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CELLULOSE AND SULFATE DEGRADATION IN A BIOCHEMICAL
REACTOR DURING TREATMENT OF MINE DRAINAGE

by

SWETA OJHA

A THESIS

Presented to the Graduate Faculty of the
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In Partial Fulfillment of the Requirements for the Degree
MASTER OF SCIENCE IN ENVIRONMENTAL ENGINEERING

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Approved by

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ABSTRACT

Seventy-two biochemical reactors were set up, and operated using a mixture of chip-bark, horse manure, and gravel as the biochemical treatment substrate. The simulated mine water containing sulfate (1000 mg/l) was pumped into each reactor at a flow rate of 0.5 ml/minute (approximate), giving an empty bed contact time of 8 days. The main idea is that the microorganisms present in horse manure would convert the cellulose in chip-bark into volatile fatty acids. The produced volatile fatty acid would enhance the metabolism of sulfate-reducing bacteria (SRB) initially present in horse manure, which would degrade (and eventually remove) the sulfate from mine-impacted water. At the end of every month, the amount of cellulose remaining in chip-bark samples were calculated using two different methods: NMR, and chemical extraction (acid-base-acid).

An objective of the experiment is analyzing the correlation between cellulose, and sulfate degradation rate. Separately, the ozonation method was evaluated as a potential surrogate for much slower biological degradation. It may be possible to predict the higher degradation rate of cellulose, and hence, degrade (or remove) the sulfate from mine-impacted water using the proposed biochemical process. The percentage of cellulose in fresh chip bark was 52%. There was degradation of 4.5 % cellulose in five months. First order kinetic equation was used to predict the time for exhaustion of cellulose. The time predicted by the designed model is 5 years to react with the maximum degradation (observed by ozone treatment) of chip bark.

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1. INTRODUCTION

1.1. MINE- IMPACTED WATER

Mine-impacted water (MIW) is water runoff from any area that has been disturbed by mining. When mine-impacted water is mixed with river water, the MIW disturbs the chemical composition of the river, which is then contaminated with minerals, varying concentrations of metals, metalloids, and several other toxic materials (Ledin and Pedersen, 1996). Mine drainage is generally formed when mining activities expose pyrite (FeS_2 , Iron disulfide), which can then mix with water and oxygen and results in sulfuric acid and dissolved iron. Mine-impacted water usually has a high concentration of metal, and iron is the most common metal in MIW. Mine drainage is liquid waste, resulting from sulfide containing materials being exposed to oxygen, and water (Nordstrom, Blowes, and Ptacek, 2015),(Akcil and Koldas, 2006), (Sheoran and Sheoran, 2006).

Mine-impacted water is found in over 10,000 miles of receiving waters in the United States (EPA, 2014). The United States produces 42.5 million metric tons of iron ore, worth \$3.8 billion and has more than 500,000 abandoned mine sites (U.S. Geological Survey, 2016). Mine-impacted water with a pH less than 7 is acid mine drainage. Mine drainage with a neutral pH (around 7) is known as neutral mine drainage.

Acid mine drainage(AMD) has a greater environmental impact than neutral drainage, which makes AMD the focus of more attention than neutral mine drainage. Scientific and applied research focuses more on acid mine drainage, although it may have low concentration of acid and iron. Neutral mine drainage typically has a high concentration of sulfate and dissolved metals (J Rinker, Nicholson, A Venhuis, and Swarbrick, 2003).

1.2. EFFECTS OF MINE DRAINAGE

Drainage emanating from mine waste deposits are serious threat to the quality of groundwater and surface water(Lindsay, Ptacek, Blowes, and Gould, 2008). Any unregulated mining has the capacity to release harmful substances to surface water, groundwater, air, and land (Chang, 2010). Contamination of water from metals is an immense environmental problem that can affect the hydrochemistry of the water, and the sediment chemistry of the surrounding soil deposits. The drainage from these mines also affect aquatic ecosystems, which in turn affects primary and secondary production, nutrient cycling, energy flow, and decomposition processes(ITRC). It is difficult for plants and animals to inhabit the areas where mines have been established, and where tailing dams used to be situated for many years. MIW can create negative environmental impact, downstream impact, community impact, and all of which the mine owner can be held liable for. All land can be affected by MIW including state, federal, public, and private lands (Lindley, 1903) (Kulyk, Transportation, and Development, 2004).

Mine-impacted water is a grave issue because mining occurs in all continents, with the exception of Antarctica (Jacobs, Lehr and Testa, 2014). Some countries even practice mining in floodplains, and shallow ocean areas around large land masses, resulting in a direct contamination of the water source (Dill, 2008). The exact scale of environmental pollution caused by mine drainage is difficult to measure. In 1989, scientists estimated that approximately 19,300 km of streams and rivers, 72,000 ha of lakes, and reservoirs throughout the world were severely affected by mine drainage. (D. Johnson and B Hallberg, 2005).

Mine drainage creates long term pollution in water (Gunson, Klein, Veiga, and Dunbar, 2011). Leaching also may cause mine impacted water. Leaching results in a stream draining away from the ore with the action of mine drainage. In the United States, leaching of products(reacted) into the surface water hamper over 20,000 km of streams (Mayda, 2013). There are two treatment options for MIW: prevention and remediation (Johnson et al., 2003).

1.2.1. Prevention. Remediation is very expensive. If proper care is taken to prevent the negative effects of MIW, then there will be fewer negative ecological and financial effects. To prevent the harmful effects from mine drainage, the following can be done as preventive measures:

- Flooding/sealing of underground mines
- Underground storage of mine tailings
- Land-based storage in sealed waste heaps
- Blending of mineral wastes
- Total solidification of tailings
- Application of anionic surfactants
- Microencapsulation (coating)

Prevention is a better technique than curing, but if it is not possible to prevent the effects of mine drainage, proper remediation should be done to prevent MIW's long-term effects.

1.2.2. Remediation. There are two types of treatment systems for mine drainage: *active systems*, and *passive systems* (Figure 1.1.). Active treatment systems usually are used only at operational mines. Passive treatment systems are used at

closed mines (Kulyk et al., 2004). Active systems require the continuous input of resources to sustain the process, whereas passive systems require little resources input during operation: the resources needed for passive treatment occur when originally implementing the system (Taft, Ricciato, 2012) .

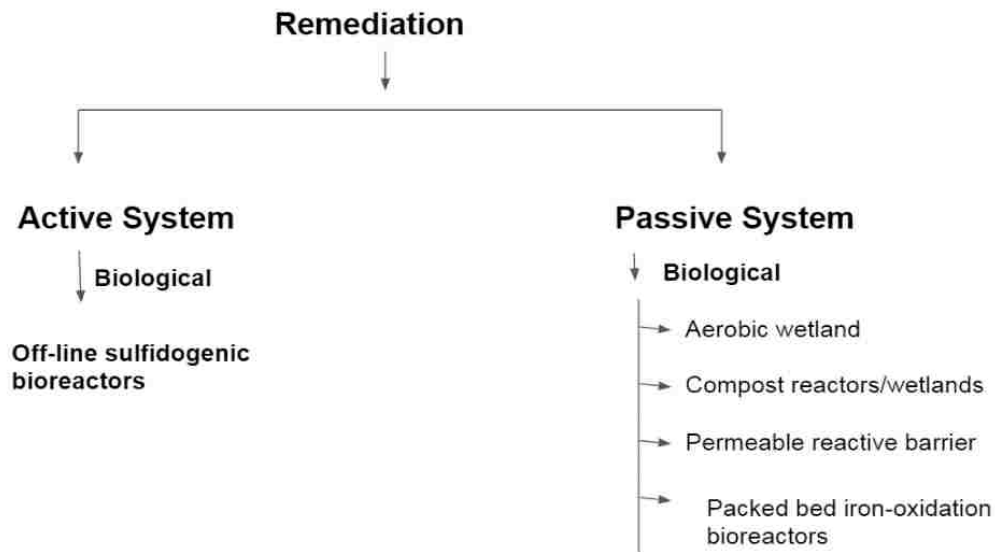


Figure 1.1. Different remediation methods for mine drainage (Klein, Tischler, Mühling, and Schlömann)

The two methods used in remediation process are *chemical (abiotic)* and *biological* methods. When using a chemical method, chemicals are added into the system. Typically, chemicals are added in wetlands to remediate the water that passes through those wetlands.

In the biological method, microorganisms are induced in wetlands to reduce concentration of harmful chemicals (Nehring and Brauning, 2002). When active or

passive system uses biological treatment, the remediation system is more effective compared to a chemical method (Means and Hinchee, 2000).

1.3. BIOCHEMICAL REACTORS (BCRs)

One passive biological treatment option is known as anaerobic cells or sulfate-reducing bioreactors (SRB) (P. Blumenstein and Gusek, 2010). The structures of BCRs is set up as a pond-like system that is used to biologically treat mine-impacted water by reducing sulfates with help of microorganisms like sulfate-reducing bacteria, cellulose degrader bacteria and microorganisms. The efficiency of sulfate degrading bioreactors depends on the sulfate-reducing bacteria (Fitch, 2015). The growth of sulfate-reducing bacteria is controlled by the composition of the reactive mixture present in the reactors. Among the reactive mixture, the most important substrate present in the reactor is the organic carbon source (Neculita, Zagury, and Bussi re, 2007). In BCRs, organic substrate is used by microbial organisms as an electron donor with sulfate as an electron acceptor. In bioreactors, organic carbon and sulfate are energy sources, and a terminal electron acceptor for sulfate-reducing bacteria (Gibert et al., 2004; Kaksonen et al., 2004; Zagury et al., 2006). BCRs remove high concentrations of metals at low pH. Many mechanisms like adsorption and precipitation of contaminant removal occur in bioreactors (Neculita et al., 2007). The reduction of sulfate helps to increase the pH, which assists in the reaction of precipitation and creates preferable conditions for metal hydroxide precipitation (Gadd, 2010). Manure of different animals, mushroom compost or hay are also used a substrate in BCRs. The generated carbonate and hydroxide anions in BCRs may also contribute to metal removal (Dvorak, MacGlashan, Morgan, and Lichtenstein, 1996). In

addition, S^{2-} reacts with metal present in mine drainage, and produces insoluble metal sulfide. The degradation of organic substrate is a rate-limiting step (Logan et al.2005).

Advantages of BCRs

- BCRs requires low operation costs and minimum levels of energy consumption.
- The flexibility in the materials that can be used causes the operation of BCRs to be less expensive than other techniques that are used to treat mine drainage.
- The BCR system is designed to function for numerous years without having to replenish construction material.

Disadvantage of BCRs

- BCRs have slow degradation rate.

As BCRs consumes sulfate they generate a high net alkalinity in the effluent.

Based on the site situation and characteristics, different designs of sulfate reducing reactors can be used.

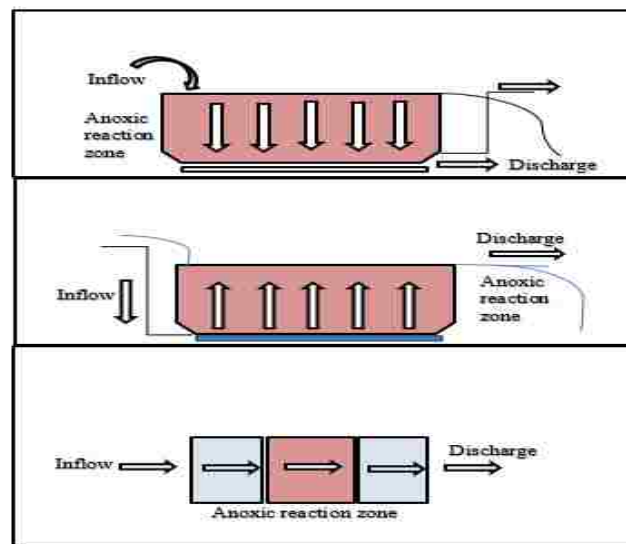


Figure 1.2. Various system configurations of BCRs (a) Downflow, (b) Upflow and (c) Horizontal flow bioreactors

All designs of bioreactors have same mechanisms for mine-impacted water to enter and distribute through the systems of BCR. All biochemical reactors have an anoxic zone, where water, microbes, and substrate react, and leads to precipitation of metals and metalloids. The anoxic zone is also the place where sulfides get reduced and pH gets increased. An anoxic zone is also the way for water to leave the system. Different types of bioreactors according to flow are shown in Figure 1.2.

The three types of BCRs are downflow BCR, upflow BCR, and horizontal flow BCR as shown in Figure 1.2. In a down-flow biochemical reactor, water enters the reactors from the topmost part of reactor. Water moves down passing through the reaction zone and then goes out through the bottom of the reactors. In up-flow biochemical reactor, water enters the reactors from the bottom of reactor. Water moves upward passing through the reaction zone, and then exit through the uppermost part of the reactors. In horizontal-flow biochemical reactor, water enters the reactors horizontally and then goes out horizontally.

Sulfate-Reducing Bacteria (SRB): All SRBs respire anaerobically by using sulfate as their electron donor. SRBs are characterized in four different taxonomic groups: (1) *Gram negative bacteria*, which have an optimum growth temperature of 20°C to 40°C, such as, *Desulfovibrio* and *Desulfomicrobium*, (2) *Gram positive bacteria*, which can withstand higher temperatures, such as, *Desulfotomaculum*, (3) *Thermophilic SRB*, which has an optimal growth temperature of 60° C to 70° C, an example is *Thermodesulfovibrio* and (4) *Archaeal thermophilic SRB*, which have an optimal growth temperature more than 80°C, example: *Archaeoglobus* (De Castro et al., 2000). SRBs usually grow in environments that have been impacted by humans, such as, rice fields, paper mills, and

streams affected by mine drainage (Campbell and Postgate, 1965). The largest populations of SRB are found in wetland and rumens.

Figure 1.3. shows how to identify a treatment process for MIW, including BCR used in the research, First, flow rate is identified, which in this research was 0.15 ml/min. The water used was neutral with pH around 7, and has around 1 mg/l of iron and aluminum. So, it is not acidic. Due to sulfate being present, water flows through the biochemical reactor, and then could go through an aerobic wetland or pond. In this research, the aerobic wetland or pond is the drainage system in which effluent drips through air down the 2-m drain pipe. Gravel was present for water distribution in reactor. The gravel happened to be limestone.

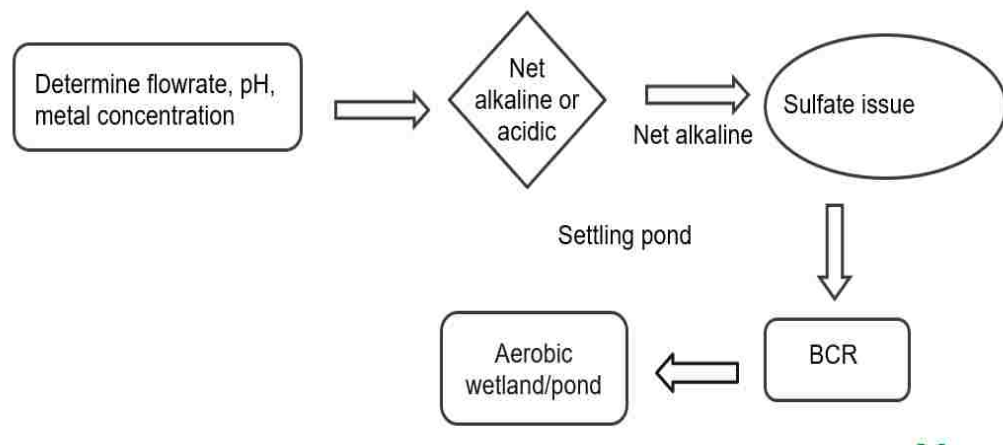


Figure 1.3. Process to choose BCR

1.4. OBJECTIVES

The objective of BCR is to convert the sulfide (soluble in water) to metal sulfide (insoluble in water) Sulfate-degrading bacteria helps in converting sulfide to metal

sulfide which helps to get rid of sulfide which is soluble in water. Though, this is the main objective, metals were not added to reactors for it is very expensive. Hence, the concentration of the metals was not evaluated. The main objectives of this research are as follows:

- *To determine the cellulose degradation in each reactor through chemical analysis:* Each month a reactor is assayed for the measurement of cellulose content, and from this to estimate degradation rate. Chip bark was taken out from each reactor to do chemical analysis of chip bark.
- *To determine the sulfate degradation every month:* Sulfate was measured with help of a Hach colorimeter to measure degradation of sulfate and examine the correspondence to cellulose degradation.
- *To determine the degradation rate of wood by treating with ozone:* Chip bark with water was treated with ozone. This method was evaluated as a potential surrogate for biological degradation done in a BCR. Two similar experiments were done by treating chip bark with ozone. Different doses of ozone were observed to result in similar degradation in the two similar experiments.
- *To determine the reproducibility to ozone treatment using dye experiment:* Dye solution was treated with ozone for 20 minutes to check the reproducibility of ozone measurement by the ozone analyzer,
- *To use the NMR method to check the results of chemical analysis for cellulose degradation:* NMR method was done to check chemical analysis result.

- *To predict time for the degradation of cellulose in biochemical reactor:* First order kinetics were used to predict degradation of cellulose in biochemical reactors

2. LITERATURE REVIEW

Mine-impacted water is waste from mines, which is a potential source of contamination to the environment by releasing large amounts of heavy metals and acid (Ledin and Pedersen, 1996). Mine drainage that encounters receiving waters is a serious threat to the quality of groundwater, and surface water. This water, whose chemical composition is affected by mining or mineral processing, is known as mine-impacted water.

There are an estimated 46,000 abandoned mines on public lands, and some of them are top-priority Superfund sites. The western part of the United States has a rich mining history, which makes mine drainage a very common issue in this area (Gault et al., 2001). MIW occurs in the places where sulfide containing metalliferous ore is deposited, sulfide-rich coal is situated, and locations where weathering of metalliferous black shales occurs (Guilbert, Park, and Park, 2007). This can occur where extraction is currently happening or where extraction used to take place years or even decades before (Conservation, 1984). When it comes to the consequences of mine drainage, a few of the main factors include: the pH of the environment, concentration, and variety of sulfur contained in the rocks in mine drainage, variety, and concentration of carbonate minerals present in mine drainage, surface area of minerals, local resources available for the reaction, size of the particles present in the waste, minerals liberation extensions in the rock, availability of water, and oxygen, and the biological environment (Jacobs, Lehr and Testa, 2014).

The potential sources for formation of mine drainage are reactive sulfide minerals, and oxidation products. Other sulfides that are found in ore deposits are included in Table 2.1., along with their chemical formula (Plumlee et al., 1999).

Table 2.1. Different sulfide minerals and their chemical formula

Sulfide mineral	Chemical formula
Pyrite	FeS ₂
Pyrrhotite	Fe _{1-x} S (X= 0 to 0.2)
Bornite	Cu ₅ FeS ₄
Arsenopyrite	FeAsS
Galena	PbS
Pentlandite	(Fe,Ni) ₉ S ₈

Among all sulfide containing materials shown in Table 2.1., pyrite (FeS₂) is the most abundant constituent found in mine drainage (Pérez-López, Delgado, Nieto, and Márquez-García, 2010). The major reason for acidity in mine drainage is the oxidation of pyrite ores. When the ions of minerals are exposed to air, and water, the oxidation of minerals is the natural chemical reaction (Harding, and Boothryd et al., 2004), (Harding, 2017). Due to the presence of pyrite, mine drainage affects the biogeochemical cycles of materials found in mine drainage: iron, sulfur, and oxygen (Couvidat, Neculita, Benzaazoua, Genty, and Chatain, 2017). This impact increases sulfate concentration,

precipitation of iron oxyhydroxide, and with some time will decrease the pH of waters (Neculita et al., 2007).

MIW is generally found in active or abandoned surface mines, underground mines, and processing industries' waste-disposal areas (haulage roads or tailing ponds (Méndez-García et al., 2015). One of the main causes of mine drainage is due to the by-products of different industrial operations such as galvanic processing and scrubbing of flue gases at different power plants that produce sulfur-rich wastewater, which becomes mixed in various streams (Johnson et al., 2000). Because of this, when resource extraction through mining occurs, it not only disturbs the land, but also often perturbs surface waters. Some of the serious complications created by MIW are mine drainage, metal contamination in various sources (groundwater and surface water), increased sediment levels in streams, and that MIW can be very expensive to clean-up (M. Jerrald et al , 1997); (Vasquez, Escobar, Neculita, Arbeli, and Roldan, 2016) . In 2008, the Canadian mining industry estimated a cost of \$2 to \$5 billion dollars for the reliable remediation of the identified acidic drainage known to have considerable environmental liability. (Group RR. 2008, U.S. Fish, and Wildlife Service). In order to follow EPA regulations and to avoid an expensive clean-up, mining industries must implement management strategies in order to reduce the risk of significantly altering ecological health, and biodiversity in receiving waterways (Winterbourn and Kettle, 2000).

There are three types of mine drainage: *acid mine drainage* (AMD), *saline drainage* (SD), and *neutral mine drainage* (NMD). AMD has a pH below 6 whereas NMD and SD have a pH values greater than 6 (USGS, 2010). More attention is usually given to acid mine drainage, whereas neutral mine drainage is a less documented system

(Heikkinen, Räisänen, and Johnson, 2009; Mayes, Potter, and Jarvis, 2009),(Heikkinen et al., 2009; Mayes et al., 2009). The characteristics of three different types of mine drainage are as follows:

A. Characteristics of acid mine drainage (Rimstidt and Vaughan, 2014):

- pH less than 7
- Contaminated with heavy metal
- High sulfate concentration. Example: Figure 2.1.



Figure 2.1. Example of acid mine drainage: Gauteng, South Africa

B. Characteristics of saline drainage (Molenda, 2014):

- pH can be alkaline or acidic
- It contains very low metal concentrations
- Less sulfate concentration
- Treatment is required for sulfate removal, and sometimes metal removal

An example of saline mine drainage: The coal mine located in the upper Silesian coal basin of southern Poland, with an area of 7400km² (Kędzior, 2009). The coal basin is the largest coal basin in Europe. It is concentrated by other mines like methane, cadmium, lead, silver and zinc (Graniczny, Colombo, Kowalski, Przyłucka, and Zdanowski, 2015).

C. Characteristics of neutral mine drainage (Heikkinen et al., 2009):

- Neutral pH
- Low to moderate metal concentrations
- May be highly contaminated by zinc, cadmium, manganese, or selenium
- Moderate sulfate concentration



Figure 2.2. Example of neutral mine drainage, Baia region, Romania (Modoi, Roba, Torok, and Ozunu, 2014)

Even though neutral mine drainage is known to be in the pH range of 6.11 to 7.42, it still cannot support as many organisms as regular water because it is catalyzed by bacteria like *Thiobacillus thioparus* in the unsaturated zone of mine drainage, and there will still be a high concentration of metal or sulfate in the water (Kirby and Cravotta, 2005; Nordstrom et al., 2015; Soucek, Cherry, Currie, Latimer, and Trent, 2000). Figure 2.2. shows an example of neutral mine drainage.

Mine drainage does not only affect the water that it meets but also affects nearby land. Mine drainage affects the health and quality of plant, and animal communities situated nearby riverbanks. The negative effects are caused by the high toxicity of reactive metals in the water column, sulfate concentration, and the acidity of the water (Jarvis and Haygarth, 2002) (Chapa, Vargas and Robinson, 2013) (Schmilt et al., 2007, Sola et al., 2004). When the land is contaminated by mine drainage, the fertility of land will diminish. Figure 2.3. shows the contamination of land in the municipality of Santa Cruz, Zambales, a province in the Philippines. The mine drainage was caused by the nickel extraction effect, and caused a rapid degradation in agriculture, and fishery sectors around the area. Along with affecting the fertility of soil, mine drainage can also cause corrosion which may affect the infrastructure of nearby buildings (A.Kumar et al., 2013).

Mine drainage can cause economic implications, and long-term environmental effect. It contaminates water, as it produces an acidic environment in water with various metal ions like iron (Roy Chowdhury, Sarkar, and Datta, 2015). Metal mine drainage has resulted in severe degradation of the quality of many rivers across the

globe(Ramani et al., 2014) (Olías and Nieto, 2014),(Jacobs et al., 2014) (Gunson, Klein, Veiga, and Dunbar, 2011).



Figure 2.3. Effect of mine drainage: Philippines

When released to streams, the concentration of the MIW with heavy metals declines as it flows further downstream from its contaminated source. The precipitation of iron hydroxide, and other metals can cover whole river beds as shown in Figure 2.3. When this occurs it significantly degrades the quality of the breeding and feeding areas of many aquatic organisms. The contamination of these breeding, and feeding grounds has caused the extinction of several endangered aquatic animals(Batty, Atkin, and Manning, 2005), (Mayes et al., 2009).

Acidic water may cause issues in the reproduction systems of aquatic life, which decreases populations. From the sulfide minerals, water infiltration becomes acidic, and



- Ferric iron is produced, when ferrous iron reacts with acid and oxygen.

Third step: Oxidation of ferric iron



- Iron oxide and sulfuric acid are produced when ferric iron reacts with pyrite, and water.

Fourth step: Hydrolysis of ferric iron



- Iron oxide and acid are produced when ferric iron reacts with water.

In the process of the weathering of pyrite in metal mine drainage, the four equations are shown above. The first equation shows the oxidation of pyrite by oxygen and water, present in the atmosphere. The result of this equation is dissolved ferrous iron and sulfuric acid. The second equation shows the oxidation of dissolved ferrous iron which then produces a ferric iron ion. The third equation displays the hydrolysis of ferric iron. In this equation, ferric iron reacts with water which results in producing iron hydroxide precipitate, and acidity. The final equation shows the oxidation of additional pyrite by ferric iron which is generated in the reaction.

2.1. TREATMENT OF MINE DRAINAGE

2.1.1. Active System. Active technologies are related to treatment technology in which the addition of a neutralizing agent has taken place (Coulton et al., 2003) (Skousen et al., 2017)(Gaikwad, 2010). The neutralizing agent may be an inorganic chemical or an organic chemical. Neutralizing agents like alkaline materials will raise the pH of mine drainage which prevents the water from becoming acidic. The other function of alkaline materials is to accelerate the chemical oxidation of ferrous iron present in mine drainage. The result of this technology is an iron-rich sludge that may contain various other metals. Alkaline materials like slaked lime, calcium carbonate, sodium hydroxide, magnesium oxide, and magnesium hydroxide can be used in this technology (Weiss and Nihon, American Institute of Mining, and Petroleum, 1976). An active treatment system has advantages of high metal removal at low pH, recovery of metal and stability of sludge. The different mechanisms that occur in active treatment are ion exchange, adsorption, and reverse osmosis of metal. Selection of resins and operation of parameters are two important parameters (Gaikwad, 2010). Continuous care is required for active treatment system. The different methods of active treatment system are gas injection, addition of caustic soda, lime dispensing dozer, hydrated lime, soda ash, oxidation, and aeration, reverse osmosis, and ion exchange. Active treatment method is expensive method which requires continuous input of resources (Trumm, 2010).

2.1.2. Passive System. Passive technologies generally sequentially remove metal, and increase the pH of MIW in an artificial bio-system that capitalizes on ecological and geochemical reactions. Passive system uses both biological and chemical processes. This technique requires less power than an active system and no chemicals

after construction and will continue to function for a long time. One passive technology is anoxic limestone drains used to reduce the acidity of mine drainage (Kleinmann, Hedin, and Nairn, 1998).

For the treatment of landfills leachate, passive methods require more land area than conventional chemical treatments (Ouakibi, Loqman, Hakkou, and Benzaazoua, 2013). There are three types of passive technology: aerobic wetlands, anaerobic substrate, and anaerobic limestone drains (Skousen et al., 2017) (Alhamed, 2016).

The different mechanisms that are responsible for the removal of metals in passive treatment system from mine impacted are sorption by organic matter, formation of carbonates, association with Fe oxides, catalyzation by bacteria either in acidic condition or neutral condition, reduction to stable forms, formation of sulfides of various metals, and biological methylation. (Sobolewski, 1999). For the treatment of mine drainage contaminated water on a small scale (pilot or lab), and full scale projects, passive bioreactors have been successfully used for over 20 years (Baştuğ and Kuyucak, 2006), (Neculita, Zagury, and Bussièrè, 2007), (Kim and Benson, 2004). Anaerobic passive system is a promising approach in which the microbial sulfate reduction process is used to precipitate metals, and sulfate is reduced to H₂S by complex microorganisms. Metals, and H₂S will interact with each other to give metal sulfide (H. Gammons, E. Duaiame, Parker, R. Poulson, and Kennelly, 2010; H. Gammons et al., 2010).

2.1.2.1. Aerobic wetland. The aerobic wetland is the simplest passive treatment system. The system is used to treat lightly acidic or alkaline water which contains by Fe. This system has a much-defined capacity to control acidity in water

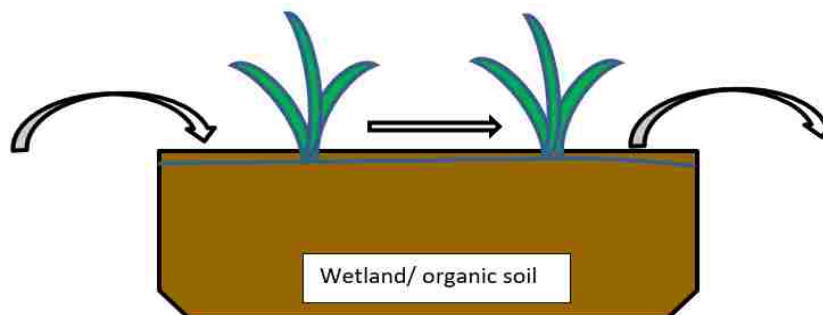


Figure 2.4. Example of aerobic wetland

The function of this system is to allow aeration of the mine water which is flowing among vegetation. Aeration helps dissolved Fe to become oxidized. The aerobic system also helps in precipitation of the oxidized Fe. But precipitation increases H^+ , which generate acidity of the water. That is why highly acidic water cannot be treated in aerobic wetlands. Schematic of a surface flow wetland with plants is shown in Figure 2.4. (Vasquez et al., 2016).

2.1.2.2. Anoxic limestone drains. The other passive method of treatment of mine drainage is to use anoxic limestone drains as shown in Figure 2.5. In this treatment method, long- narrow ditches are filled with limestone and covered to prevent air from entering as shown in Figure 2.5. Mine drainage with a low pH is directed to flow through the trench. Limestone produces bicarbonate alkalinity via dissolution. The outlet is held in a settling pond for pH adjustment, and metal precipitation to be complete before being discharged to natural water sources (Newcombe and Brennan, 2010).

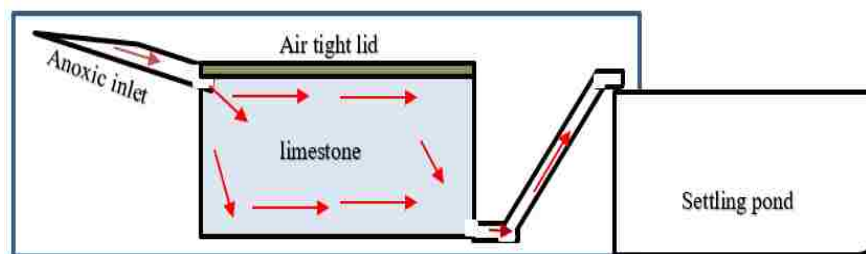


Figure 2.5. Example of Anoxic limestone wetland

2.1.2.3. Anaerobic system. Anaerobic wetlands are also known as passive biological systems or compost bioreactors. In an anaerobic wetland, reactions occur without the presence of oxygen. Anaerobic wetlands are generally made below ground level (Johnson and Hallberg, 2005). The experiment, described in this paper is a passive biological system that receives no air as shown in Figure 2.6. In this system, a microbial catalyzed reaction occurs, which gives out net alkalinity, and biogenic sulfide. Electron donors are derived from the organic substituents present in the wetland. The choice of organic substance depends on the local availability.

In this experiment, an anaerobic wetland was used with wood as the organic substrate. Horse manure is used as it has rich bacterial ecosystem. A long-term provision of appropriate substrate for sulfate-reducing and iron-reducing bacteria was used. Limestone was used to have proper distribution of water flow.

An anaerobic environment is beneficial for decaying plant materials, especially the cellulose of plant materials. Cellulose is the insoluble component of wood. Although the cellulose in wood is insoluble, it can be decomposed by complex interacting microorganisms (S. Leschine, 1995) Bacterial, and fungal degradation occurs extracellularly with the outer cell envelope of plant. (S. B. Leschine, 1995). BCR is one of

the passive treatment systems. BCR is also known as biogeochemical reactors. BCRs is a system that produces microorganisms that transform contaminants, and produce chemicals that assist in the remediation of water.

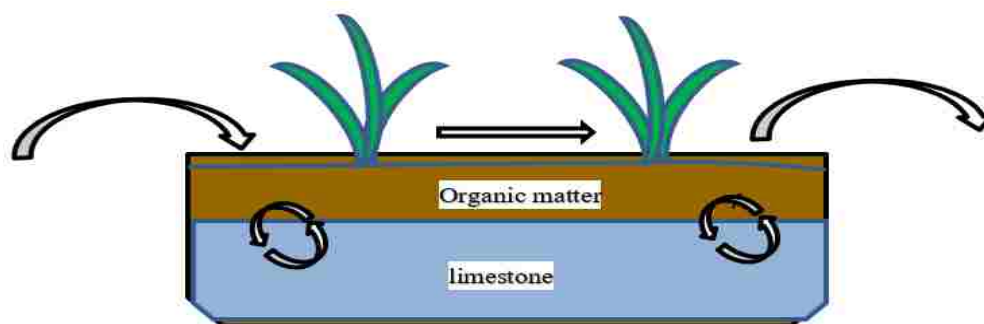


Figure 2.6. Example of anaerobic wetland

2.1.2.4. Biochemical reactor. BCRs are reliable biotechnology for mine treatment because they can control the problem of acidity, high sulfate concentration, and also high concentration of metals (Fitch, 2015). BCR use mixtures that have an organic carbon source like cellulosic waste, a bacterial source like river sediment or animal manure, solid porous media like gravel or sand, generally also a neutralizing agent like limestone, and often a nitrogen source like urea (Cocos et al., 2002; Zagury et al., 2006) (Neculita et al., 2006). Many researchers have completed many unsuccessful attempts to predict the biodegradability of organic components present in reactors by the extraction of chemicals (Zagury et al., 2006, Gibert et al., 2004) (Machemer et al., 1993).

Different organic sources were used as a remediation of mine drainage. Bacteria found in Red lake were used to treat Mine-impacted water. Carbon sources, like sweet

potato, were seen to be highly utilized by bacteria which increased the pH of mine drainage, and decreased the sulfate concentration from mine-impacted water. The result of this experiment showed that sulfate-reducing bacteria could not utilize horse manure enough in low pH. So, sulfate concentration was not decreased by horse manure in low pH. It was found that horse manure works better in high pH rather than low pH.

For stimulation of removing ferrous iron, wetlands have been constructed for the treatment of mine-impacted water, which develop a steady state model that is based on decomposition, kinetics, and reaction stoichiometry. An anaerobic environment with entire organic substrate, constant temperature, no addition of organic matter input, and subsurface flow will have a model simulation which indicates that wetlands have readily decomposable substitutes that are rich in organic carbon. Wetlands rich in organic carbon will remove iron more than wetlands that have less biodegradable substitute. It is considered that it will take three to five years to have equal decrement (Tarutis and Unz, 1994).

2.1.3. Decomposition of Organic Matter in BCRs. By comparing volatile solids over different time periods, the decomposition rates of woody materials are determined in a lab scale wetland (Ye, 2006). Volatile fatty acids formed in a BCR almost certainly limits the rate of sulfate degradation due to an excess of sulfate (Welz, Ramond, Cowan, Prins, and Burton, 2011). In biochemical reactors, microorganism use SO_4^{-2} as electron acceptors. As influent has large concentration of sulfate, woody (chip-bark) or organic materials will slowly degrade. The simultaneous reduction of sulfate is the source of energy for the organism (Welz, Ramond, Cowan). There are different substrates used in biochemical reactors. The role and example of substrates are described in Table 2.2.

2.1.4. Substrate used in SRB. Figure 2.7 explains the mutualistic relation of the bacteria present in a BCR. The degradation of cellulose is a key feature of this anaerobic microbial process (McDonald et al., 2013). As the cellulose degrades it serves as an electron donor which after additional oxidation is used in sulfate reduction within the reactor (Lindow and Borden, 2005). Chip bark is the most effective source of cellulose. Table 2.3. shows the percentage of cellulose found in different wood (Rabemanolontsoa, Ayada, and Saka, 2011) (Raisanen and Nurmi, 2014).

Table 2.2. Role of different components of bioreactors (Sun and Cheng, 2002)

Role	Example of components
Long term electron donor	Chip bark, walnut shell, Horse manure, cow manure, pig manure, Other compost, rice hulls
Short term electron donor for begininng	Hay, straw, yard waste, brewery waste, pulp , acetic acid, ethanol
Alkalinity source	Limestone seashells , fly ash , kin dust, seashells
Microbial ecosystem	Manure of different animals(cow, pig, horse, hen), publicly owned treatment works sludge, sludge pond or slime dams at mining sites, septic system products . Fresh manure from browsing animal has high capacity for reduction of cellulose.
Better flow through bed	Gravels sand, chip bark, walnut shell, horse manure, cow manure, pig manure, other compost, rice hulls, and crushed rock fractured nut shell
Solid surface sites for microbial growth	Presence of all the component listed above.

When organic matter from cellulose, such as cellobiose is used as a carbon source by heterotrophic and anaerobic bacteria, the organic matter breaks into simplified carbon compounds, such as glucose, lactic acid, and lactate act as food for other microorganisms such as SRB.

In BCRs, there are various ecosystems of microorganisms. Fermentative and sulfate-reducing bacteria have a mutualistic relationship as described in Figure 2.7., which is essential in a lignin-cellulose based system, and are important in the passive treatment of mine drainage

First, cellulose-degrading bacteria degrade cellulose from wood to cellobiose. The degraded cellulose is then converted to glucose. After that, fermentative organisms convert glucose to lactic acid which is also known as fermentation. Lactic acid is converted to lactate by deprotonation. Lactate is converted to acetate and carbon dioxide by acetogens.

Acetate is used by sulfate-reducing bacteria as an electron donor. Sulfate-reducing bacteria reduce sulfates for electron acceptance. The reduced sulfur is left as sulfide which is metal sulfides.

Metal sulfides generally are solids that do not dissolve in water. The more rapidly cellulose degradation occurs, the more rapidly sulfate will degrade from the mine drainage, and the greater the amount of metal precipitated.

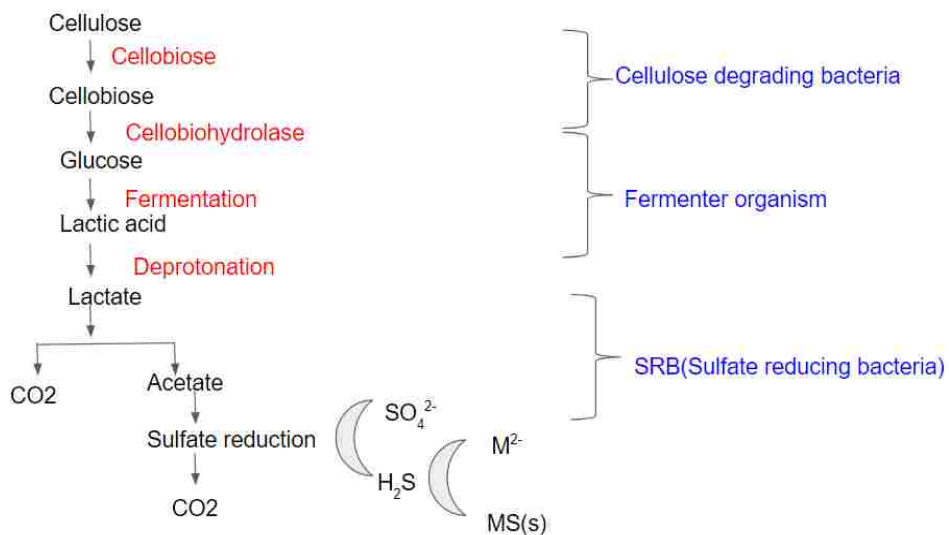


Figure 2.7. Food chain of bacteria in bioreactor

Table 2.3. Chemical composition of hard and soft wood

Species	Extractives (%)	Lignin (%)	Hemicellulose (%)	Cellulose (%)
European oak	4.4	25	29	38
French oak	3.8-6.1	25-34	19-26	39-42
Chestnut oak	6.6	22	30	41
Scot pine (bark)	5.0	27.0	26.9	40.7
Norway spruce	2.0	27.4	27.3	42.0

2.2. WOOD COMPOSITION

Wood has a complex chemical composition. Chemically, wood is described as composed of extractives, lignin, hemicellulose, holocellulose, and cellulose. Extractives, cellulose, and hemicellulose are the biodegradable organic contents of wood (Figure 2.8.). Lignin is a complex, and un-degradable component. The percentage of cellulose is higher than lignin, hemicellulose, and extractives. The lowest fractional organic compound is extractives or extraneous materials, generally found to be less than five percent of wood components (Reighard and Olesik, 1996).

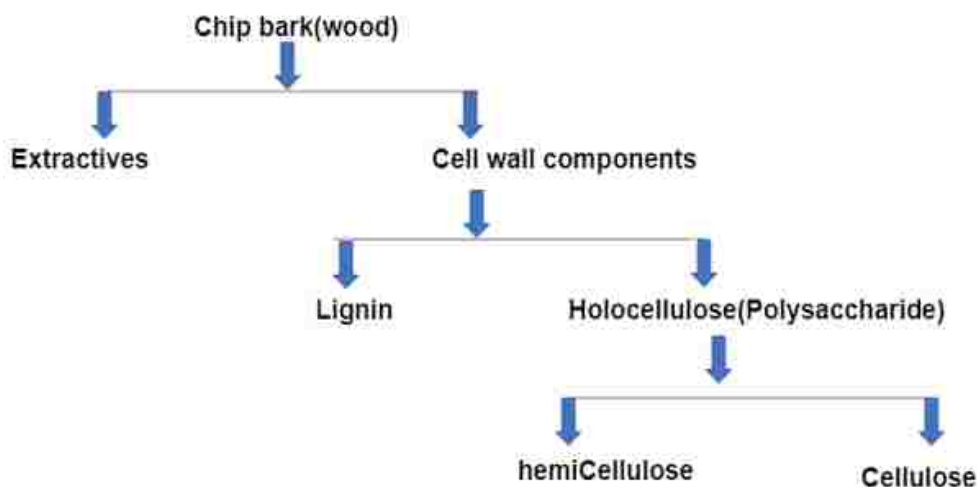


Figure 2.8. The composition of wood (Chandrasekaran, Hopke, Rector, Allen, and Lin, 2012)

2.2.1. Extractives. Extractives are the components of wood that are soluble in neutral organic solvents and water. Extractives have a lower molecular weight amongst the natural chemical products which are found in wood and are volatile with steam.

Extractives are non-structured, non-polymer components of wood biomass. Major chemicals reported to comprise extractives are fats, fatty acids, terpenes, resins, alcohols, phenols, steroids, and waxes. Extractives are subdivided by solvation (wegener, Fengel, Dietrich, and Gerd, 1989). Water and other solvents like acetone, toluene, and ethanol are used to extract extractives (wegener, Fengel, Dietrich, and Gerd, 1989).

2.2.2. Lignin. The organic substance which binds the cells, vessels, and fibers of wood are known as lignin. Lignin is a dendritic network polymer of a phenyl propane basic unit, and shows a variation in its chemical composition (Lo, Baird, and Hanson, 1983). An example of lignin structure is shown in Figure 2.9.

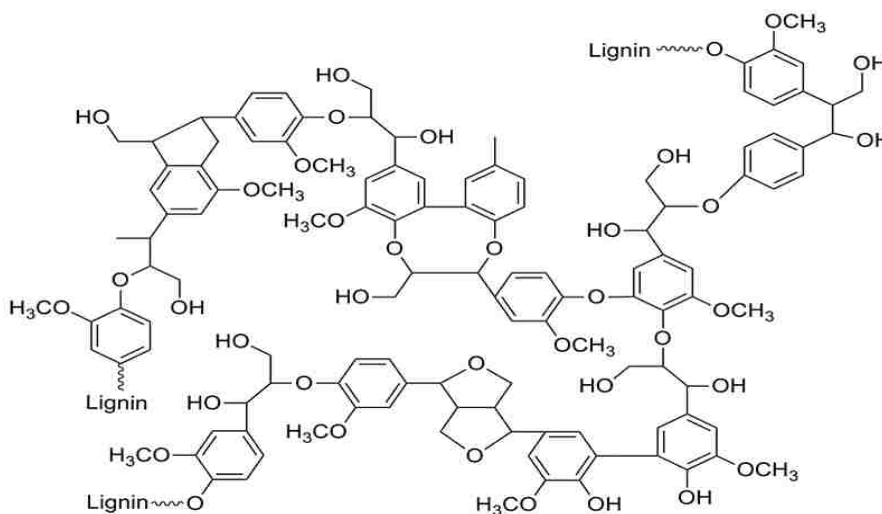


Figure 2.9. Typical chemical structures of lignin (Chandrasekaran et al., 2012)

2.2.3. Hemicellulose. Hemicellulose is composed of polysaccharides which contain various kinds of sugar monomers such as glucose, D-Pentose sugars, small amounts of L-sugars, xylose, mannose, galactose, rhamnose, and arabinose. (Sinnott and

Royal Society of, 2007). Acidified sugars (glucuronic acid, and galacturonic acid) are also found in hemicellulose. Generally, the structure of hemicellulose is a random, amorphous structure with very little strength in Figure 2.10. (Saxena and Brown, 2007)

2.2.4. Holocellulose. Holocellulose is a mixture of hemicellulose, and cellulose. Holocellulose does not contain extraneous materials, nor lignin. Holocellulose is the polysaccharide fraction of wood materials that is insoluble in water (Lv, Wu, and Lou, 2010).

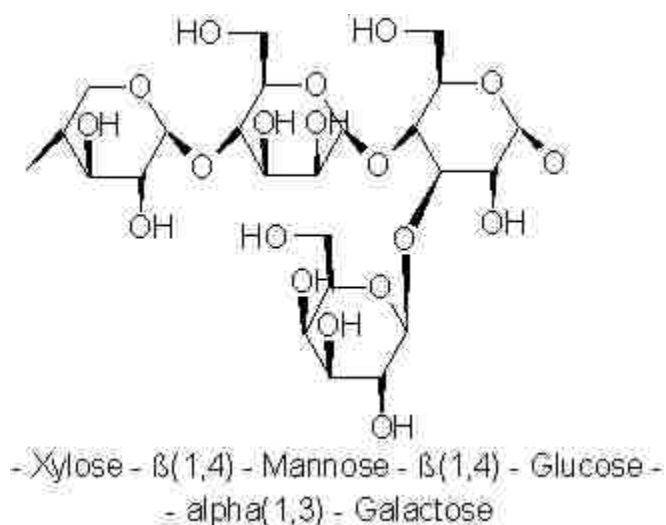


Figure 2.10. Typical chemical structures Hemicellulose

2.2.5. Cellulose. Cellulose is the main components present in plant cell walls, and comprises 40-50% of the mass of dry wood (Delmer, and Haigler et al., 2002). Cellulose is a linear polymer with crystalline structure, making it strong, and resistant to hydrolysis. Cellulose ($C_6H_{10}O_5$) is made of glucose, but is a polysaccharide consisting of a linear chain with many D-glucose units. It has 500 to 14000 β -D-glucoses connected

(1→4) (Somerville et al., 2006). These polymer chains are connected to each other by hydrogen bonds thus forming insoluble micro fibrils as shown in Figure 2.11.

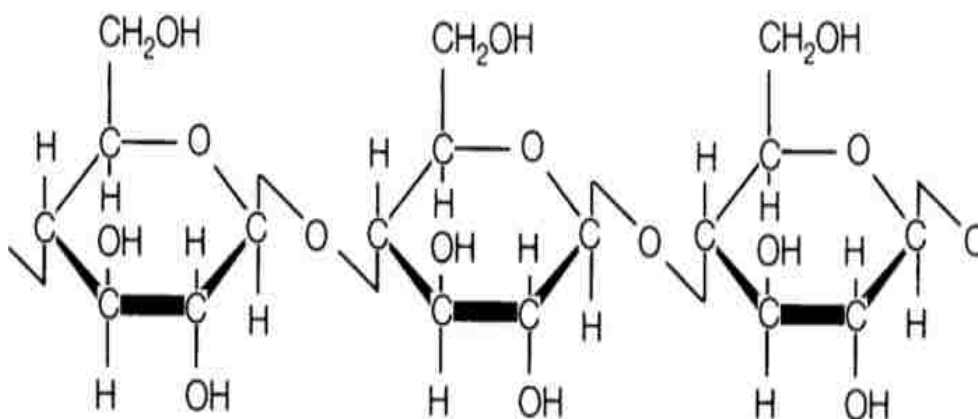


Figure 2.11. Typical chemical structures cellulose

Chang Ye et al. (2006) determined the decrement of organic substrate present used in biochemical reactors. Three different wetlands (Horizontal, Downflow, and Upflow) were designed. After 4.9 years, for the horizontal wetland, it was found that the organic substrate decreased from 34% to 17%. The decomposition rates of organic matter were calculated in lab scale wetlands based on the volatile solid present in wetlands over time. For the description of oxidation of organic matter, the Monod rate equation has been used (Ye, 2006). The cellulose of chip-bark slowly degrades over time, and in turn provides an electron donor. There will be degradation of cellulose from chip bark. The following form was used in order to modify the Monod equation (Ye, 2006).

$$R = R_{\max}(C_{\text{org}} - C_{\text{refrac}} / K_{\text{org}} - (C_{\text{org}} - C_{\text{refrac}})) \quad - (5)$$

In equation 5, R is the rate of decomposition of organic substrate (mg/mg original substrate day). C_{org} is the volatile solid, which is degradable organic substrate (g/g original substrate). K_{org} is the half saturation constant. C_{refrac} represents non-degradable organic substrate like lignin.

2.3. DESIGN CONSIDERATION OF BCR

CERCLA (Comprehensive Environmental Response Compensation and Liability Act) generally governs for the selection of systems at different remediation sites. In the CERCLA (superfund) process, effectiveness, implementation, minimum reliable cost, and the community's regulatory acceptance is considered. From the US EPA (2005), the main information required knowing the effectiveness of passive treatments or bioreactors are the followings:

- *Source characterization*: influent quality of MIW, flow rates, loading of acid, and metal, geochemistry, sulfate concentration, presence of bacteria.
- *Site characterization (geography)*: topography, weather, risk of creation of additional sources.
- *Environmental target*: contamination concern, discharge standard, criteria of human, and ecological risk, and applicable rules, and regulation
- *Available technologies*: source control, active treatment, passive treatment based on substrates present in the area
- An estimation of the amount of reduced sulfate sufficient to precipitate metals as sulfides.

- A bench-scale test is performed in the lab to know the loading range, thickness of the substrate, residence time, degradation rate of the substrate, and metal removal efficiency.
- Pilot-scale testing is performed at the site for some time, performed at the loading rate with the substrate mixture
- Effluent quality is evaluated with applicable discharge standards which include parameters like nitrate/nitrite, ammonia, phosphorus, and BOD.

2.3.1. Challenges of Biochemical Reactors. There are some challenges for the functioning of bioreactors. Some of these possible challenges are variations of seasonal temperature in the mine drainage area, changes in the rate of metal loading, short-circuiting, gas lock-ups, and effects of disasters. Other challenges may occur such as having less bacteria present in the source of bacteria used in the biochemical reactor, flowrates may differ which may cause problems in the degradation of sulfate and cellulose.

2.3.2. Previous Work. Two bioreactors (identical suspended and immobilized bioreactors) were set to check the capacity of removal of heavy metals by microorganisms present in bioreactors by S. R. M. Kutty in Malaysia (S R M Kutty, 2017). Immobilized bioreactors contained rice husk. For an effective microbial activity of some microorganism, heavy metals are essential. But if water has too high concentration of heavy metal, microorganism will not be able to sustain. Too high concentration of metal in water affects microbial activities by blocking functional groups on microorganism, which will displace essential metal ions. The metal removal was found by comparing the rate of substrate removal for bioreactor. The highest metal was

removed by 75% and 90% in CS and IM respectively. The rate of substrate removal was found to be 1.85g/L.d for immobilized bioreactor and 4.2693 g/L.d for identical suspended bioreactor. The highest removal of metal was seen in immobilized bioreactor due to presence of organic substrate (rice husk) which increases the ability of microorganism (S R M Kutty, 2017).

In study done by Figueroa et al., seven pilot scale sulfate-reducing bioreactors were set up. All bioreactors had varying ratios of alfafa hay, pine woodchips and sawdust. These experiments were done to analyze the influence of substrate composition on zinc removal and microbial community structure for 500 days.

Figure 2.12 shows that 18 mg of zinc was removed per gram of substrate in 500 days in column containing higher percentage of alfafa hay. Flow rates in each column was 0.4L/day. For the column that contain alfafa and sawdust, there was degradation of 14 mg of zinc removed per gram of substrate.

The minimum efficiency was seen in the column which contained only saw dust. In the column which contained wood chips and alfafa hay, there was degradation of 12 mg of metal per gram of substrate. Lowest removal of zinc was found in the column which contained only saw dust.

Dr. Fitch and his students performed an experiment of three types biochemical reactors for 2500 days. Three different bioreactors with different flow were set up. The first set up was horizontal wetland, second wetland was downflow and the other wetland was the wetland which has up flow bioreactor. Horizontal wetland has very low flowrate. The degradation of horizontal wetland is first order kinetics as shown in Figure 2.13.

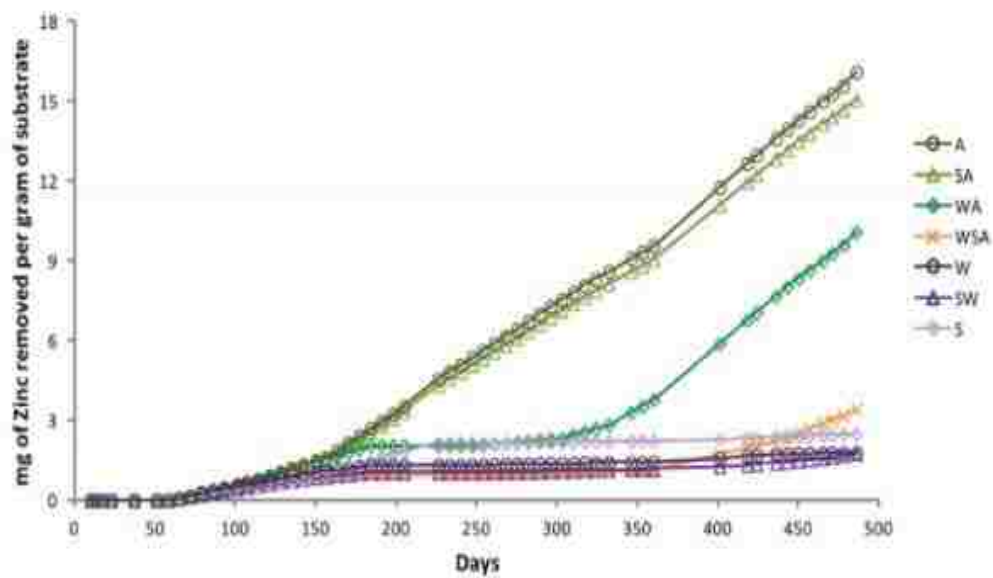


Figure 2.12. Zinc removed per gram of substrate

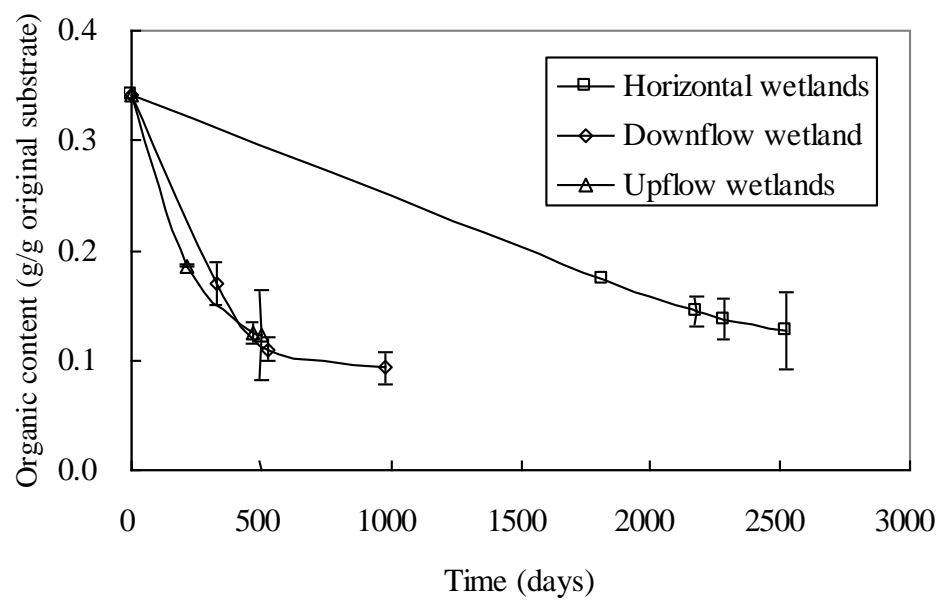


Figure 2.13. Organic content degradation in Chang Ye et al, 2006

2.3.3. NMR. Solid state NMR (Nuclear magnetic resonance) is a reliable method to get structure of characterize of the composition of wood. The main components of wood are carbohydrate, and lignin which has differing spectra as shown in Figure 2.14.

Cellulose has six signals seen in CP/MAS ^{13}C -NMR spectra. The spectra are made up of anhydroglucose unit which is split into fine structure clusters. The cluster present in spectra is due to supramolecular structure of cellulose (Piotto, Saudek, and Sklenář, 1992) (Tokoh, Takabe, Sugiyama, and Fujita, 2002).

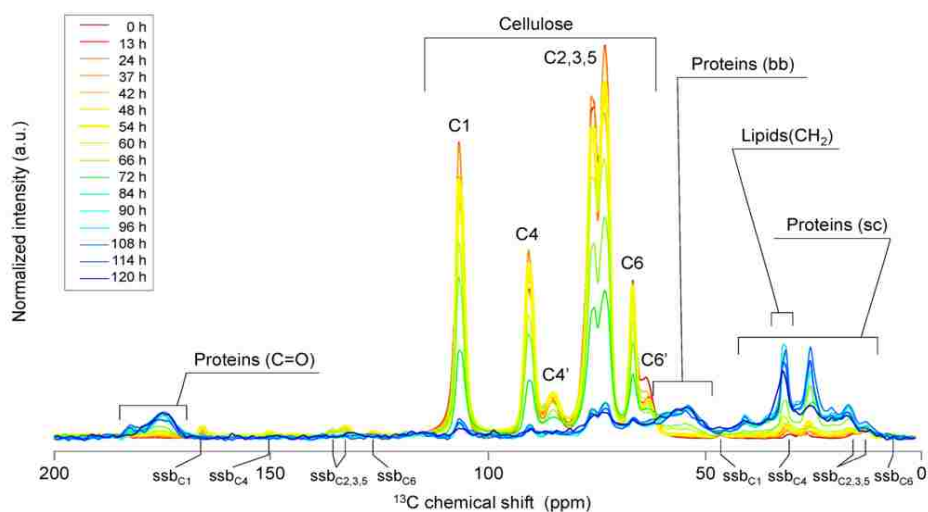


Figure 2.14. Example of NMR spectra of wood

The signal at 95-110 ppm is from Carbon-1 of cellulose. The spectra present at 60-90 ppm are for carbon-4, which are glycosidic bond carbons. (Zhu et al., 2015). This carbon-4 signal is used to estimate crystallinity, and lateral dimensions.

The spectrum present in 68-76 ppm arises from other carbons, carbon-2, Carbon-3, and carbon-5. The peak at 63 ppm represents carbon-6 of the cellulose molecule. The spectrum at 53 ppm represents lignin present (Zhu et al., 2015).

3. MATERIALS AND METHODS OF THE BIOCHEMICAL REACTORS

3.1. DESCRIPTION OF BIOREACTORS

In these bioreactors, wood chips, and horse manure were used as substrates. Horse manure has an ecosystem of bacteria including cellulose degrading bacteria, sulfate-reducing bacteria, and other microorganisms, which in the BCR aids in reducing sulfate from mine drainage. The purpose of the wood chips is to provide cellulose for cellulose degrading bacteria, which in turn will increase the activity of sulfate-reducing bacteria. The bottommost and topmost parts of the bioreactors were covered with limestone gravel. This might increase the pH, and alkalinity of the bioreactor, but was added to have proper distribution or flow of water. The diagram of a bioreactor, used in this research is shown in Figure 3.1.

3.2. DESCRIPTION OF OVERALL SETTING

A translucent round deli container 6 inches in diameter, and one foot in height was used as a reactor. Seventy-three reactors were set on two plastic shelving units. Each reactor contained 3 kilograms of limestone gravel, plastic mesh (to separate gravel from the chip bark), 200 grams of wood chip bark mixed with 150 grams of horse manure, plastic mesh again and then gravels respectively as shown in Figure 3.1. The ratio of horse manure, and wood was 2/1.5. Two peristaltic pumps (Cole Parmer Model No. 7553-70) were used to pump water. Two tanks were used in this experiment. 60 grams of Na_2SO_4 were added to 10 gallons of water in the smaller tank making it 1000 mg/L of sulfate concentrated water like mine drainage. The bigger tank represents a river into which sulfate concentrated water is pumped. The second pump was used to deliver the river concentrated with sulfate water to a main manifold. From the manifold, water

flowed to all biochemical reactors by way of six sub-manifolds. Figure 3.2. shows the BCR on the shelving and water delivery system.

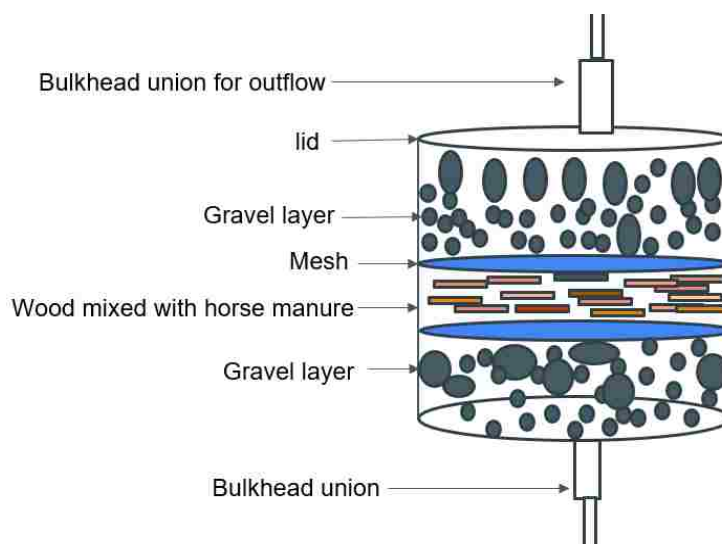


Figure 3.1. A bioreactor with its internal diagram

Every month, one reactor was taken out in order to measure percentage of cellulose and sulfate concentration. Drain system is 2m high which is situated just back of shelf. Flowrate of outflow was measured. All reactors are up-flow bioreactor system as water is flowing from down to up.

The dry weight of the chip bark (sample) was measured by keeping the chip bark in an oven at 121 ° C for 24 hours.

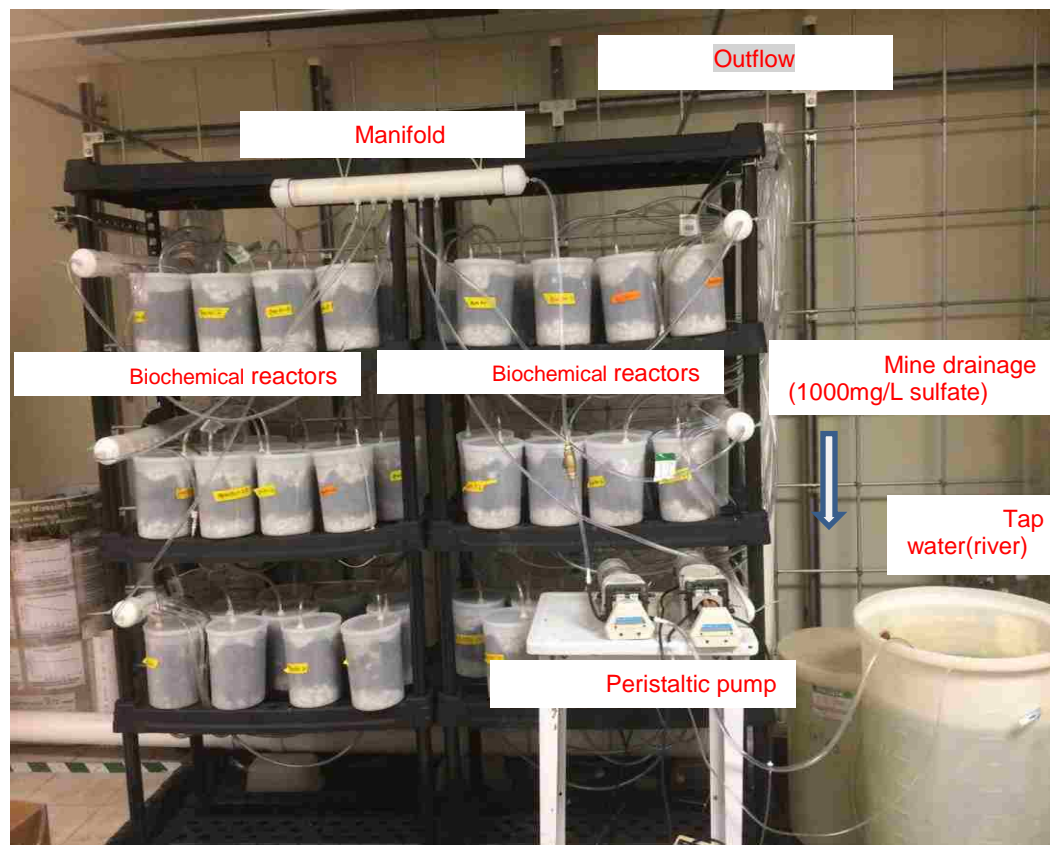


Figure 3.2. Bioreactor set up in research

Trial done for the experiment: Before, container only with chip bark was placed on the shelf. Water could flow through container. Excessive compression was seen, after sometimes, it flooded. That is why graves were kept for proper distribution of water. Five reactors were placed in the shelf with the flow rate of 0.15 ml/min for trial. After that, seventy-two reactors were placed in shelving.

3.3. CHEMICAL METHODS

A schematic of the extractions required to quantify wood composition is shown in Figure 3.3.

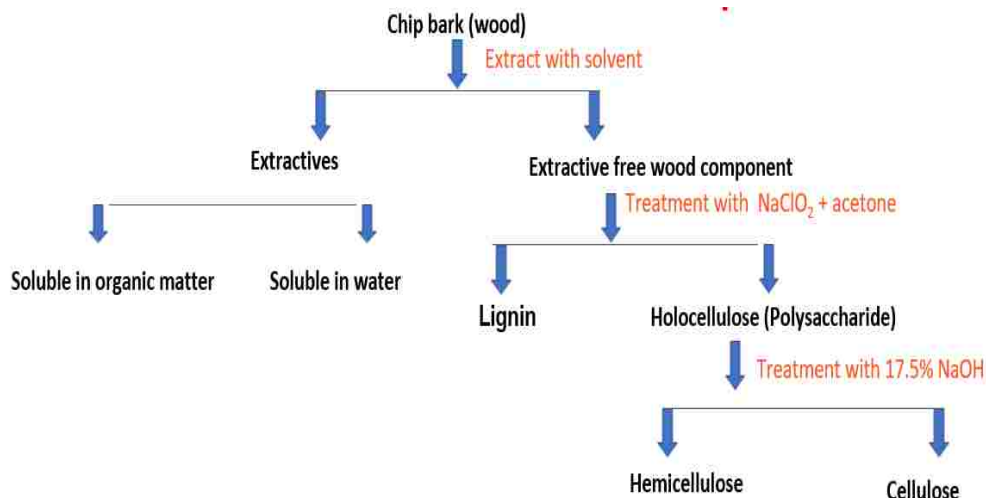


Figure 3.3. Process to get chemical composition of wood (Basu, 2013)

3.3.1. Extractives. For the determination of the extractives, the methods were followed as per Rowell (2005). First, a sample of blended wood was dried at 105°C in an oven for 24 hours. The sample was blended in a blender, and then passed through a sieve of 0.44 mm size. Three grams of the sieved solid was taken in an extraction thimble. A Soxhlet extractor was made ready by heating water in a pan. A round bottom flask was placed in the hot water bath, adding the extraction thimble with 3 grams of sample to the Soxhlet extractor. Then Soxhlet extractor, and condenser was connected to the round bottom flask. 200 ml of acetone (98% concentrated), and 50 ml of DI water were added to the round bottom flask and the water bath was heated to 450°C. The extraction was carried out in a well-ventilated chemical fume hood for 6 hours. After the extraction, the thimble was removed from the extractor to drain the excess solvent. The excess solvent was kept in a bottle to re-use later on. The solid sample was washed with 50 ml ethanol, and then filtered by filter paper. The residue was then dried in the oven at 105°C for 24

hours. After being dried, the sample was cooled in a desiccator for an hour. Thereafter, being dried, the weight of the sample was taken. This mass of solid is the mass of the extractives-free saw dust.

3.3.2. Holocellulose. The water-insoluble carbohydrate fraction of wood is known as holocellulose. To extract holocellulose, the chlorination method was applied to remove the lignin, with the remaining solid characterized as holocellulose. From the solid remaining after the Soxhlet extraction, a 2.5-gram sample was placed in a 250-ml Erlenmeyer flask. 80 ml of hot distilled water, 0.5 ml of acetone, and one gram of sodium chlorite (NaClO_2) were added to the flask. The mixture was heated in a water bath at 70°C for one hour. After every hour an additional dose of 0.5 ml of acetone, and 1 g of NaClO_2 were added until six hours had passed, and a total of seven dosings (including the original dosing) had been added. The mixture was left in a desiccator for 24 hours, and then it was filtered through filter paper. The residue was washed again with acetone, and left in an oven to dry at 105°C for 24 hours. The solid whitish residue remaining, the lignin-free holocellulose, was weighed.

3.3.3. Hemicellulose. The solid whitish residue from the previous extraction, (lignin free) holocellulose, was used for determination of cellulose. A 2-gram dry sample of holocellulose was placed in a 250 ml Erlenmeyer flask. 10 ml of 17.5% NaOH was added to the flask, and a glass lid was placed on it. The flask was kept in a water bath at 20°C , and stirred by using stirrer machine (Orbit shaker-3520). Every 5 minutes, another 5 ml of 17.5% NaOH was added to the flask until 15 minutes had passed, and 25 ml of 17.5% NaOH in total had been used. After the addition was done, the mixture of the sample, and the 25 ml of 17.5% NaOH was kept in a desiccator for 30 minutes. After 30

minutes had passed, 33 ml of water was added to the mixture, and then filtered through filter paper. The residue cellulose was then washed in 100 ml of 8.3% NaOH solution, and was subjected to treatment with acid by adding 15 ml of 10% of acetic acid. The cellulose was then again washed, and filtered water, and dried at 105°C for 24 hours.

3.4. DETERMINATION OF SULFATE CONCENTRATION

To determine sulfate concentration, a Hach Colorimeter is used. To get sulfate concentration, program 91 is set. First, 10 ml of sample is collected in a vial. The “Zero” key should be pressed after the sample is filled in vial. SulfaVer sulfate reagent powder pillow is added to the solution inside the vial. The vial should be shaken to let it dissolve. After five minutes, the “READ” key is pressed. And the concentration of sulfate in water is reported.

3.5. DETERMINATION OF FLOWRATE

Flowrate was measured with help of graduated cylinder and timer. Each out flow was taken out from the drain system. The water from each outflow was collected in graduated cylinder for one minute. Hence in this was flowrate of each reactor was calculated in ml/min. Second flowrate was taken by undergrad research intern, Kala Morgan.

4. BCR RESULTS

The protocol which is followed in this research was gravimetric analysis (P.Basu et al., 2008), performed twice in order to check accuracy. The results with fresh chipbark are shown in Table 4.1.

Table 4.1. Chemical composition of fresh wood

Reactor	Extractives (%)	Lignin (%)	Hemicellulose (%)	Cellulose (%)
Fresh chip bark	5	20.0	23	52
Second time	5.0	19.38	22.62	53

Fresh chip bark was taken, and chemical analysis was performed. From the experiment we got 52% of cellulose, 23% of hemicellulose, 20% of lignin, and 5% of extractives. Same experiment was done for 2nd time. For second time, there was same % of extractives. Percentage of lignin was found to be 19.38. Hemicellulose was found to be 22.62%, and cellulose was 53%. There is only slight change in lignin, and hemicellulose.

Each month, one reactor was sacrificed for determination of cellulose, and sulfate concentration. The result is shown in Table 4.2. To take out a sample flow was cut off using a clamp. Water in the reactor was emptied. After that, we take out lids, gravel, and finally mixture of wet horse manure and wood is taken out. Horse manure was dumped,

and chip bark was taken in a clean tray. The chip bark was dried in an oven for 24 hours at 103°C.

In the beginning, the percentages of extractives, lignin, hemicellulose and cellulose of oak and pine was determined. Pine is soft wood, and oak is hard wood. The result that we got from chemical analysis is described in Table 4.2. Percentage of cellulose of pine was found to be more than oak.

Table 4.2. Percentage of chemical composition of pine and oak

	Lignin (%)	Hemicellulose (%)	Cellulose (%)	Extractives (%)
Oak	29	21.25	48.75	1
Pine	31.1	18.97	50	0.9

From Table 4.3. shows the first month, the percentage of cellulose in the first reactor degraded to 48% from the zero- month values of 52%. In Table 4.3., lignin percentage was found to be increasing. In the second month reactor, cellulose percentage was found to be the same as the cellulose percentage of first month reactor. But there was an increase in lignin. In the third month cellulose percentage was recorded to be 46%, and again lignin was slightly increased. In fourth month, cellulose percentage reached 46.5%. The percentage of lignin increased to 27%. In fifth sampled reactor, the cellulose percentage was recorded to be 45%. Lignin was recorded to be increased in fifth reactor. In sixth month, cellulose increased than cellulose of fifth month reactor. In 6 months, it

was recorded that there remained 47.5% of cellulose and 25.5% of lignin. The graph in Figure 4.1. shows the degradation of cellulose from 1st month to 6th month reactor.

Table 4.3. Chemical composition of wood in reactor (1st to 6th) month

Reactor	Month	Extractive (%)	Lignin (%)	Hemicellulose (%)	Cellulose (%)
Reactor 1	1	6	23	23	48
Reactor 2	2	5	25	22	48
Reactor 3	3	7.6	27	20	46
Reactor 4	4	6.5	27	20	46.5
Reactor 5	5	7	28	20	45
Reactor 6	6	7	25.5	20	47.5
Reactor 7	7	7	28	20.5	44.5

The degradation of cellulose each month is shown in Figure 4.1. The graph looks like cellulose is following first-order degradation. For six months the interval of time, lignin may be assumed to be constant in biochemical reactor. Lignin is non-biodegradable (Ye, 2006). A graph of cellulose/ lignin vs time is shown in Figure 4.2. From Table 4.3., the percentage of cellulose has decreased but lignin has started

increasing. The reason is that microorganism like cellulose most. They only feed cellulose; because of that lignin may have increased more in 3-gram sample used for chemical analysis. In the first month, the ratio of cellulose, and lignin was found to be 2.5. In sixth month, cellulose/ lignin was found to have slightly from previous months, but the trend is a decreasing ratio.

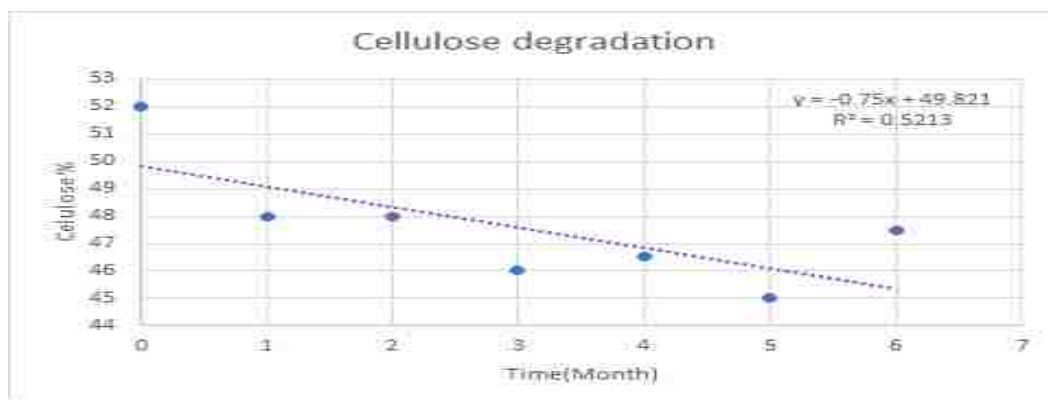


Figure 4.1. Degradation of cellulose in bioreactors

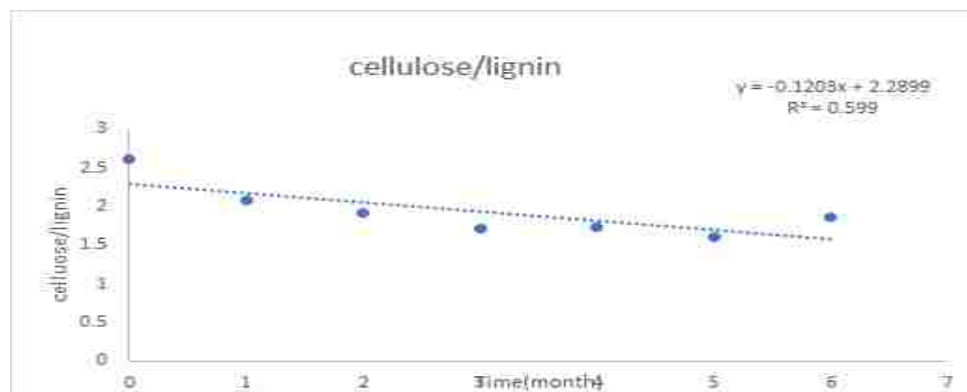


Figure 4.2. Graph for Cellulose/lignin vs time

Figure 4.2. shows the normalization of cellulose for lignin. First, original lignin was calculated with help of Table 4.3. Lignin was calculated in original total mass of 3-g. Remaining cellulose mass was calculated from Table 4.1. Original cellulose mass was calculated by keeping lignin constant.

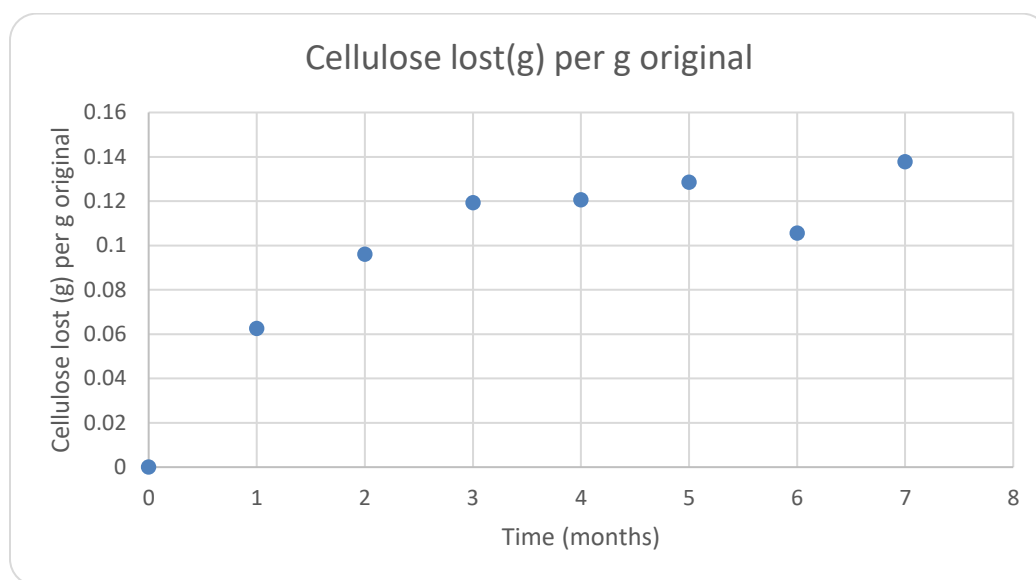


Figure 4.3. Graph for Cellulose lost/ original mass (g/g)

The cellulose lost per gram per gram of original mass of wood. In first month, 0.06 gram of cellulose was lost per 300 grams of wood. In second month it was found to be 0.1 grams. In second, third, fourth and fifth month, the decrement was found to be 0.12gram, 0.121-gram, 0.13 gram respectively. In sixth month, the decrement was found to be only 0.1gram. In seventh month, it decrements was found to be 0.138gram per original mass.

4.1. PREDICTION OF TIME FOR MAXIMUM DEGRADATION

From Figure 4.4., it was found that there is first-order degradation. By using a first order equation, Figure 4.4. was the result. The first point is the percentage of cellulose in 1st month reactor. The first six points are the result of this experiment. From Figure 4.4, it is concluded that in 8 years, there will be less than 10% cellulose present. The red point data in Figure 4.4. represents the result that we have done for four months. The blue points data is the prediction that we got from first order kinetics equation.

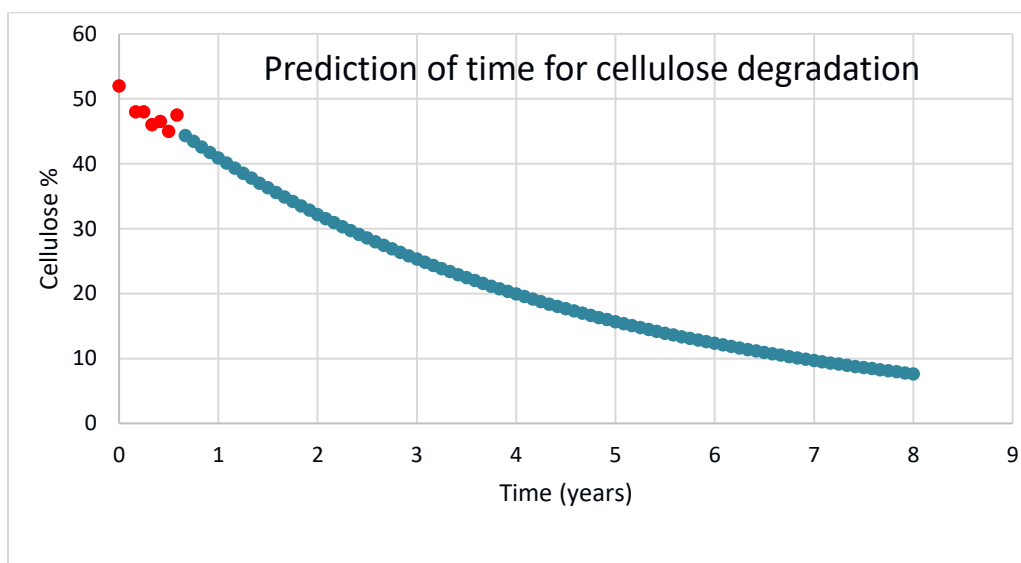


Figure 4.4. Prediction of cellulose degradation

4.2. SULFATE CONCENTRATION REDUCTION

According to Figure 4.5., the sulfate concentration was found to be around 100 mg/L, when percentage of cellulose in reactor was 52%. Whenever a reactor was taken out, influent sulfate concentration of the water in the reactor was measured. In the first month, it was found to be degraded to 70 mg/l. In second month, percentage of cellulose was 48%.

In sixth month it was degraded to 45 mg/L of sulfate concentration in the outflow, when percentage of cellulose was found to be 44.5%.

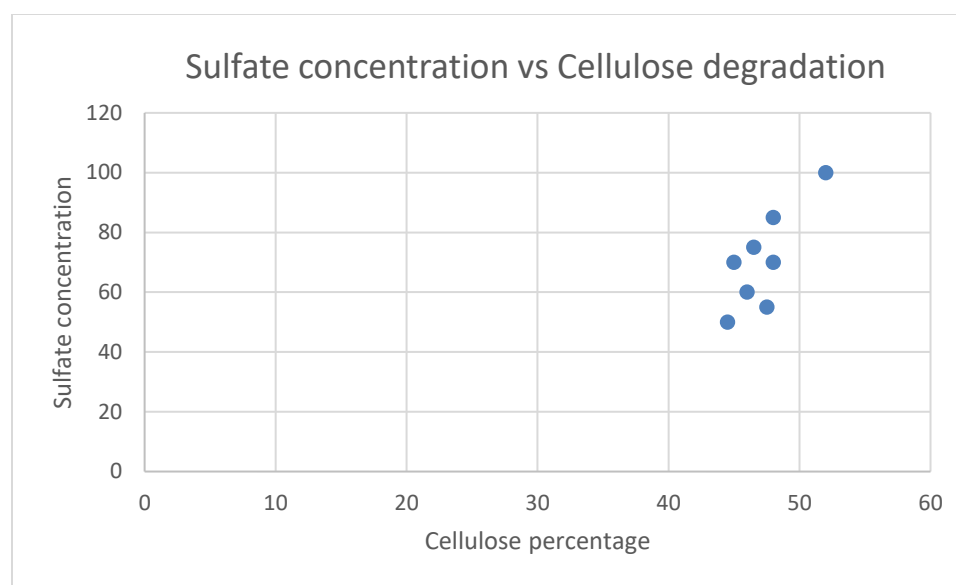


Figure 4.5. Degradation of sulfate concentration vs cellulose degradation

According to Figure 4.6., in fifteen days, concentration of sulfate was 100mg/L. Effluent of first month reactors has sulfate concentration of 70mg/L. The sulfate concentration of effluent of second month reactors was found to be 85mg/L. Effluent of third month has little lower than third month reactor, which was examined to be 60mg/L. Effluent of fourth, fifth and sixth month reactors was found to be decreasing to 55mg/L. In seven-month reactors, the effluent has sulfate concentration of 50mg/L.

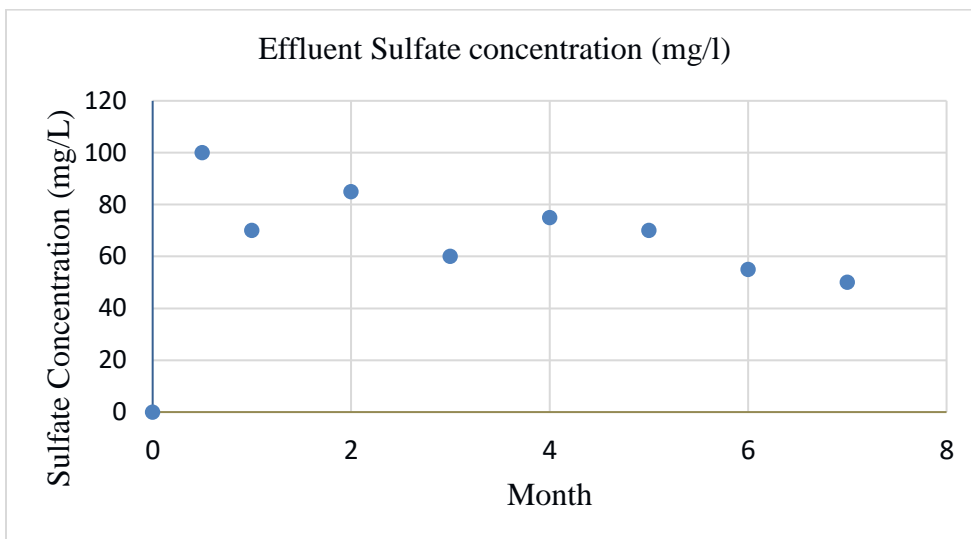


Figure 4.6. Sulfate reduction vs time

There is inconsistent in the result as all bioreactors do not have consistent flowrate. The inconsistency is due to failure of adjustment of flow rates to all the reactors. The other reason is the leakage in bulk head union and lid. Poor quality and cheaper reactors were used, which resulted in continuous failure in adjustment of flowrates.

The flow rate was predicted to be 0.15mg/L. The flowrate could not be maintained due to the failure of biochemical reactors. The sulfate reduction data is shown in Figure 4.7., if its flow rate is 0.15 mg/L.

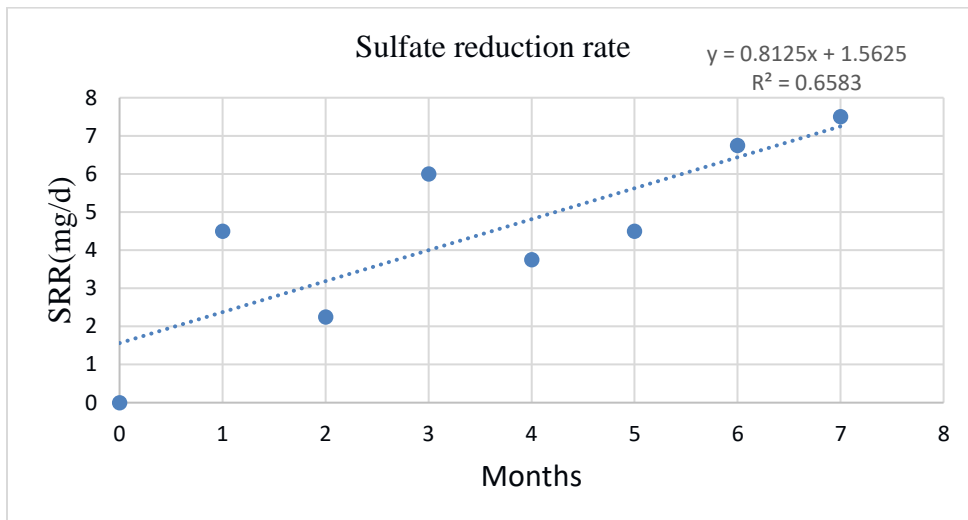


Figure 4.7. Sulfate reduction rate data

5. CELLULOSE DEGRADATION BY OZONE

The ozone experiment was evaluated as a potential surrogate for much slower biological degradation. It may be possible to predict the higher degradation rate of cellulose with help of ozonation experiment. Ozone is strong reactive species (Liu, Wu, and Chen, 2007). Ozone treatment was found to be effective for degrading cellulose in wood components. (Kobayashi, Asano, Kajiyama, and Tomita, 2005). Applied ozone oxidizes complex structure of cellulose, and hence degrades cellulose from wood. (Lee, Hamid, and Zain, 2014).

5.1. OZONE GENERATOR

Ozone produced is a very powerful, and can oxidize odors, smoke, harmful contaminants, and the organic materials in oxidant chip bark. An Ozonia, Model 03V9-AR/ was used to generate ozone. An oxygen cylinder was connected to ozone generator. The flow of ozone was maintained with the help of a flow meter, which was already present in the ozone generator. To vary the doses of ozone, a variety of flow rates, and exposure times were tested. The flow rates were 1 LPM (Liter per minute), 2 LPM, 3 LPM, 4 LPM, 5 LPM, and 7 LPM. The high voltage corona present in the ozone generator ionizes water flowing through the generator, and converts oxygen(O_2) to ozone(O_3).

5.2. OZONE ANALYZER

The ozone analyzer (Figure 5.2.) used in this experiment is a “PCI ozone and control system ozone monitor HC-12”. The monitor is used to determine the percent by weight of ozone produced by the ozone analyzer. The analyzer has an inlet for ozone as shown in Figure 5.3. Ozone produced from the ozone generator passes to a stainless-steel

container(6L) from the ozone generator, then to the analyzer. The container (Figure 5.4.) contains the sample and 4 liters of water. After passing through the analyzer, the gas passes to the fume hood. Nitrogen gas was used as the inert gas, which was connected to the ozone analyzer. The ozone analyzer gives the percent by weight of ozone flowing through the ozone analyzer.

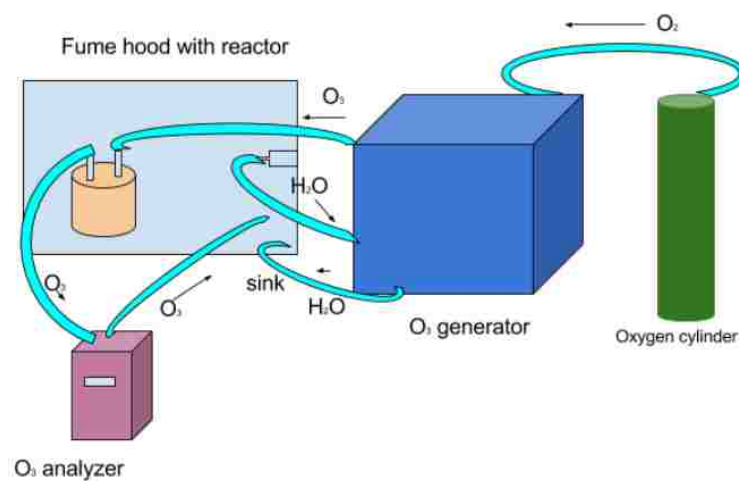


Figure 5.1. Set up of ozone experiment



Figure 5.2. Ozone generator



Figure 5.3. Ozone analyzer



Figure 5.4. Container for ozone experiment

The ozone produced in water, based on percent by weight ozone is:

$$\text{Ozone output (g/hr)} = F * T * (14.3 \text{ g/m}^3 * (\text{perc. by wt.}) \dots (6)$$

where,

F is the flow rate of ozone analyzer (in milliliter/minute)

T is the total time for which the ozone analyzer was active.

14.3 g/m^3 is the density of oxygen in g/m^3 .

Perc. by wt is the ozone content as measured by the ozone analyzer.

5.3. METHODS OF OZONATION

The flow of ozone-containing gas results in pressure within the reactor, which should be strong enough to bear the pressure. When an Erlenmeyer flask with a rubber stopper was first used, the rubber stopper broke into pieces within two minutes. First, a plastic container was used. The plastic container worked well for 1 LPM but due to the high pressure the plastic container did not work properly for 2 LPM of ozone flow. The lid of the plastic container was swollen, and opened making loud sound. Therefore, a reactor made of stainless steel with a proper lock system was used.

In each experiment, a differing dose of ozone was applied to a mixture of water, and chip bark for several hours. A set flow rate (1 LPM to 6 LPM) was allowed to flow for a different number of hours. 7LPM was also tried, but due to high pressure, the container started shaking. Weight of the dry sample was recorded at the end of the experiment. At a given ending hour, the weight of sample was measured. The sample(chip-bark) was removed with the help of sieve. The sample was kept in the oven for 24 hours to dry it. Thereafter the remaining mass was measured. Cellulose content was then determined.

5.4. RESULT OF OZONE TREATMENT (CHIP BARK)

Two separate experiments were done in which ozone was dosed to chip bark, and 4 liters of water. When 0.2-gram of ozone was dosed, only 15% of the wood was degraded (Figure 5.4.). When 0.35 gram of ozone was dosed, 25% of wood was found to

be degraded. The maximum degradation was found to be around 35%. When only 0.8 gram of ozone had been dosed to the container containing chip bark.

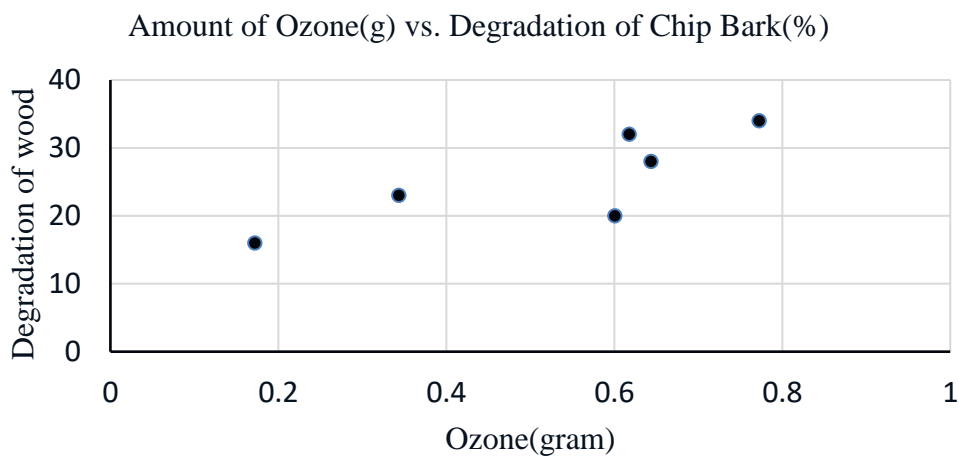


Figure 5.5. Degradation of cellulose by ozone in first set of experiment.

In second experiment, same procedure was followed for ozone treatment. In this experiment, different doses were given. 4-g of ozone could reduce 15% of chip bark. The maximum dose, treated was 11-g of ozone. 11-g of ozone degraded 35% of ozone as shown in Figure 5.5.

Different results were observed between the experiment in two different sets of experiment. The reason behind these differences may be due to the following reasons:

- Differences in dose of ozone reported by the ozone analyzer
- Differences in degradability of wood

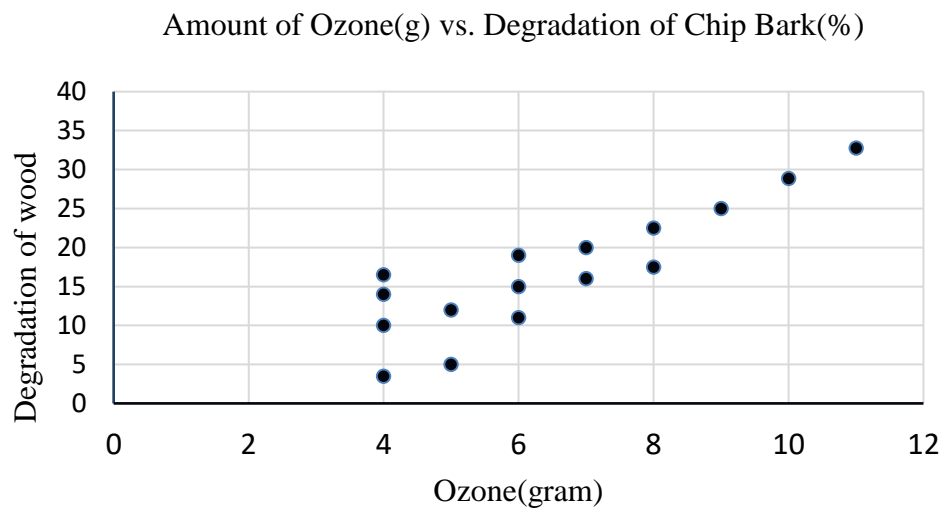


Figure 5.6. Degradation of cellulose by ozone during second set of experiments

6. DYE EXPERIMENT

To check precision or accuracy of the ozone analyzer, an experiment with dye was done. First, to determine the proper wavelength measurement (less than 0.8 cm^{-1}) of dye, 10 ml of dye was mixed with 10 ml of DI water. In 4 liters of water, 0.6 ml of the mixture of dye, and water was added. The maximum absorption wavelength was checked using a Cary UV scanning spectrophotometer.

After checking the result for each 0.1 ml was added, it was found that after adding 0.6 ml of water in 4-Liters of water, the result (Figure 6.1.) was good, which was an absorption between 0.8 cm^{-1} , and 0.7 cm^{-1} .

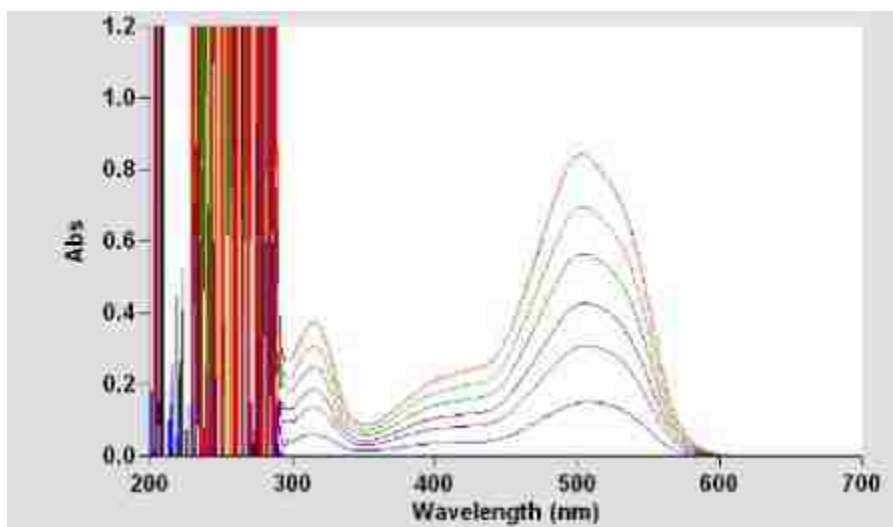


Figure 6.1. Checking appropriate drops to make 0.8 cm^{-1}

Two experiments were done. First, 0.6ml of the mixture of dye, and water was added to 4 liters of DI water. The mixture was treated with ozone flowing at 2 LPM.

After ten minutes, 2 ml of sample was taken, and checked using Cary UV scanning spectrophotometer.

For the first experiment, it took 40 minutes for the adsorption at 0.8 cm^{-1} to fall below 0.02 cm^{-1} . Initially, an absorption of 0.8 cm^{-1} was recorded (Figure 6.2.). After treating with ozone for 10 minutes, the absorption of dye had decreased to 0.2 cm^{-1} . Treating with ozone for more 10 minutes, it was observed that absorption decreased to 0.1 cm^{-1} . The final treatment was to use 40 minutes of ozone exposure. The last result was an absorption of 0.001 cm^{-1} .

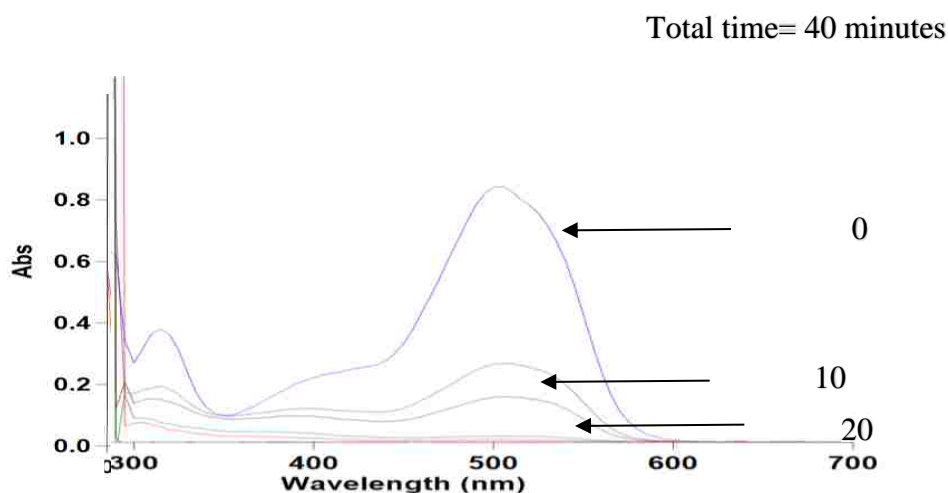


Figure 6.2. First dye-ozone test

For the second dye-ozone experiment, the same protocol was followed. For the first experiment, it took 70 minutes for the adsorption at 0.8 cm^{-1} to fall to 0.02 cm^{-1} . Starting at 0.8 cm^{-1} , after 10 minutes, absorption had decreased to 0.5 cm^{-1} (Figure 6.3.). Again, treatment with ozone was given for 10 more minutes, 0.2 cm^{-1} was observed. But

after 10 minutes no degradation was seen. The experiment was done for a total of 70 minutes. After 70 minutes, the absorption was found to be 0.002 cm^{-1} .

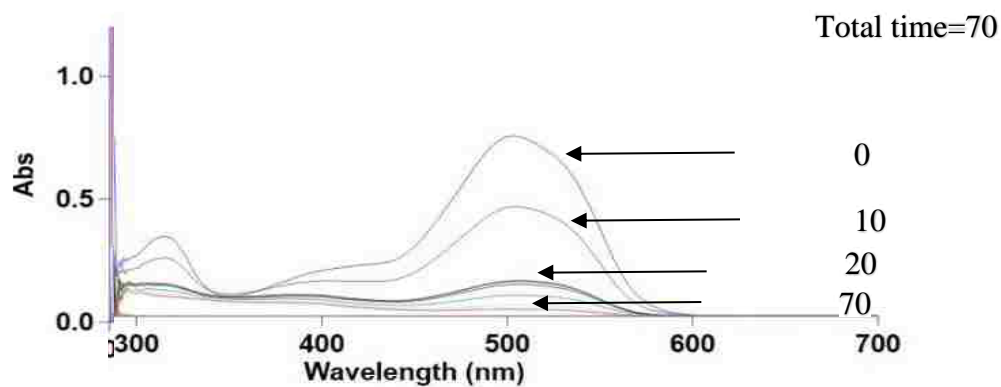


Figure 6.3. Second dye-ozone test

The two experiments showed two different degradation rates despite having the same reported flow rate and ozone dose. Therefore, it was concluded that there was a difference in doses coming from the ozone generator and therefore degradability of wood is reliable.

7. NMR (NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY)

NMR is one of the analytical methods used in this study. NMR is applied by chemists, and physicists in order to find structural information from various chemical including natural organic materials(NOM) (Hult, Larsson, and Iversen, 2000). NMR allows nondestructive determination of subunits much like chemical extraction, Raman spectroscopy, and electron paramagnetic resonance can. In this study NMR was used to confirm, and quantify the cellulose content of the wood samples.

NMR uses the property of the nuclear spin in order to determine the chemical structure of chemical(Earl and VanderHart, 1980). The nuclear spin is the property of any nucleus which has an odd number of protons, and/or neutrons (Cano-Barrita et al., 2015).

The two options for NMR procedures to measure the degradation of cellulose are liquid-state NMR or solid-state NMR. Overall, solid-state NMR is more beneficial when measuring the degradation of organic compounds. Liquid-state NMR, although very high quality, does not always accurately reflect the structural information of the material (Maunu, 2002).

7.1. NMR FOR CELLULOSE

Cellulose is the most important component of wood since it is the most prominent constituent found in plant cells. It is found to exist in various polymorphic crystalline forms (Xia, Petti, Williams, and DeBolt, 2014). To understand the composition and sequence of the polysaccharide unit of cellulose, solid state NMR resonance is a most informative technique. (Duffy, Pandit, and Ruban, 2014), (Tynkkynen et al., 2012) (Holtman et al., 2010)The NMR technique is very useful in studying the supramolecular structure of cellulose (Grunin, Grunin, Nikolskaya, Sheveleva, and Nikolaev, 2017). The

high resolution of solid state NMR also aids in the study of crystalline cellulose.

Overall, NMR determines the quantity of functional groups in complex components as all equivalent nuclei gives rise to signal of equal intensity (Hammes, Smernik, Skjemstad, and Schmidt, 2008)

7.2. METHOD

Samples used for this research were kindly analyzed by Suraj Dhantula, Dr. Nicholas Leventis's current Ph.D student! The procedure to get spectra from NMR was reported by Xiao et al., (2009).

First, sample was blended to sawdust in a blender. A 5 gram of oven-dried chip bark sawdust was extracted using 250 ml of acetone. The extraction was done with the help of Soxhlet extractor. The acetone solutions used for extraction were 200 ml of acetone, and 50 ml of water.

The extracted sawdust was ground to a powder of about 150 μm , and stored in phosphorus pentoxide. It was given to Dr. Dhantula. High resolution for NMR was used by him to get a spectrum of the sample, using a Bruker AVANCE AV 400 spectrometer, operated at 400 MHz. The spectrometer contains a double-tuned solid-state probe which is equipped with a 7-mm spinner (outer diameter). The extracted sawdust was loaded into the NMR rotor cell, and weighed using an analytical balance. Almost 200 mg of sample was enclosed in a cylindrical cell machined from an aluminum nitride ceramic rod inside the 7mm NMR rotor. The ^{13}C CP/MS were recorded. The strength of ^{13}C is 62.5 kHz, and it spins at a rate of 15 kHz. The various spectra were obtained by applying a 2-ms contact time, 25.4 ms reacting time, and 2 s recycle delay. The spectra have 30 kHz sweep

width. There were 1024 scans of CP/MAS spectra. This fitting was performed by the built-in procedure in the software, known as MestReNova.

Cellulose-I has six signals seen in the CP/MAS ^{13}C -NMR spectra. The spectra are made up of an anhydroglucose unit which is split into fine structure clusters. The cluster present in spectra is due to the supramolecular structure of cellulose. (Lambert, Davies, and Neivandt, 2005), (Tokoh et al., 2002).

7.3. RESULT OF NMR METHOD

Figure 7.1. shows the degradation of cellulose over every six months. Compared to result of chemical analysis, there is slight like the result of NMR analysis. Calculation of cellulose degradation by NMR method: The cellulose percentage in NMR method was calculated by keeping spectra of lignin constant. There is difference between cellulose degradation graph (chemical analysis), and Cellulose degradation (NMR method). Percentage of cellulose from NMR method was found by taking integration (Figure 7.2). Spectra of lignin was kept being constant.

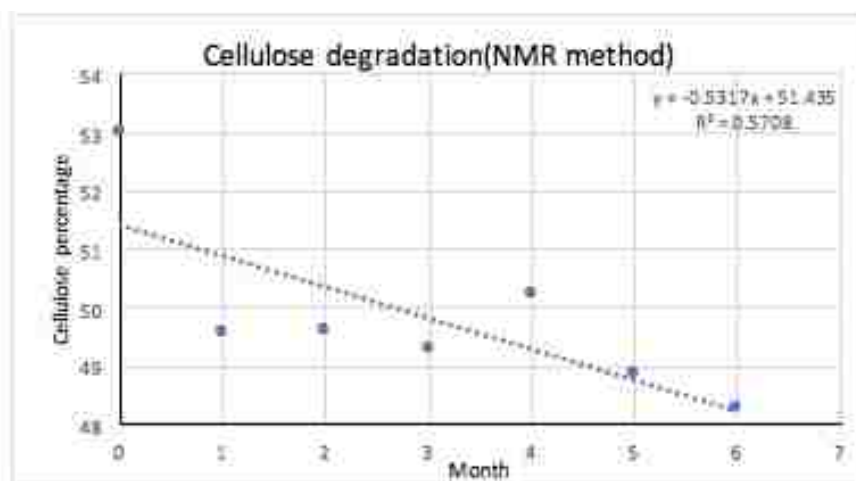


Figure 7.1. Cellulose degradation in NMR method

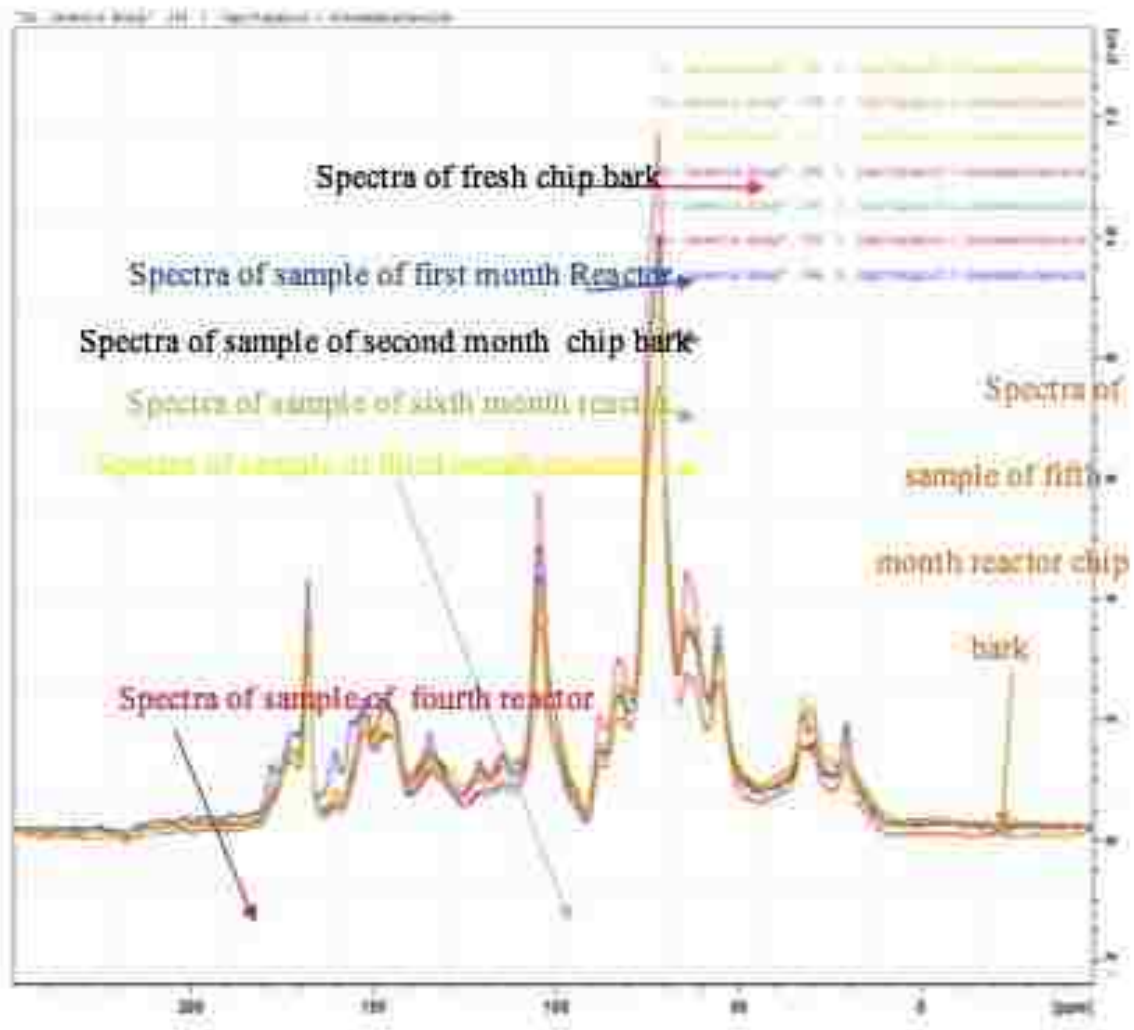


Figure 7.2. Degradation of cellulose over six months

8. DISCUSSION AND RECOMMENDATION

Degradation of cellulose, and sulfate by cellulose degrading bacteria, and sulfate-reducing bacteria was seen. Sulfate was found to be degraded by 45%, cellulose 4% over six months. Still there is an absence of consistency in the degradation of cellulose and sulfate. For all reactors, there is not a consistent flowrate as we assumed to be. Some reactors had no water effluent at the time the flow rate was taken but it is quite impossible to maintain flowrates without a flowmeter in each reactor without flow meter.

Other reasons for a disturbance in the flowrate is leakage of lids, and reactors. Almost 20 reactors were changed in the beginning due to a leakage in the bulk head union and bottommost part of reactors. Furthermore, more than 40 lids were changed due to leakage in lids caused by the pressure of the water needed to create an anaerobic environment. Sometime lids used to get punctured, and leakage used to be in floor. Once lab was flooded due to puncture in 1 reactor. The plastic that is used in this research is of poor quality and requires much maintenance due to the constant punctures and leakage that occur. Better reactors should be used to avoid these issues.

APPENDIX

A.1. NMR SPECTRA

A.1.1. Fresh Chip Bark

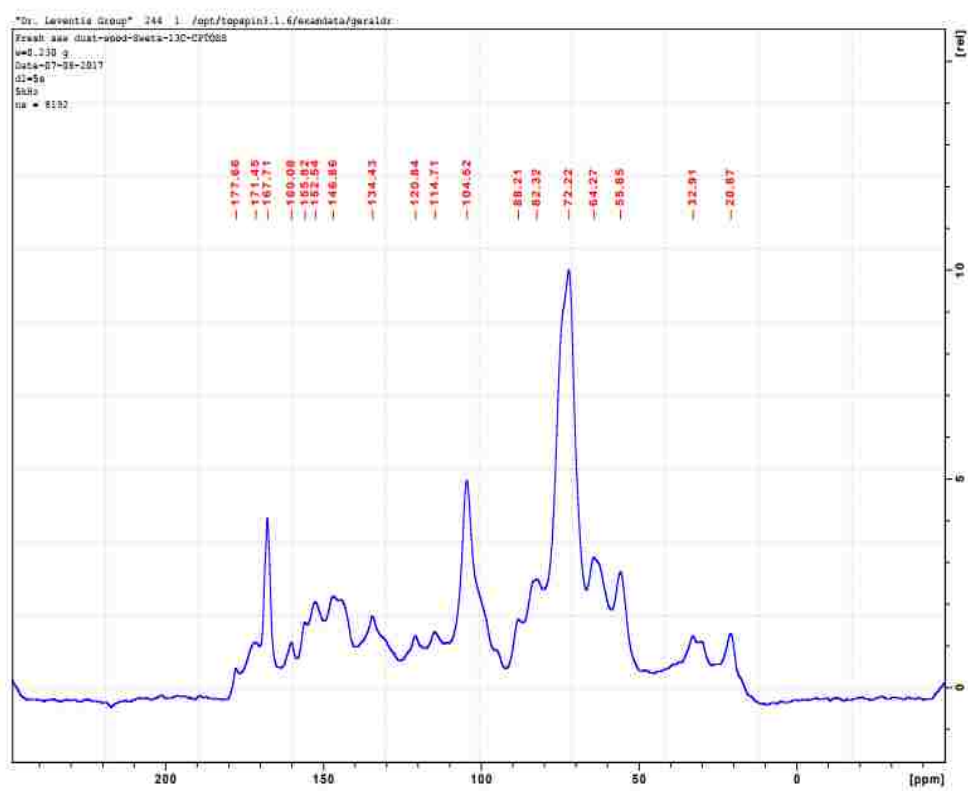


Figure A.1. NMR spectra of fresh chip bark

A.1.2. First Month

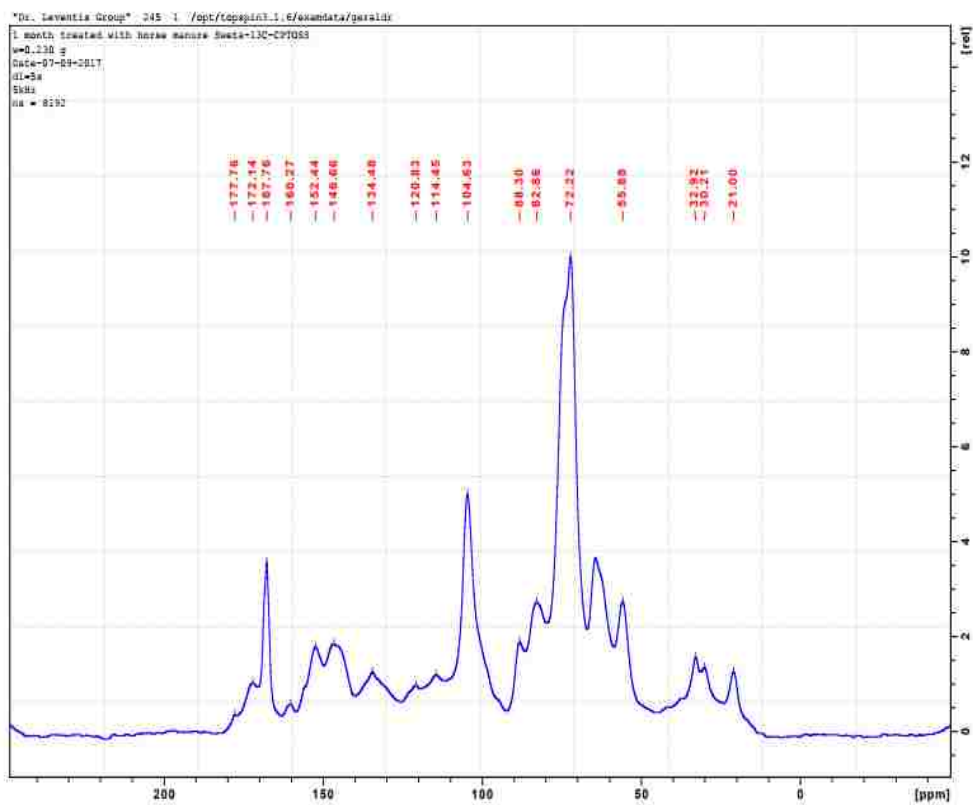


Figure A.2. NMR spectrum of sample of first month BCR

A.1.3. Second Month

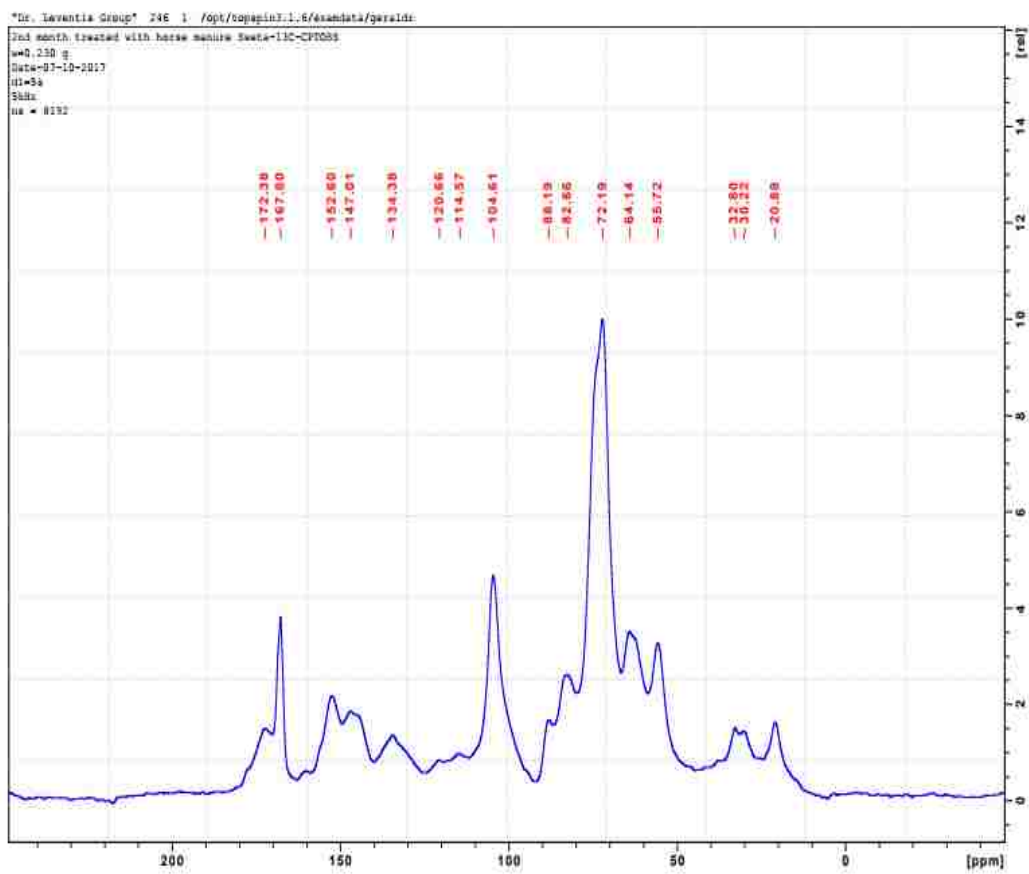


Figure A.3. NMR spectrum of sample of second month BC

A.1.4. Third Month

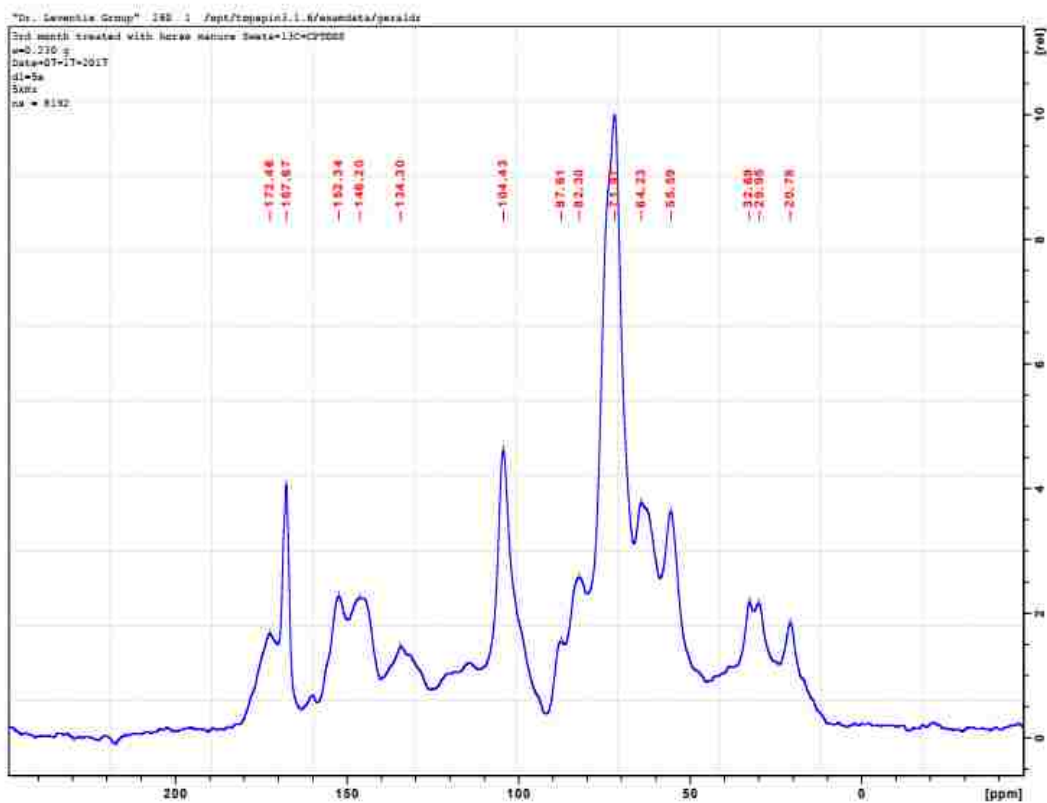


Figure A.4. NMR spectrum of sample of third month BCR

A.1.5. Fourth Month

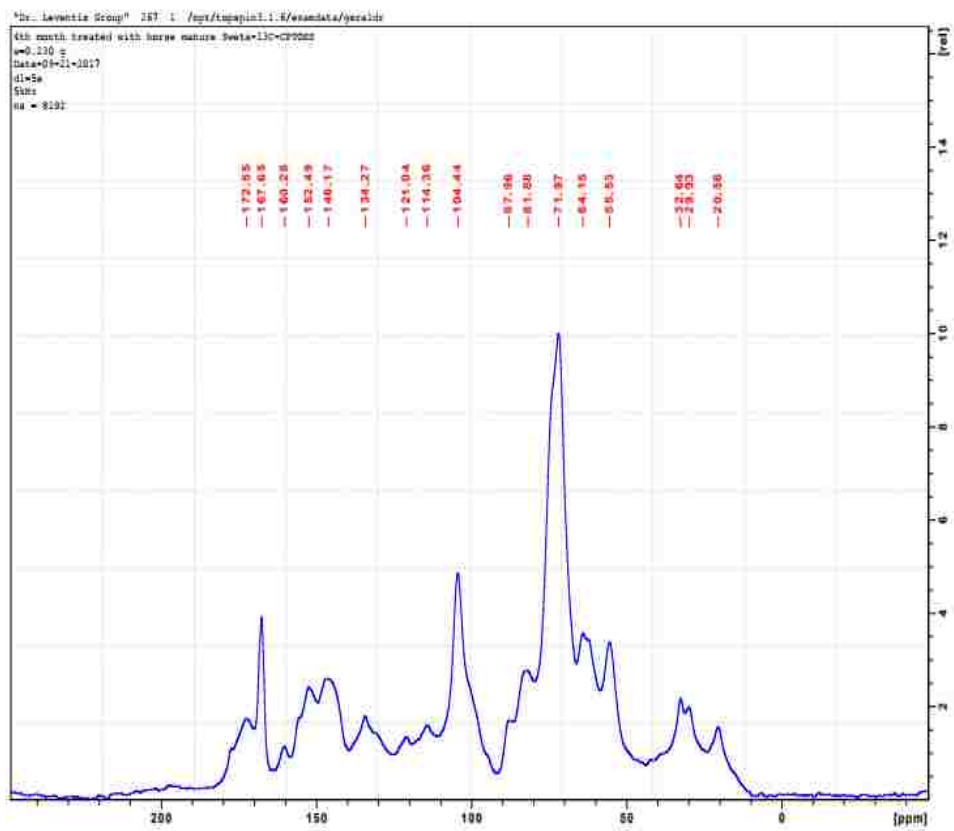


Figure A.5. NMR spectrum of sample of fourth month BCR

A.1.6. Fifth Month

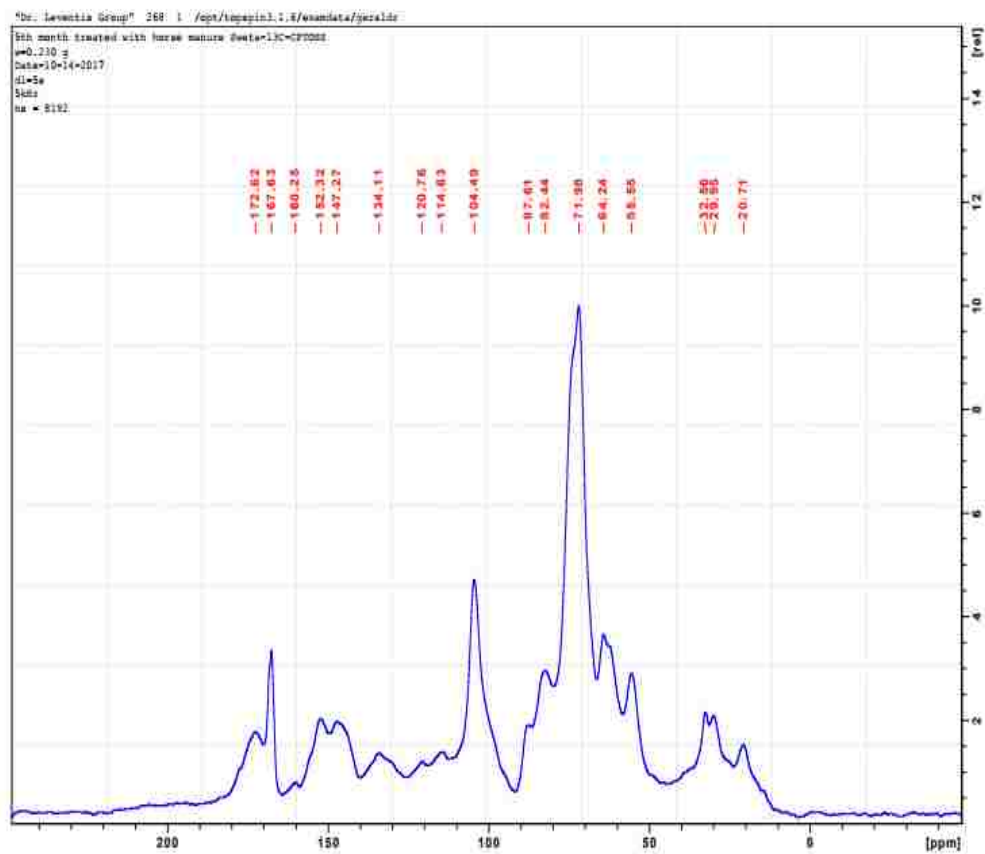


Figure A.6. NMR spectrum of sample of fifth month BCR

A.1.7. Sixth Month

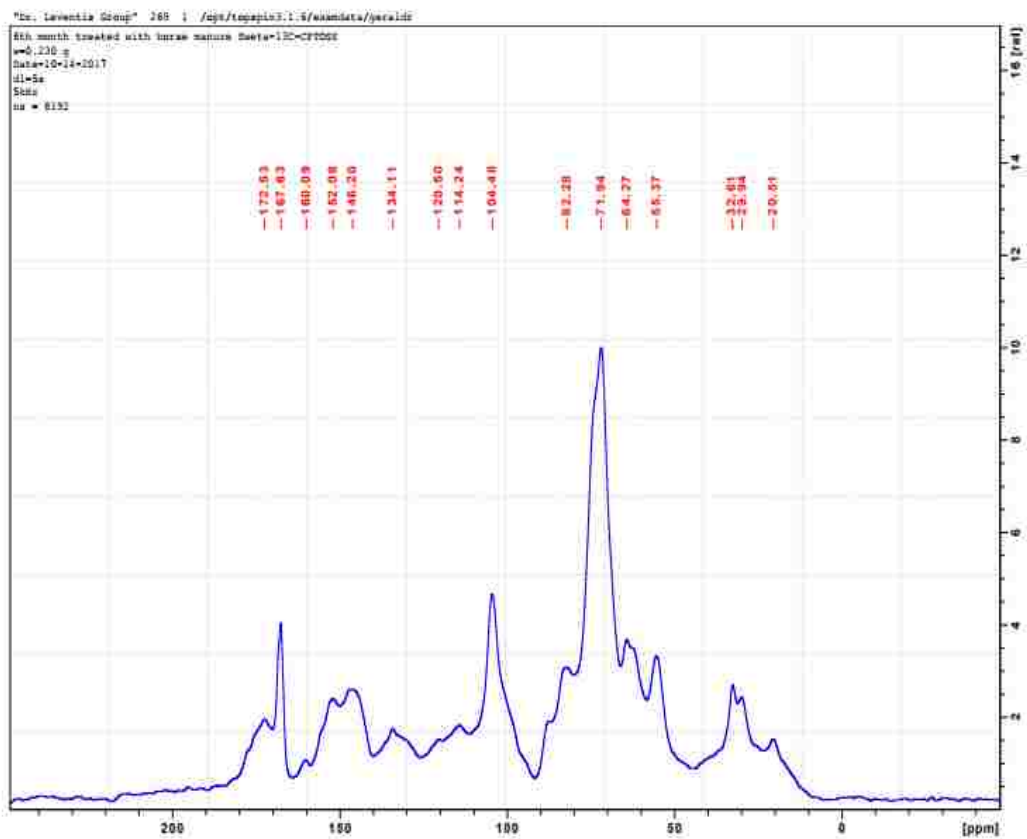


Figure A.7. NMR spectrum of sample of sixth month BCR

A.2.2. First Month

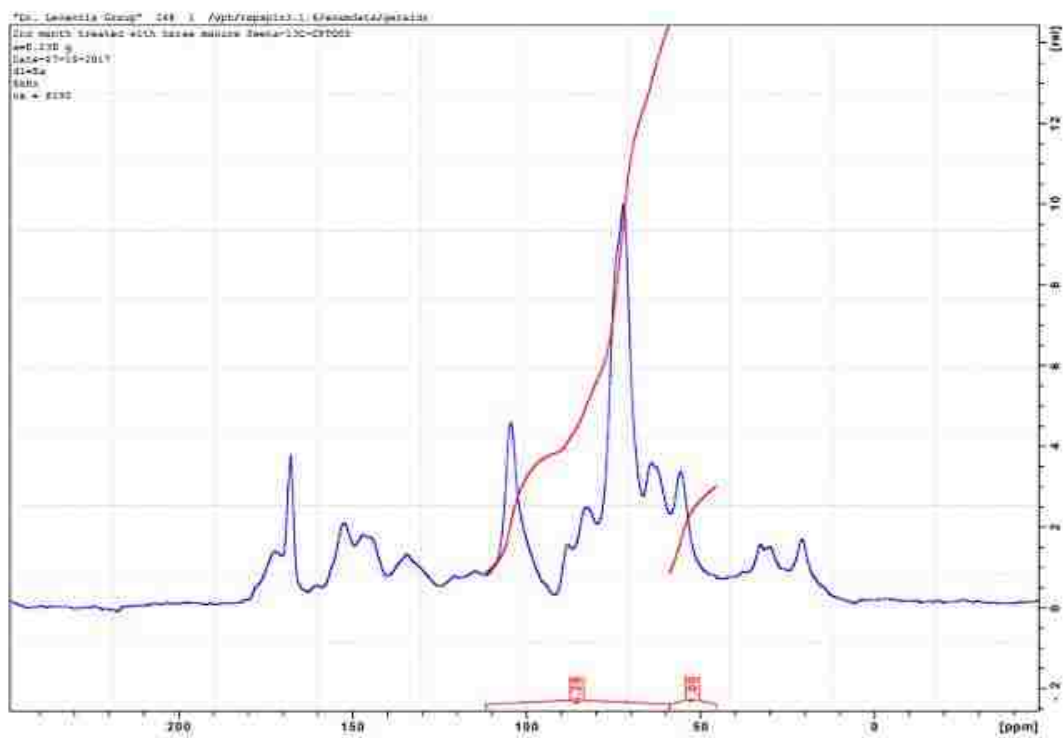


Figure A.9. Integration of spectrum of sample of first month reactor

A.2.4. Third Month

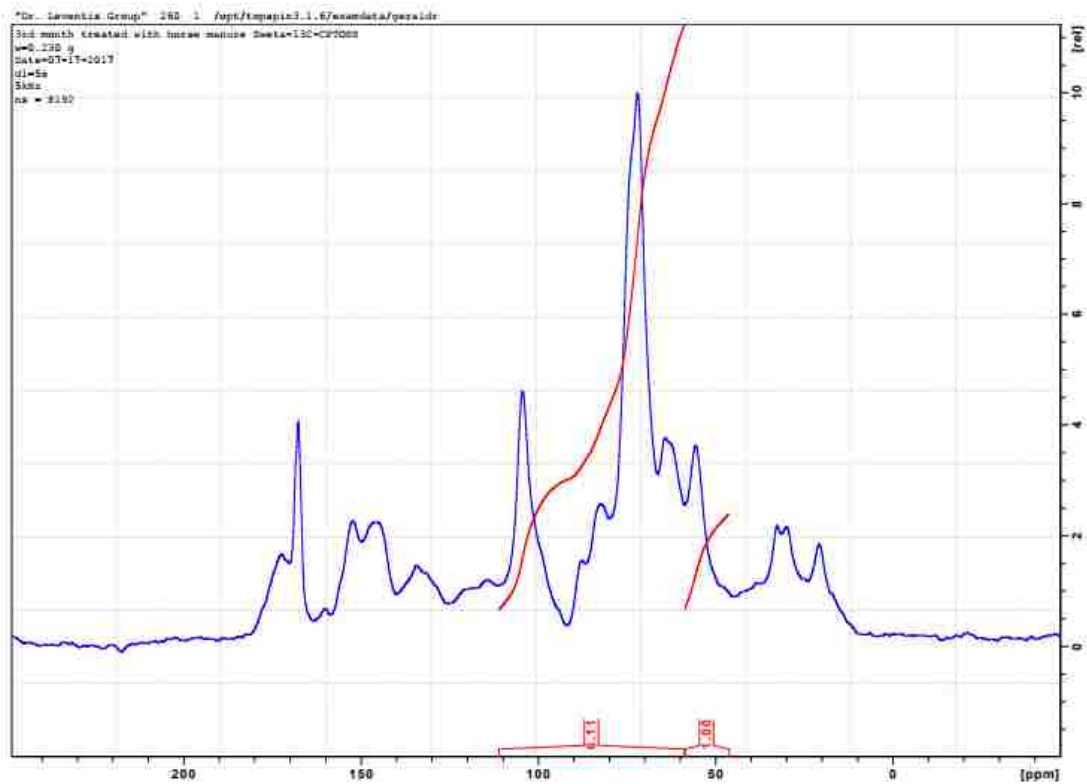


Figure A.11. Integration of spectrum of sample of third month reactor

A.2.5. Fourth Month

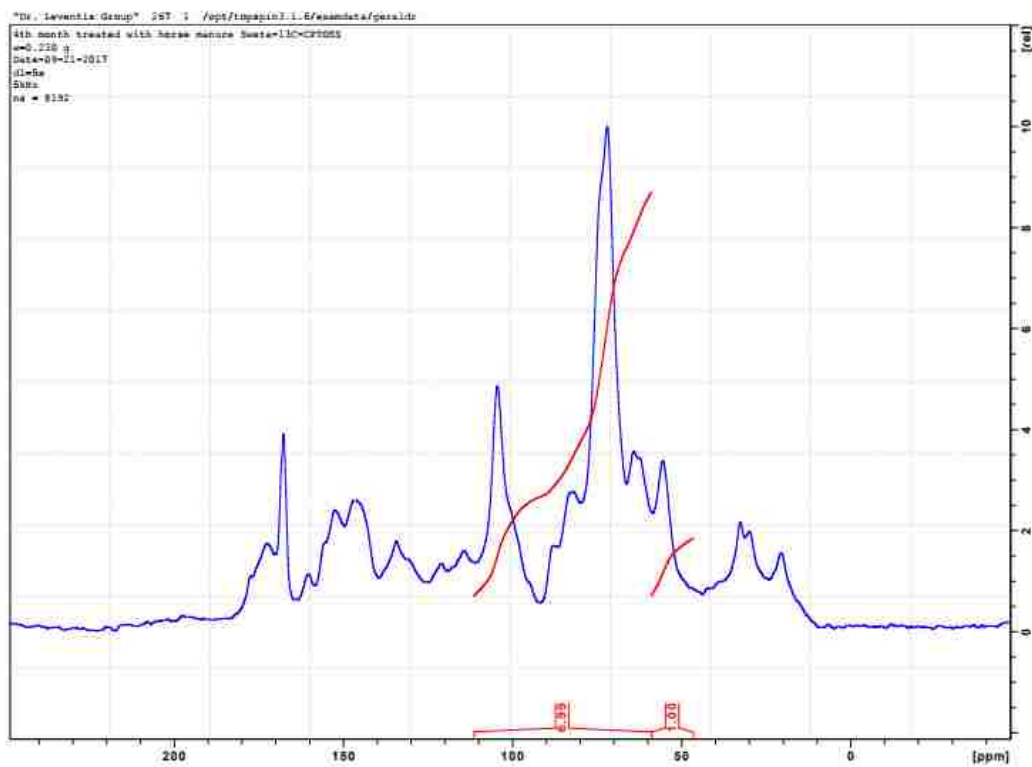


Figure A.12. Integration of spectrum of sample of fourth month reactor

A.2.6. Fifth Month

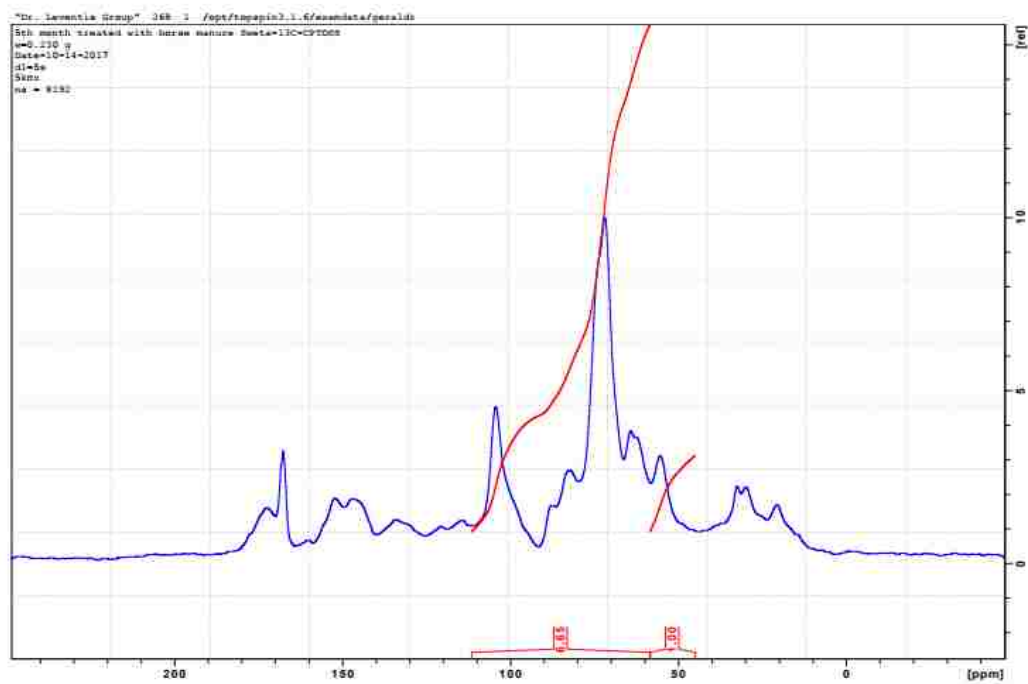


Figure A.13. Integration of spectrum of sample of fifth month reactor

A.2.7. Sixth Month

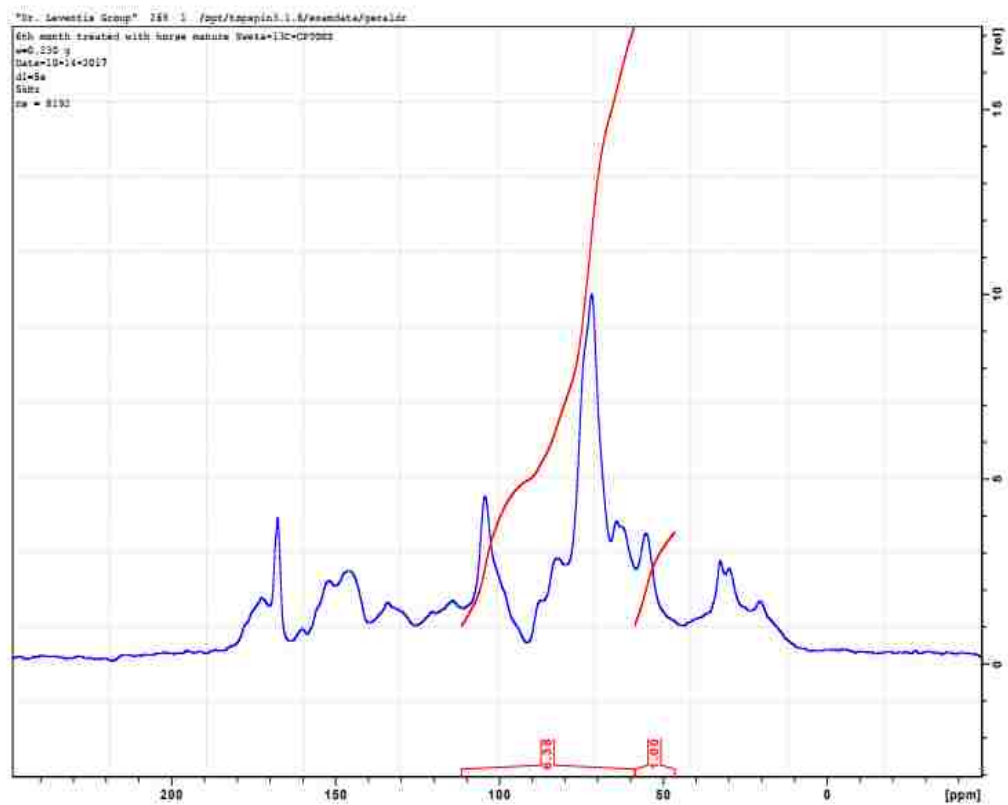


Figure A.14. Integration of spectrum of sample of sixth month reactor

A.3. NMR SPECTRA (FRESH CHIP BARK-SAMPLE OF 6 MONTH)

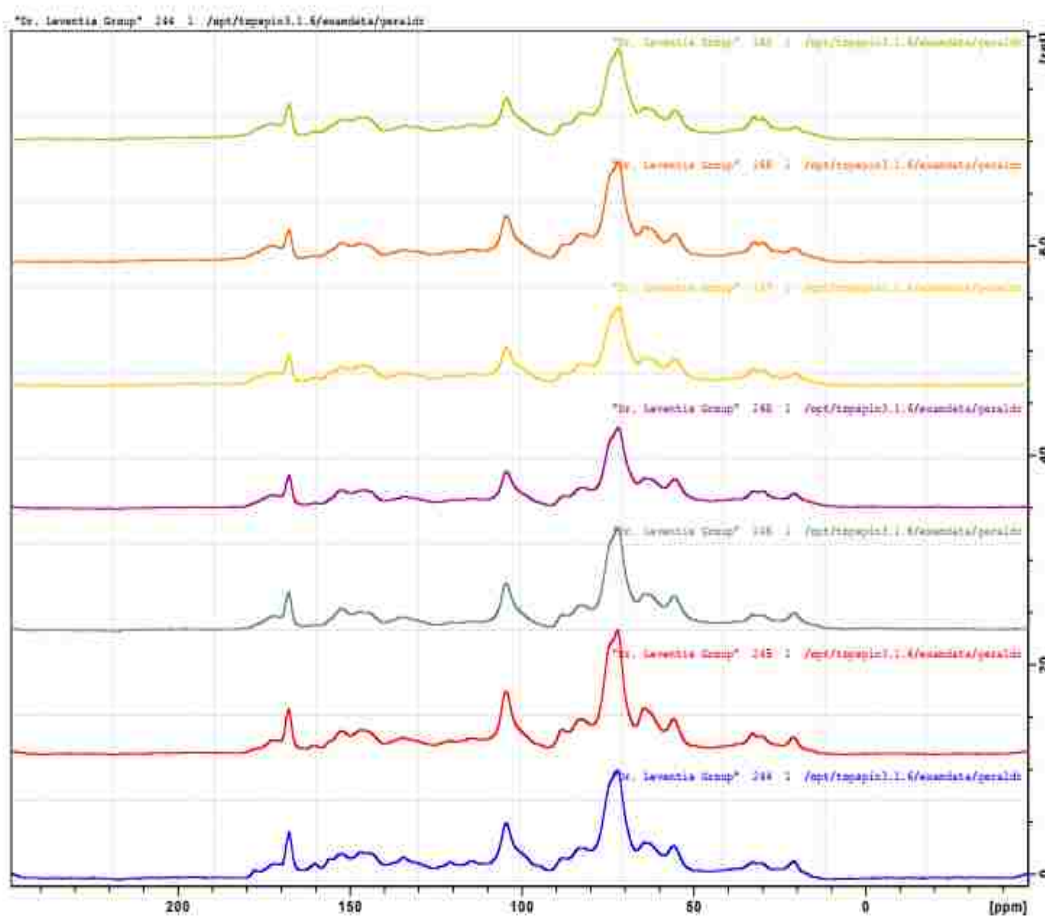


Figure A.15. NMR spectra of sample of all reactors.

Blue is spectra of fresh chip bark, red is spectra of first month reactor, dark green is spectra of second month reactor, purple is spectra of third month reactor, yellow spectra is spectra of fourth month reactor, orange is spectra of fifth month reactor, and light green is spectra of sixth month reactor

A.4. COMPARISON OF RESULT OF NMR AND CHEMICAL ANALYSIS

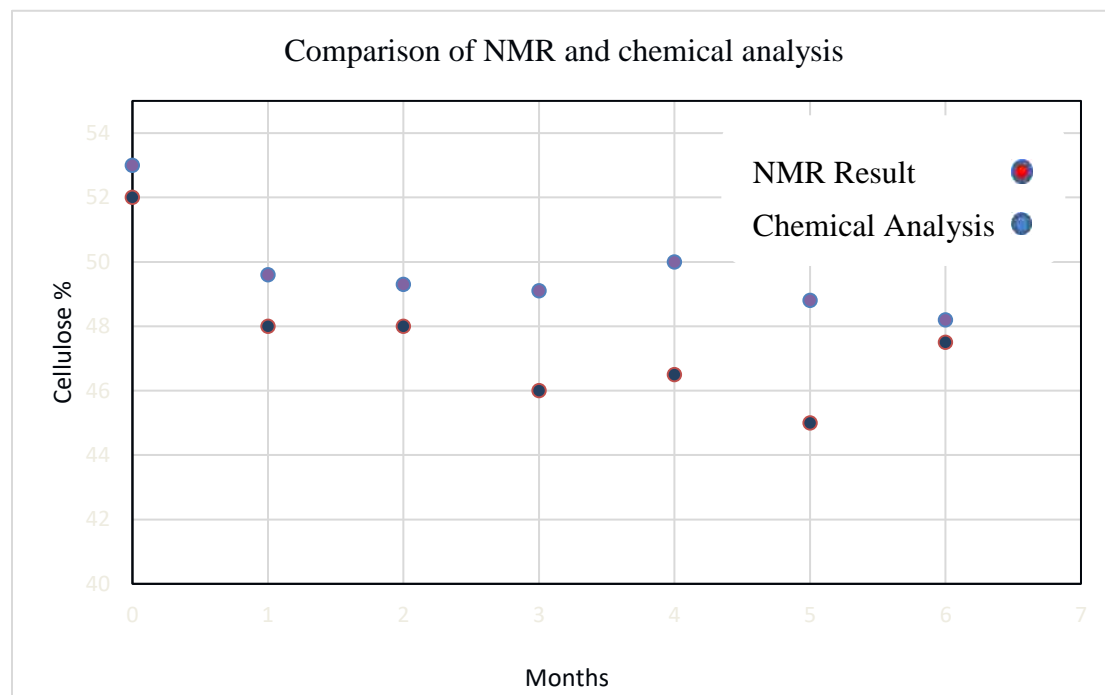


Figure A.16. Comparison of result of NMR and chemical analysis

A.5. FLOWRATE AFTER THREE MONTHS

Table A.1. Flowrates of reactors (Determined in 08/20/2017)

Reactor	Flowrate(ml/min)
1	50
2	0
3	0
4	50
5	20
6	0
7	0
8	0
9	0
10	0
11	0
12	0

13	0
14	60
15	0
16	0
17	10
18	0
19	0
20	0
21	0
22	0
23	0
24	50
25	0
26	0
27	0

28	0
29	0
30	0
31	0
32	0
33	0
34	0
35	0
36	0
37	0
38	60
39	0
40	0
41	0
42	0

43	0
44	0
45	0
46	0
47	0
48	0
49	0
50	0
51	0
52	0
53	0
54	20
55	0
56	0
57	0

58	0
59	0
60	0
61	0
62	0
63	0
64	0
65	0
66	0
67	0
68	40
69	Gone
70	Gone
71	Gone
72	Gone

A.6. FLOWRATE AFTER FIVE MONTHS

Table A.2. Flowrates of reactors (determined in 10/26/2017)

Reactor	Flowrate(ml/min)
1	33
2	6
3	0
4	8
5	4
6	18
7	14
8	50
9	0
10	23
11	0
12	4

13	6
14	14
15	50
16	5
17	14
18	0
19	4
20	0
21	50
22	9
23	50
24	50
25	0
26	0
27	0

28	6.5
29	0
30	4
31	0
32	0
33	0
34	0
35	0
36	50
37	11
38	4
39	4
40	0
41	0
42	0

43	2
44	4
45	0
46	6.5
47	0
48	0
49	0
50	0
51	0
52	0
53	0
54	50
55	25
56	0
57	50

58	0
59	50
60	13.5
61	9.5
62	0
63	0
64	50
65	50
66	Gone
67	Gone
68	Gone
69	Gone
70	Gone
71	Gone

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