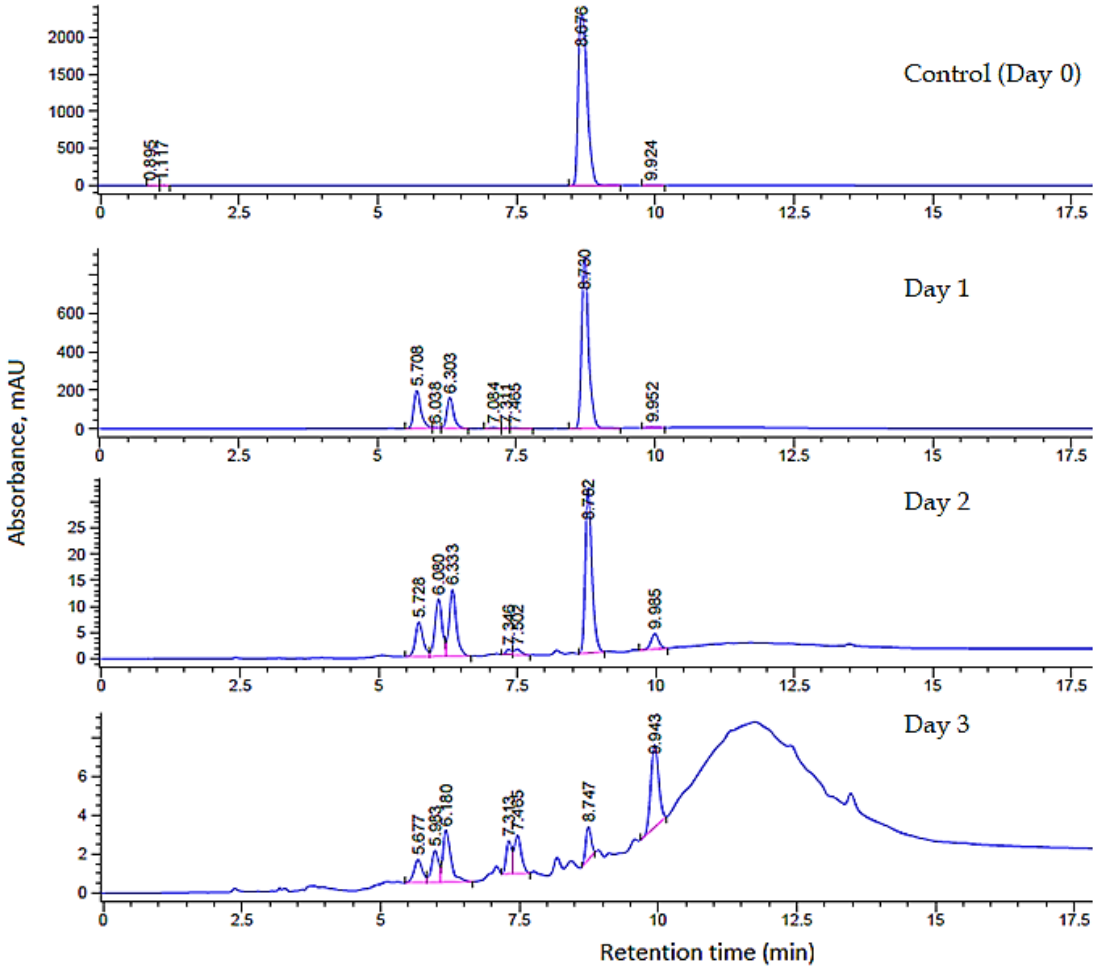

Figure 3.5 **HPLC chromatograms showing degradation of benzoate in strain RL17-337-BIC-C. UV detection is at 280 nm. Benzoate peak is shown at time 8.7 min. Note the differences in y-axis range for each chromatogram.**

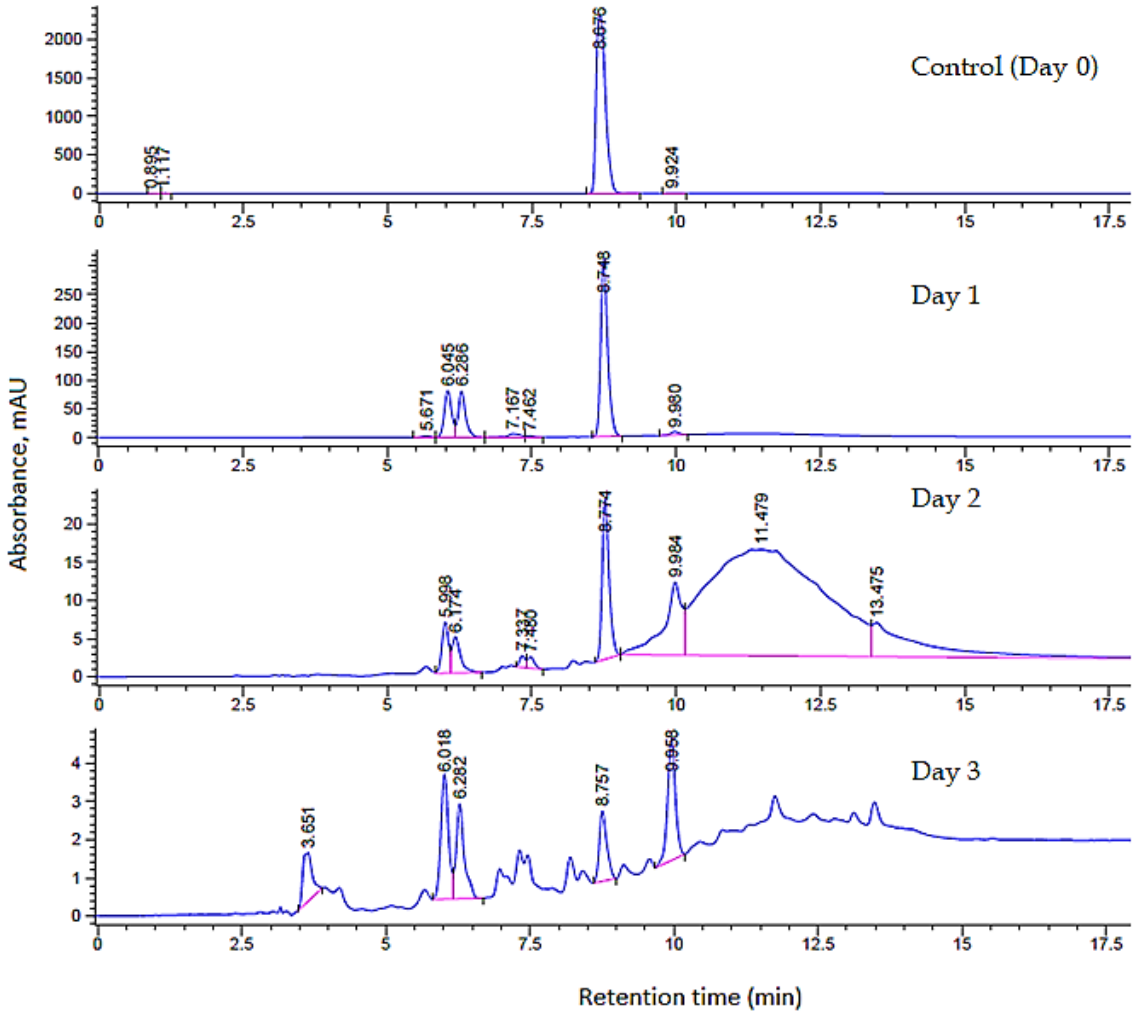

Figure 3.6 **HPLC chromatograms showing degradation of benzoate in strain RL16-009-B52D-A. UV detection is at 280 nm. Benzoate peak is shown at time 8.7 min. Note the differences in y-axis range for each chromatogram.**

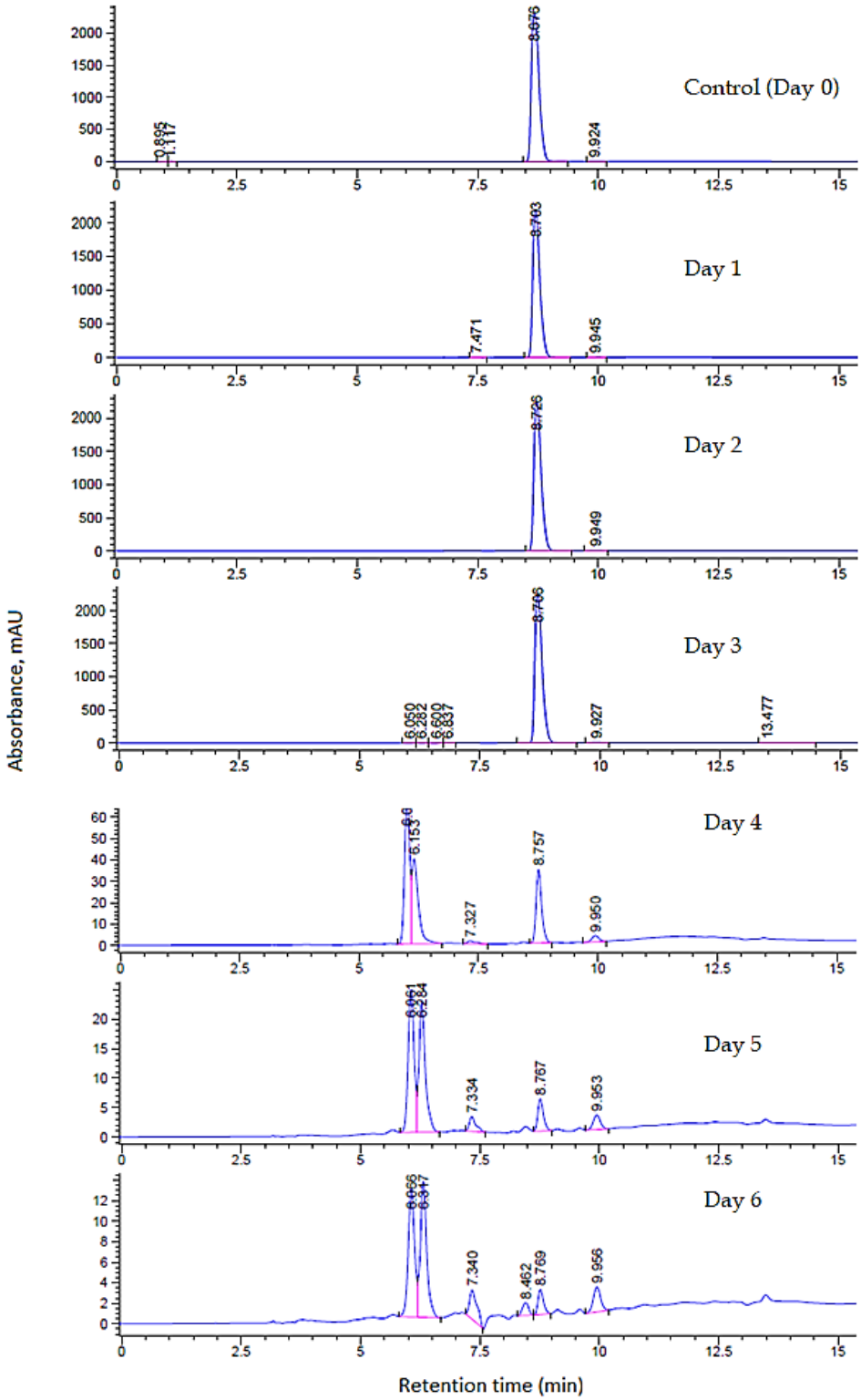

Figure 3.7 **HPLC chromatograms showing degradation of benzoate in strain RL16-009-BSH-A – slow degradation. UV detection at 280 nm. Benzoate peak is shown at time 8.7 min and starts to degrade at day 4. Note the differences in y-axis range for each chromatogram.**

3.6.4. HPLC time-course analysis for benzoate degradation by *B. xenovorans*

For strain RL16-009-B52D-A, time-course HPLC analysis was carried to determine when the benzoate signal starts to decrease, when metabolite peaks begin to appear, and when metabolite peaks decay. Samples were taken for HPLC analysis at 16, 18, 20, 22, 24, and 48 hours and were ran side-by-side with control sample. No change in benzoate peak was observed for control sample. The results show that the benzoate peak began to degrade at 16 h and detectable metabolite peaks begin to appear at 24 hours (Figure 3.8). The metabolite peaks are very low in intensity, and at 48 h (i.e., when the cells have reached the stationary phase), the metabolite peaks had almost disappeared.

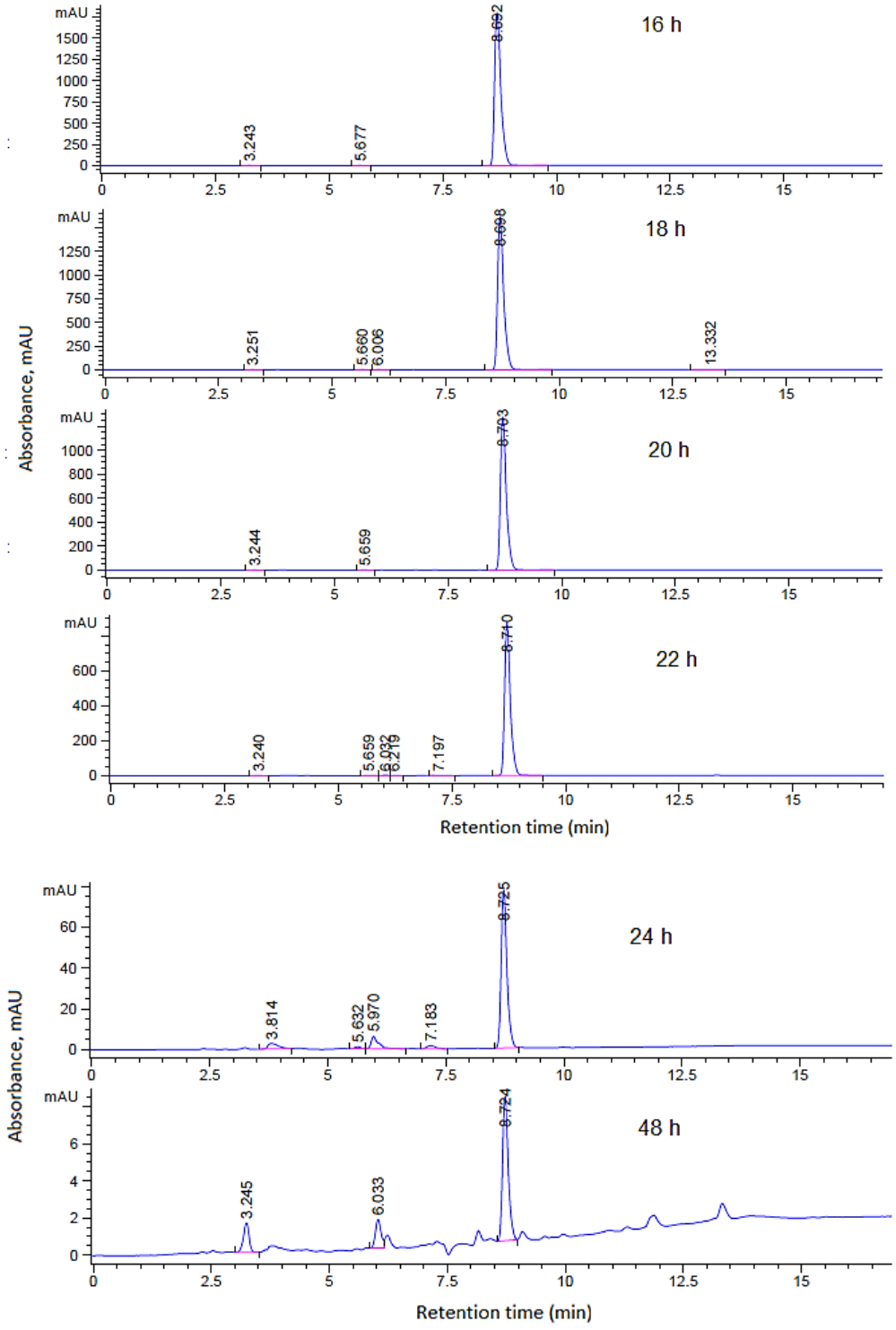

Figure 3.8 **Time-course HPLC chromatograms showing degradation of benzoate in strain RL16-009-B52D-A. UV detection is at 280 nm. Benzoate peak is shown at time 8.7 min. Note the differences in y-axis range for each chromatogram.**

3.6.5. Yields and identification of degradation intermediates

A large culture (1 L containing 1 g of benzoate) was degraded by the strain RL16-009-B52D-A to produce 0.262 g of crude product mixture. About 130 mg of the crude product was taken and analyzed using HPLC. Separation of products by HPLC showed the appearance of metabolites with retention times at: 3.03, 12.07, 16.23, 18.02, and 23.2 min with gradual decrease of parent substrate (benzoate) with retention time of 19.50 min (Appendix B, Figure B1). The major metabolite was identified as catechol (12.07 min) whose ^{1}H NMR spectrum matched with its authentic standard (Appendix B, Figure B2) with a yield of 31.21 mg. Identification of the other metabolic products were unassigned by NMR analysis and their yields are presented in Appendix B, Table B1.

3.7. Discussion

The growth curves shown in Figures 3.2 and 3.3 indicate that the bacteria were able to utilize another carbon source other than glucose in the M9 medium for growth. They attained a maximum OD_{600} of 0.3 in M9 medium, and a maximum OD_{600} of 0.66 in the presence of 1 mg mL^{-1} benzoate. The degradation studies by HPLC confirms that the *B. xenovorans* utilized the carbon source from the benzoate as the amount of benzoate present in the media was reduced. To our knowledge, most studies of benzoate degradation have been conducted at concentrations less than 1 mg mL^{-1}, and most bacteria that have been studied do not effectively grow at this concentration. Interestingly, our new *B. xenovorans* strains, RL17-337-BIC-C and RL16-009-B52D-A, displayed a rapid growth rate and degrade benzoate at 1 mg mL^{-1} within 24 hours at 30°C. Furthermore, the degradation studies reveal the presence of extracellular metabolites that are likely intermediates in the degradation of benzoate. Benzoate degradation by the bacteria strains reveals a similar pattern of biodegradation involving the destabilization of the benzene ring to form intermediates. Intermediate compounds are usually utilized by bacteria for their growth, which can also be observed due to decrease in the metabolite peaks shown in the HPLC chromatograms. The *ben-cat* pathway is most likely demonstrated by the new *B. xenovorans* strain as catechol is the major metabolic product formed and is the central intermediate. There are three well known dihydroxy-aromatic central intermediates that result from biodegradation of aromatic compounds and they include: catechol, protocatechuic acid and gentisic acid.[37] Catechol has emerged as the

most frequently encountered metabolite in most bacteria. The other metabolite peaks shown in low amounts could be because of further catabolism of the catechol via lower pathways (aromatic ring cleavage) to intermediary metabolites (Figure 1.9). The intermediary metabolites were further utilized by the bacteria for their growth. The metabolite at retention time 3.03 min has NMR peaks at lower ppm peculiar to a *n*-alkanes or alkenes with no aromatic peaks (Appendix B, Figure B3) but is inconclusive. Based upon the structure of the observed metabolite, a catabolic pathway for the breakdown of benzoate in *B. xenovorans* RL16-009-B52D-A is shown below:

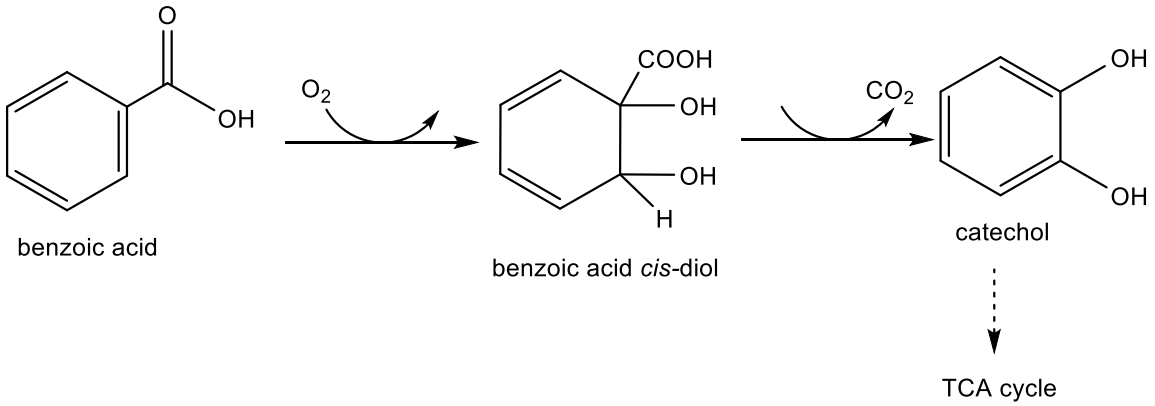

Figure 3.9 Mechanism of benzoate degradation in *B. xenovorans* RL16-009-B52D-A.

The results presented in this Chapter suggest that the *bet-cat* pathway is highly active in these *Burkholderia* isolates. At present, we do not have any evidence that the *box* pathway is active. As noted above, the *box* pathway is implicated in PCB metabolism. The absence of *box* pathway activity could be one reason that we do not observe growth on lignin or biphenyl (see Chapter 2). This is an interesting contrast to the genetically related *Burkholderia* LB400. Further work is needed to delineate the specific genetic and proteomic differences, and how they are related to substrate metabolism, in these two classes of bacteria.

3.8. Conclusions

This study shows that the new *B. xenovorans* strains were able to grow and utilize benzoate as a carbon source. The level of benzoate degradation between these strains differ. A bacteria strain of interest (RL16-009-B52D-A) demonstrated rapid growth in benzoate, and this strain was found to degraded benzoate to produce catechol as the

major catabolic product. The results obtained in this project may prove useful in the enhancement of aerobic degradation of benzoate for bioremediation studies.

3.9. References

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Chapter 4.

Growth of novel *Burkholderia sp.* strains on common petroleum hydrocarbon pollutants

The major source of energy and the raw materials used in products common in daily life are obtained from petroleum. Activities associated with the petrochemical industry have contributed to many of today's major environmental problems, one of which is hydrocarbon contamination of soil and water. The release of hydrocarbons into the environment can be an accident of nature or due to human activities, which are the root causes of water and soil pollution.[1] The different classes of petroleum hydrocarbons known as SARA (saturates, aromatics, resins and asphaltenes) and their biodegradation were introduced in Chapter 1 (sections 1.5 and 1.6.2). Many hydrocarbon components are carcinogens, mutagens, and/or neurotoxic compounds, making amelioration of their release, or remediation, especially important.[2] One of the primary ways by which petroleum and other hydrocarbon pollutants can be removed from the environment is through biodegradation involving natural populations of organisms.[1]

Petroleum products are widely in use in a vast array of industrial and consumer applications. The most common applications that spring to mind are transportation, in the form of gasoline and diesel. This widespread application also means that petroleum components are ubiquitous pollutants. These pollutants contain a complex blend of hydrocarbons making their biodegradation difficult. For instance, gasoline hydrocarbons contain 4 to 12 carbons. In comparison, diesel is a mixture of saturated hydrocarbons (75%, including *n*, *iso*, and cyclo-alkane) and 25% aromatic hydrocarbons.[3,4] Many of the monoaromatics (e.g., ethylbenzene) and *n*-alkanes are known to be readily biodegraded,[5] while highly branched alkanes are much more challenging for naturally occurring systems to degrade.[6,7]

Due to a great many environmental concerns, regulations on use of aromatics in fuel oil composition were established.[8] As a consequence, fuels now contain far fewer aromatic compounds. However, to compensate for the reduced energy rich aromatics, branched alkanes are widely used. A key component of gasoline formulations presently is isooctane (2,2,4-trimethyl pentane).[9] Isooctane has a high-octane rating (100%), which is

a standard for modern internal combustion engines to benchmark their efficiency.[10] While isooctane can be found in crude oil, it is primarily produced synthetically from isobutane and isobutene.[10] In the future, the use of isooctane as an additive will only increase, making investigation into biodegradation routes especially important.[6]

In the context of environmental pollution, the quaternary carbon of isooctane is the main feature that makes it more recalcitrant; only few cases of its biodegradation have been reported.[9] The mechanism of isooctane biodegradation is mostly reported to be catalyzed by oxygenases, but in selected cases oxygen is not required.[9,11,12] To the best of our knowledge, only one bacterial strain is capable of utilizing it as a sole carbon source: *Mycobacterium austroafricanum*.[13] Consequently, the discovery of new organisms that can remediate soil or water contaminated with isooctane is of great interest.

The endophytic bacterium *Burkholderia phytofirmans* PsJN was reported to degrade diesel-contaminated soil and to promote plant health in said soils.[14] The relationship between the plants and the bacteria is mutually beneficial, wherein plants provide nutrients for their associated endophytic bacteria. In exchange, the endophytic bacteria can promote plant growth by different mechanisms. For example, the enzymatic action of 1-aminocyclopropane-1-carboxylate deaminase modulates ethylene levels in plants.[15] Other important plant-health aspects promoted by bacteria include the reduction of pathogenic activity and growth through competition for nutrients and space,[16] stimulation of plant resistance mechanisms,[17] and the synthesis of biosurfactants and enzymes responsible that play roles in hydrolysis reactions.[18]

In this Chapter, the recently isolated *Burkholderia sp.* strains were screened to investigate their growth on minimal media (M9) plates containing some major hydrocarbon components of gasoline and diesel oil such as naphthalene, heptane, decane, and isooctane. The low enthalpy of sublimation (i.e., volatility) of each compound was utilized and the hydrocarbon was assayed for use by bacteria strains as the sole carbon source for growth. The *Burkholderia* species used in this work are the *B. xenovorans* (U86373) and *B. phytofirmans* (AY497470). The *B. phytofirmans* isolates were identified based on the 16S rRNA gene similarity as a novel strain of *B. phytofirmans* PsJN, while the *B. xenovorans* isolates were those used in Chapters 2 and 3.

4.1. Materials and methods

4.1.1. Preparation of M9 plates and bacteria culture

The M9 plates were prepared according to the procedure in section 2.6.1 except some few modifications. 15 g of agar was added to 500 mL of the M9 salt mix (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl) and sterilized by autoclaving at 120°C for 20 min. After allowing to cool to about 50°C, vitamins and trace elements were added, but no glucose was added. The M9-agar liquid mix was poured on petri dishes and allowed to solidify before storage at 4°C.

The M9 plates were streaked with the bacteria strains of interest using wire coil sterilized with flame. The cover of the petri dish was filled with about 50 mg (solid) or 50 µL (liquid) of the respective hydrocarbon compounds, and the inoculated M9-agar plate was inverted to allow the hydrocarbon compounds to sublime onto the surface. The plates were incubated under aerobic condition at 30 ^oC for at least 3 days. The *Burkholderia phytofirmans* strains include: RL17-361-BIB-A, RL17-379-BIF-B, RL16-011-BIC-C, RL17-374-BIF-A, RL17-373-BIF-A, RL17-336-BIF-A, RL17-328-BIF-A, RL17-347-BIC-D, RL17-350-BIC-A, and RL17-351-BIF-A, while the *Burkholderia xenovorans* strains include: RL17-337-BIC-C, RL16-009-BSH-A, RL17-329-BIC-A, and RL16-009-B52D-A.

4.2. Results

4.2.1. Growth trials of the selected *Burkholderia* strains on M9 plates containing common hydrocarbons as the sole carbon source

For *in vitro* biodegradation studies of water-insoluble pollutants, a vapour/sublimation technique was previously reported and has been applied here.[19] The compound, which serves as substrate for the bacteria growth, is sublimed from a dish (heated or not) containing the compound (pollutant) onto the surface of an inverted, inoculated agar petri dish.[19] Volatile or high vapour pressure compounds do not require heat, while very low vapour pressure compounds require heat to carry out sublimation. This method has been applied for the screening of hydrocarbon-degrading bacteria,[9,20] and was utilized in this study.

The growth trial experiments for selected *B. phytofirmans* and *B. xenovorans* strains were carried out on M9 plates containing a representative group of hydrocarbon pollutants, as presented in Table 4.1. These substrates were chosen as a representative set of hydrocarbon pollutants. Comparison for heptane and isooctane, which have similar vapour pressures (Table 4.1) is the most straightforward and offers a comparison of reactivity of linear and branched hydrocarbons. Growth of colonies were observed for some of the *B. phytofirmans* strains in the presence of isooctane and heptane, as well as the *B. xenovorans* strains in the presence of heptane. The colonies appeared within one to two days of incubation. None of the bacteria strains were able to grow using naphthalene, biphenyl, or decane. No bacteria colonies were observed for the control M9 plates without any hydrocarbons added. The test results show that some of the *B. phytofirmans* and *B. xenovorans* strains were able to utilize the carbon source from isooctane and heptane to grow.

Table 4.1. Growth tests of the B. phytofirmans and B. xenovorans on M9 plates containing common hydrocarbons as the sole carbon source

Strains	**Substrates**					
	Control	**Naphthalene**	**Biphenyl**	**Heptane**	**Decane**	**Isooctane**
Vapour Pressure	**N/A**	**8.94 Pa**	**0.7 Pa**	**5.3 kPa**	**0.2 kPa**	**5.5 kPa**
B. phytofirmans						
RL17-361-BIB-A	–	–	–	–	–	+
RL17-379-BIF-B	–	–	–	–	–	+
RL16-011-BIC-C	–	–	–	–	–	+
RL17-374-BIF-A	–	–	–	–	–	+
RL17-373-BIF-A	–	–	–	–	–	–
RL17-336-BIF-A	–	–	–	–	–	–
RL17-328-BIF-A	–	–	–	+	–	–
RL17-347-BIC-D	–	–	–	–	–	–
RL17-350-BIC-A	–	–	–	–	–	–
RL17-351-BIF-A	–	–	–	–	–	+
B. xenovorans						
RL17-337-BIC-C	–	–	–	+	–	–
RL16-009-BSH-A	–	–	–	+	–	–
RL17-329-BIC-A	–	–	–	+	–	–
RL16-009-B52D-A	–	–	–	+	–	–

\+ indicate the presence of bacteria colonies on the M9 plate

– indicate the absence of bacteria colonies on the M9 plate

4.3. Discussion

The *Burkholderia sp.* (as introduced in section 1.7) is a ubiquitous bacterial species and they have been implicated in diverse reactions relevant for biodegradation and bioremediation. According to the results set out in Table 4,1, certain strains of the endophytic bacteria *B. phytofirmans* are able to grow on isooctane and heptane as the sole carbon source. The *B. xenovorans* strains were able to grow on heptane, but not isooctane. The only bacterium that is a well-characterized degrader of the recalcitrant isooctane is *Mycobacterium austroafricanum* IFP2173,[9] while *B. phytofirmans* PsJN has phytoremediation activity when grown with ryegrass in a laboratory-prepared diesel-contaminated soil system.[14] Owing to their shared phylogenomic history, this is an indication that our new *B. phytofirmans* strains could exhibit similar biodegradation capability as the PsJN. The formation of colonies by the new *B. phytofirmans* strains on isooctane and heptane indicates that the bacteria strains may readily degrade the low-molecular weight and non-aromatic hydrocarbons in oils. It is reported that most bacteria do not degrade, or are slow to degrade, high molecular weight hydrocarbons and chlorinated or nitrated compounds.[21,22]

At present, we do not have a clear explanation for the observed differences in reactivity. It is thought provoking that the only observed growth is for the carbon sources with the highest vapour pressures, potentially making them more available to the *Burkholderia*. It may be worth revisiting growth on the aromatic hydrocarbons, either using a more optimized approach based on the literature protocol used here or using liquid culture. The differences in growth on isooctane and heptane also are noteworthy. Isooctane is usually more difficult to breakdown than heptane, but the organisms that act on isooctane do not grow on heptane (and vice versa). This observation could mean that different pathways and/or enzymes are involved. Consequently, these organisms may be interesting starting point for finding novel redox enzymes.

4.4. Conclusions

In this work, newly isolated strains of the endophytic bacteria *B. phytofirmans* were able to grow aerobically on plates containing only isooctane and heptane as the carbon source. The observation of growth in the presence of isooctane is noteworthy given the limited reports on organisms that can degrade this recalcitrant compound. The *B.*

xenovorans strains, which also degraded benzoate (Chapter 3), were also able to grow on heptane. This result suggests diverse metabolic pathways in these *B. xenovorans* strains. These results should be further investigated This work provides the foundation for further investigation of the rate and mechanism by which these new bacteria strains degrade isooctane and heptane, which are one of the key components of gasoline and diesel.

4.5. References

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Chapter 5.

Summary and Future Directions

Biological strategies for valorization of lignocellulosic biomass and remediation of environmental pollutants show great promise and are effective in some current applications. However, many improvements are possible, and much effort is ongoing in these areas. Lignin, the recalcitrant component of lignocellulosic biomass, is rich in valuable aromatic compounds and is eliminated as waste in large amounts in the pulp and paper industry. The lignin structure is complex due to its biosynthesis and pre-treatment methods, which are a great challenge for its biodegradation. Only a limited number of microorganisms, both fungi and bacteria, have shown evidence of delignification activity.

Recent research efforts are geared toward the discovery of delignifying bacteria, which is a motivation for work described in this thesis. This will expose researchers in understanding strategies for biomass deconstruction. Discovery of new delignifying bacteria stands to expand our knowledge of substrate specificity, provide rich source of new enzymes, and a platform for understanding how bacteria break down lignin.[1] The work described in this thesis involves the screening of new *Burkholderia sp.* isolates. First, isolates of the *Burkholderia xenovorans* were screened for delignification activity using alkali lignin (Chapter 2) as well as their ability to degrade common hydrocarbon pollutants (Chapters 3 and 4). Although the new strains share evolutionary history with the polychlorinated biphenyl-degrading *B. xenovorans* LB400 based on their 16S ribosomal RNA, the results suggest that these new strains are incapable of degrading C-C bonds in biphenyls, either in the lignin or biphenyl models. Further experiments revealed that they were able to grow on and degrade benzoate. Second, new endophytic *Burkholderia phytofirmans* strains were also screened on minimal media (M9) plates to show if they can grow on common petroleum hydrocarbon pollutants (Chapter 4). The results show that some of *B. phytofirmans* strains were able to grow on isooctane, a recalcitrant hydrocarbon present in gasoline.[2]

The new *B. xenovorans* strains were unable to degrade the alkali lignin or related models, which is an indication of the diversity among bacteria of similar phylogeny.[3] Furthermore, the alkali lignin is probably not the best representative of natural lignin due

to formation of difficult-to-degrade crosslinked groups during pre-treatment.[4] Exploring the delignification capability of the new *B. xenovorans* isolates by testing them in a wide range of lignin sources is one of the experimental approaches to discover potent delignifying bacteria. However, this poses a challenge due to the complexity and heterogeneity of the lignin structure and could obscure the complete understanding of mechanism of degradation. The use of monomeric and polymeric aromatic hydrocarbons presents a simpler approach to understand the mode(s) of action of the new *Burkholderia* isolates. Therefore, as the intrinsic capabilities of biology are being utilized, lignin pre-treatment or solubilization strategies are also expected to develop. Knowledge of the former will provide an understanding of the suitable pre-treatment strategy for biological conversion in a biorefinery. Both conditions could be optimized to obtain a desired product and in high yield.

The new *Burkholderia* isolates were also tested for their growth and degradation of selected hydrocarbons that are common environmental pollutants.[2,5] First, the *B. xenovorans* strains were subjected to liquid culture containing anthracene, biphenyl, and benzoate as the carbon source. The strains were able to grow only on benzoate and at a relatively rapid rate. Chromatographic study revealed that they utilized benzoate as a carbon source, producing several metabolite intermediates. Second, the *B. phytofirmans* and *B. xenovorans* were also screened for growth on plates containing naphthalene, biphenyl, heptane, decane, and isooctane as the sole carbon source. The results showed that some of the *B. phytofirmans* strains grew on isooctane, while the *B. xenovorans* strains grew on heptane. The growth of *B. phytofirmans* using isooctane as a carbon source may indicate that the utilization of isooctane as a carbon source may not be only a feature of actinobacteria, such as *Mycobacterium austroafricanum*.

Developing understanding of the mechanism of benzoate and hydrocarbon degradation by the new *Burkholderia* strains is key. For *B. xenovorans* growing on benzoate, we have been able to show that the bacteria exhibit a *ben-cat* pathway, a knowledge which will be useful because benzoates are mostly present as intermediates in many bacteria pathways for degradation of aromatic compounds, including lignin.[6] A full genomic and proteomic characterization of the degradation pathways of these potent bacteria isolates will be a starting point for bioremediation studies. For *B. phytofirmans*, we have a unique opportunity to explore the chemistry and biochemistry of a microbe that can grow on recalcitrant isooctane.

A long-term goal, as more pathways and gene products for the breakdown of lignin and aromatic compounds are being discovered, so is the use of synthetic biology and metabolic engineering in biotechnology. For example, in lignin catabolism in the peripheral (upper) pathway, the natural intermediates of interest (e.g., vanillin) that are generated can be collected by eliminating the enzymes responsible for their further metabolism.[7] Also, to improve degradation of toxic wastes, genetically engineered microorganisms in bioremediation have been utilized under laboratory conditions.[8] However, because of environmental concerns more work is still ongoing for the use of genetically modified bacteria for *in situ* bioremediation. The work described here adds new data to be considered in these advanced applications and biotransformation strategies.

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Appendix A.

Supplementary Information for the bacteria isolates.

The data and text in this Appendix were generously provided by Mr. Jake Haeckl from the Linington lab.

Location (coordinates) of the *B. xenovorans* and *B. phytofirmans*

B. phytofirmans

RL17-361-BIB-A – 48.6114274 N, 123.6494197 W

RL17-379-BIF-B – 49.38158848 N, 121.934559 W

RL16-011-BIC-C – bamboo roots from 49.269418 N, 122.94937 W

RL17-374-BIF-A – 54.503061 N, 128.712914 W

RL17-373-BIF-A – 54.503061 N, 128.712914 W

RL17-336-BIF-A – 49.41982372 N, 123.4924181 W

RL17-328-BIF-A – 49.62579254 N, 123.1899419 W

RL17-347-BIC-D – 49.17544105 N, 123.9619669 W

RL17-350-BIC-A – 49.16487192 N, 123.9655269 W

RL17-351-BIF-A – 49.16487192 N, 123.9655269 W

B. xenovorans

RL17-337-BIC-C – 49.42034652 N, 123.4913815 W

RL16-009-BSH-A –tomato plant roots from 49.269418 N, 122.94937 W

RL17-329-BIC-A – 49.62579254 N, 123.1899419 W

RL16-009-B52D-A –tomato plant roots from 49.269418 N, 122.94937 W

Condition of the media before storage

Plate BIC and BSH are the same recipe. Plate BIB is the basal medium B52D but with 0.2 g/L $MgSO_4$ added. BIF and BIC are presented in Table A1 below. All media were prepared at pH 5.5 prior to autoclaving.

Environmental Sampling

Environmental samples were collected from shallow plant roots in Southern British Columbia (see above). Briefly, alcohol-sterilized tweezers, scissors, and scoops were used to reveal roots, a small portion of the root was cut and placed into a sterile 50-mL centrifuge tube (Falcon) with a small amount of surrounding soil. Latitude and longitude coordinates were obtained using a handheld GPS, and identification photographs taken of each plant. Tubes were sealed with the cap while in the field, but this cap was replaced with parafilm as soon as possible on returning to the laboratory. Samples were stored a 4°C prior to processing.

Samples were processed by removing small portions of root and soil material from the collection tube and transferring to a 15-mL centrifuge tube (Falcon). Each sample was diluted with sterile 1X PBS buffer to cover the soil and root material in the tube (approximately 1 mL), vortexed at the maximum setting for 30 seconds and allowed to precipitate for 30 minutes. From each sample, 100 µL aliquots were spread onto each of the 6 selection media plate types and incubated at 30°C for 5-7 days. After incubation, unique colonies were picked based on variations in color and other morphological characteristics using a sterile plastic loop, inoculated onto LB agar plates (25 g/L, Fisher Scientific) and incubated at 30°C for 48 hours. Single colonies were then picked to inoculate freshly autoclaved LB liquid media (10 mL) which were shaken at 200 rpm at ambient temperature overnight to prepare cultures for DNA purification.

DNA Purification for PCR

DNA was extracted from each turbid overnight culture using the Promega Wizard Genomic DNA Purification Kit according to the Gram-negative bacteria protocol. Simultaneously, 500 µL of culture was added to an equal volume of sterile 1:1 glycerol/water in cryo-microcentrifuge tubes for preservation. DNA concentrations were measured using a SpectraDrop Micro-volume Microplate with a Spectramax i3x plate reader (Molecular Devices). Each sample was suspended in 2 µL of resuspension buffer and this rehydration buffer from the DNA purification kit used as the blank for each plate in three technical replicates. DNA samples from each isolate were then used for PCR experiments.

Isolate Identification

16S rRNA gene PCR reactions were performed in 50 μL volumes containing 25 μL 2x MasterMix with dye (ABM), 2.5 μL of universal primers 8F and 1492R (0.15 μM final concentration), and 20 μL of nuclease-free water and template DNA (up to 1 μg DNA per reaction). PCR products were confirmed by standard 1% agarose gel electrophoresis at 100 V for 30 minutes. PCR products were purified directly from PCR reaction tubes using the QIAquick PCR Purification Kit according to the manufacturer's protocol (Qiagen). DNA concentrations were determined using the microplate reader as described above. PCR products were sent to UBC-NAPS sequencing service (UBC, Vancouver, BC) with the same 8F and 1492R PCR primers for sequencing. Contigs were generated using CAP3 (Huang and Madan 1999). BLAST searches were conducted for all contigs and restricted to the 16S ribosomal RNA sequence database. Results were tabulated for each isolate with the accession number and the percent identity of the top BLAST homolog only (Tables S9 for pilot sampling with all 20 media and Table S10 for full scale with five best candidate media).

Phylogenetic Analysis

Maximum likelihood 16S rRNA gene sequence consensus trees were created for *Burkholderia* and untargeted isolates in MEGA7 (Kumar et al. 2016). Sequences were aligned using MUSCLE and a ML tree was constructed with 1000 bootstrap replicates using the Tamura-Nei model (Tamura and Nei 1993). All positions containing gaps and missing data were eliminated. The *Burkholderia* tree was constructed of 417 sequences consisting of the 295 isolates from this study and 122 reference sequences. References sequences came from the top BLAST homolog for all untargeted organisms and for *Burkholderia* were derived from a recent phylogenomic analysis of the genus (Sawana et al. 2014). There were a total of 822 positions in the final dataset. The annotated tree was generated using iTOL (Letunic and Bork 2016).

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Table A1. Media condition for storage of the *Burkholderia* isolates.

Component	BIB (B52D)	BIC (BSH)	BIF
Yeast Extract	5 g/L	5 g/L	None
KH_2PO_4	3 g/L	3 g/L	3 g/L
$NiSO_4*6H_2O$; $CuSO_4$	292 mg/L; 85 mg/L	292 mg/L; 85 mg/L	292 mg/L; 85 mg/L
$MgSO_4*7H_2O$	0.2 g/L (not in B52D)	None	0.2 g/L
L-sorbose/hydroxyproline	None	1 g/L each	None
D-glucosamine	None	none	5 g/L
Fusaric acid/bacitracin	None	100 mg/L each	100 mg/L each
Econazole nitrate salt/cycloheximide/nystatin	100 mg/L each	100 mg/L each	100 mg/L each

Appendix B.

Supplementary information for benzoate degradation by the *B. xenovorans* strains

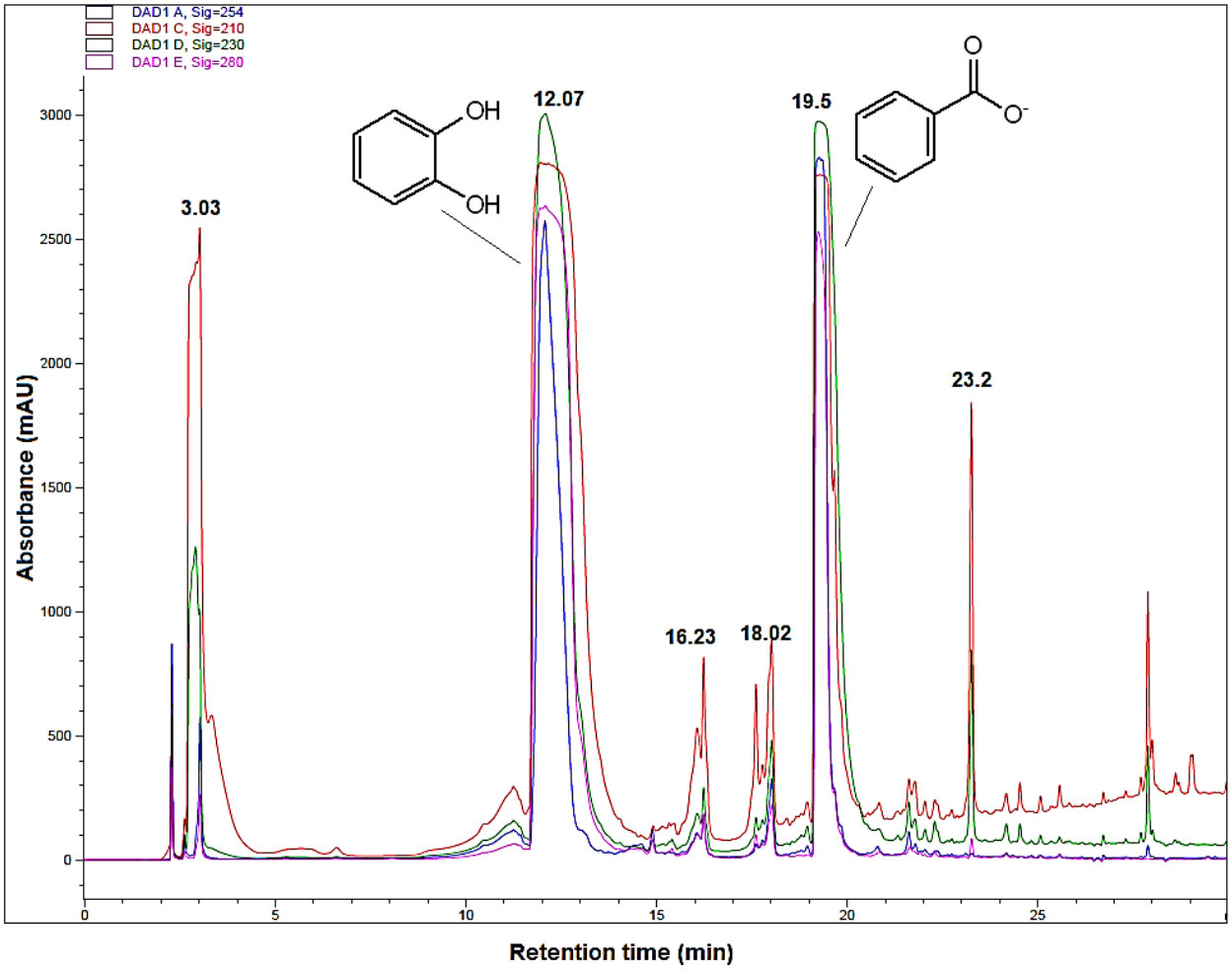

Figure B1. **HPLC chromatogram for purification of the crude benzoate degradation product in strain RL16-009-B52D-A. UV detections shown in legend. Peaks shown with retention time were collected separately for NMR analysis.**

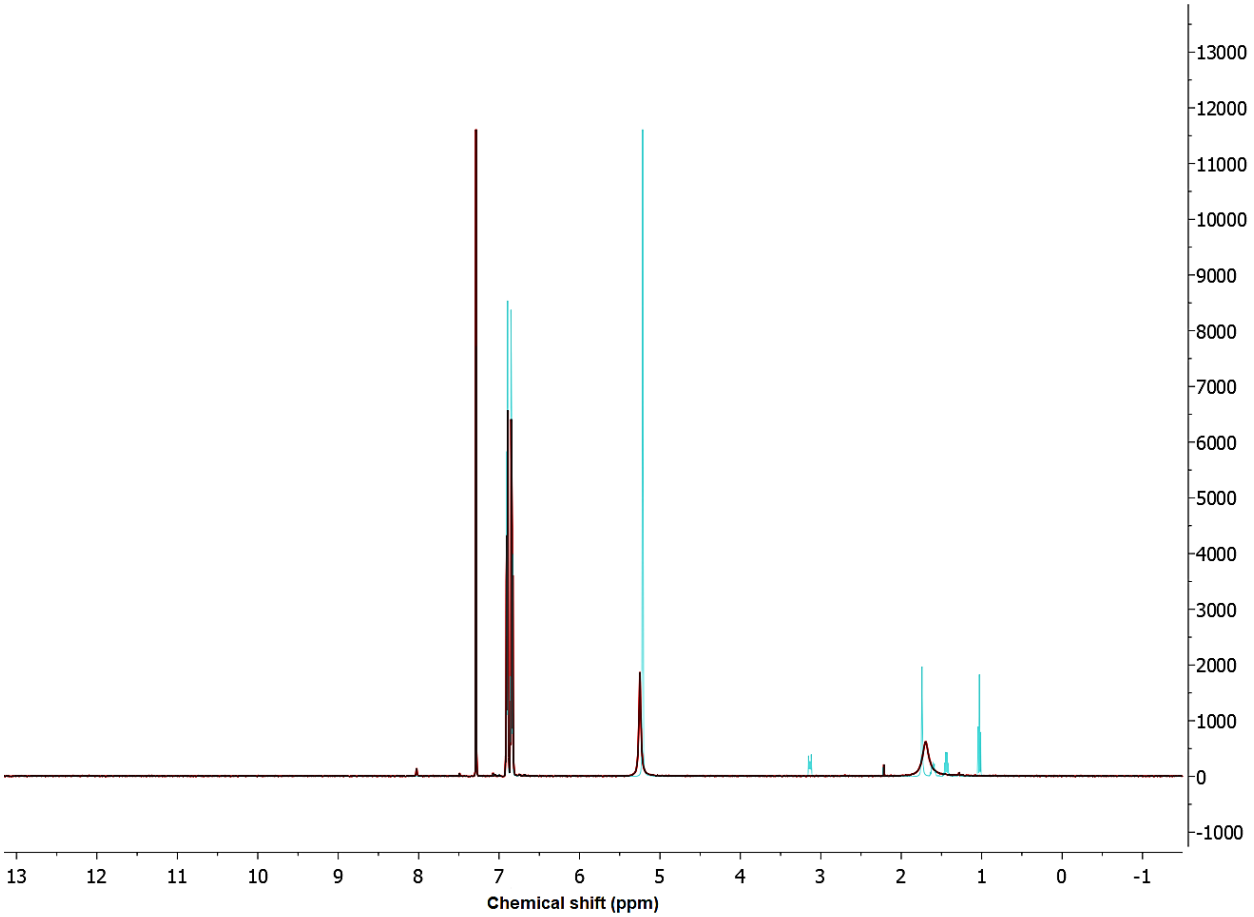

Figure B2. **^{1}H NMR signal of catechol standard (blue) and catechol obtained via the bacteria degradation (red).**

Table B1. **Yields of the collected catabolic products**

Retention time (min)	Yields (mg)
3.03	0.42
16.23	1.2
18.02	0.89
23.2	0.59

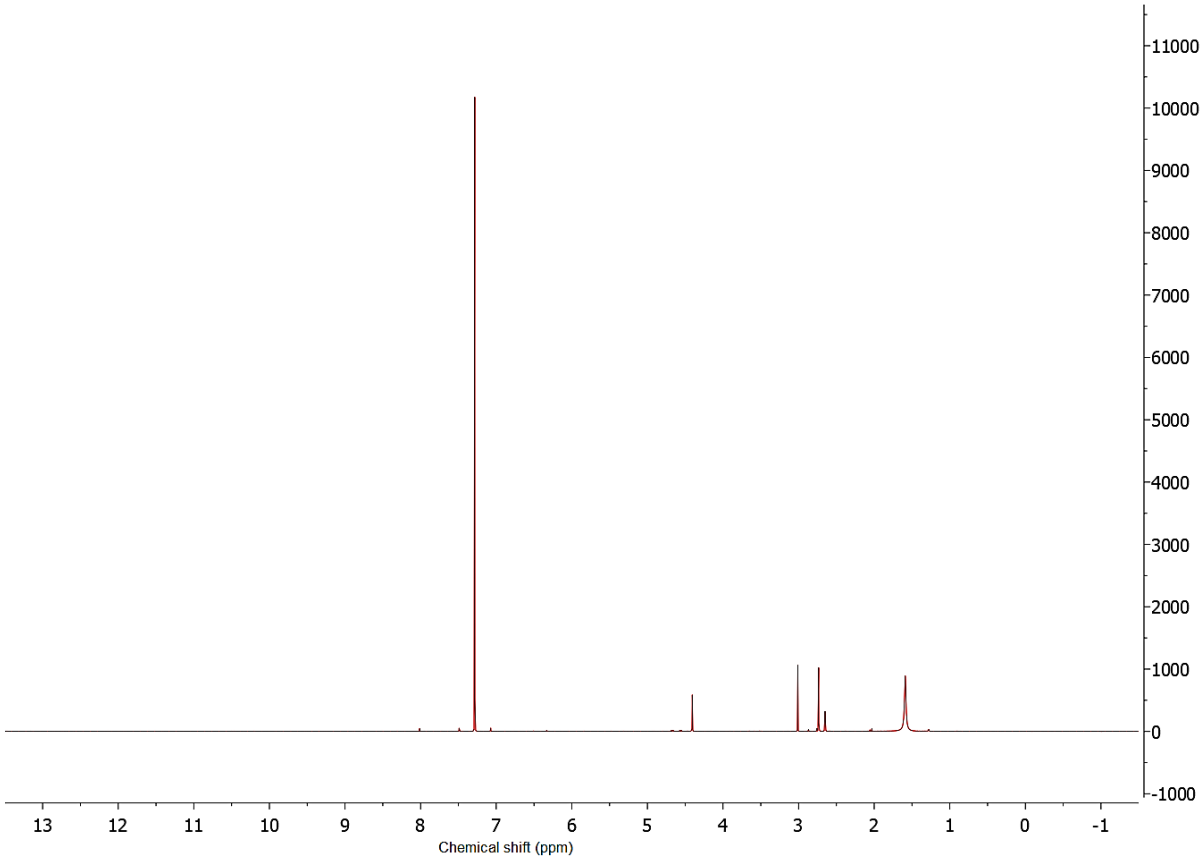

Figure B3. ^{1}**H NMR spectrum of metabolite at retention time 3.03 min.**

Appendix C

The commonly used pre-treatment processes are briefly described below:

Steam explosion

This method can be classified as a hydrothermal process. The biomass is subjected to high pressure steam (200 - 2000 psi) and high temperature (180 - 230 ºC) followed by explosive decompression that opens up the biomass fibers.[1] In some cases, catalytic amounts of H_2SO_4, CO_2, or SO_2 are added to enhance the efficiency of the process. The lignin produced from the steam explosion process is rich in low molecular weight aromatic compounds that are soluble in organic solvents.[1,2]

Dilute acid

The acid pre-treatment of lignocellulosic biomass can be done in two ways depending on the end use. It can be performed at high temperature of over 180 ºC within a short time (less than 5 min) or at low temperature below 120 ºC over a long period of time (30-90 min). The process is carried out in specialized reactors and is known to generate compounds that inhibit the fermentation step.[1,3]

Alkali pre-treatment (Kraft lignin process)

The method uses alkali chemicals such as sodium, calcium, potassium and ammonium salts to treat biomass at ambient conditions. Sodium hydroxide is commonly used and is the most effective.[1,4] Considerable amounts of sodium hydroxide together with sodium sulfide are added in a series of steps in the process at temperatures between 150 - 180 ºC for up to two hours.[5]

Organosolv process

In this method, biomass is treated with organic solvents such as ethanol, methanol, ethylene glycol, acetone etc., in water. The process conditions such as temperature, pressure, reaction time, solvent concentration and catalyst (mostly acids) used can be varied depending on the source of the lignocellulosic biomass.[1,6] The three common organosolv pre-treatments include: Battelle, formasolv, and ethanosolv and they generally

make use of solvent, acid, and water. Lignin is dissolved in the solvent while cellulose and hemicellulose are hydrolyzed.[1]

Ammonia-based pre-treatment

This method uses ammonia to pre-treat lignocellulosic biomass and there are three types: ammonia fiber explosion (AFEX), ammonia recycle percolation (ARP) and soaking aqueous ammonia (SAA). These processes can be differentiated in terms of their energy requirements.[1] During the process, cellulose undergo phase change and the resulting sugars become more reactive. At the same time, the structure of lignin is modified and overall, the process does not produce compounds that inhibit the function of fermenting organisms, making it suitable for the fermentation step.[1]

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