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## Screening, optimization and extraction of polyhydroxyalkanoates and peptidoglycan from *Bacillus megaterium*

Louis Paul-Anthony Paladino  
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Screening, Optimization and Extraction of Polyhydroxyalkanoates  
and Peptidoglycan from *Bacillus megaterium*

By

Louis Paul-Anthony Paladino

A Report

Submitted in partial fulfillment of requirements

For a degree of

MASTER OF SCIENCE IN BIOLOGICAL SCIENCES

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**Abstract:**

Bioplastics are polymers (such as polyesters) produced from bacterial fermentations that are biodegradable and nonhazardous. They are produced by a wide variety of bacteria and are made only when stress conditions allow, such as when nutrient levels are low, more specifically levels of nitrogen and oxygen. These stress conditions cause certain bacteria to build up excess carbon deposits as energy reserves in the form of polyhydroxyalkanoates (PHAs). PHAs can be extracted and formed into actual plastic with the same strength of conventional, synthetic-based plastics without the need to rely on foreign petroleum.

The overall goal of this project was to select for a bacteria that could grow on sugars found in the lignocellulosic biomass, and get the bacteria to produce PHAs and peptidoglycan. Once this was accomplished the goal was to extract PHAs and peptidoglycan in order to make a stronger more rigid plastic, by combining them into a co-polymer. The individual goals of this project were to: (1) Select and screen bacteria that are capable of producing PHAs by utilizing the carbon/energy sources found in lignocellulosic biomass; (2) Maximize the utilization of those sugars present in woody biomass in order to produce optimal levels of PHAs. (3) Use room temperature ionic liquids (RTILs) in order to separate the cell membrane and peptidoglycan, allowing for better extraction of PHAs and more intact peptidoglycan. *B. megaterium* a Gram-positive PHA-producing bacterium was selected for study in this project. It was grown on a variety of different substrates in order to maximize both its growth and production of PHAs. The optimal conditions were found to be 30°C, pH 6.0 and sugar concentration of either 30g/L glucose or xylose. After optimal growth was obtained, both RTILs and enzymatic treatments were used to break the cell wall, in order to extract the PHAs, and peptidoglycan. PHAs and peptidoglycan were successfully extracted from the cell, and will be used in the future to create a new stronger co-polymer. Peptidoglycan recovery yield was 16% of the cells' dry weight.

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## LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BGP	Best Growth/PHB production
BMIMCl	1-butyl-3-methylimidazolium chloride
DIC	Differential Interphase Contrast
DO	Dissolved Oxygen
EDTA	Ethylenediaminetetraacetic Acid
GC	Gas Chromatography
GRP	Growth and PHB production
GRNP	Growth but no PHB production
HPLC	High Performance Liquid Chromatography
LN	Limited Nitrogen
N	Nitrogen
NGR	No Growth
NRLM	Nitrate-limited Minimal medium
NRLM-G	Nitrate-limited Minimal medium with glucose
NRLM-X	Nitrate-limited Minimal medium with xylose
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PHV	Polyhydroxyvalerate
PHBV	Polyhydroxybutyrate and Polyhydroxyvalerate
RTILs	Room Temperature Ionic Liquids
SDS	Sodium Dodecyl Sulfate
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth

## Introduction

Bioplastics are polymers (such as polyesters) produced from bacterial fermentations that are biodegradable and nonhazardous. They are produced by a wide variety of bacteria and are made only when stress conditions allow, such as when nutrient levels are low, more specifically levels of nitrogen and oxygen (Nath et al., 2008). These stress conditions cause certain bacteria to build up excess carbon deposits as energy reserves in the form of polyhydroxyalkanoates (PHAs). PHAs can be extracted and formed into actual plastic with the same strength of conventional, synthetic-based plastics without the need to rely on foreign petroleum.

The focus of this project is on secondary metabolites produced from certain bacterial species after a fermentation reaction. The overall goal of this project is to successfully screen for bacteria that will be able to produce PHAs from lignocellulosic biomass. The lignocellulosic biomass contains five sugars (glucose, xylose, arabinose, mannose, and galactose) (Avérous and Le Digabel., 2006). The difficulty of screening and selecting a specific bacterium is that any bacterium chosen or screened will have positives and negatives associated with it. A variety of factors will be considered in the selection of the bacterium. For example, Gram-positive bacteria may be favored because of their ability to produce large amounts of peptidoglycan which can aid as a co polymer. However, Gram-negative bacteria have thinner cell walls and would be easier to get the PHAs extracted from, as well as produce higher PHA yields. In either case, the Gram-positive and/or the Gram-negative bacteria chosen will need to utilize specific sugars, be able to grow in non optimal pH, and able to produce high yield PHAs.

The bacterial strains ultimately selected were be used to produce PHAs and peptidoglycan as co-polymer (as explained later). This will be done in large amounts utilizing the lignocellulosic biomass. The significance this will have is that it will decrease the need for petroleum oil and help create a cleaner environment.

## **Objectives**

- 1.) Select and screen bacteria that are capable of producing PHAs by utilizing the carbon/energy sources found in lignocellulosic biomass.
- 2.) Maximize the utilization of those sugars present in woody biomass in order to produce optimal levels of PHAs.
- 3.) Use room temperature ionic liquids (RTILs) in order to separate the cell membrane and peptidoglycan, allowing for better extraction of PHAs and peptidoglycan.

## **Literature review**

### **1.1 Previous studies on PHA production from woody biomass**

It has been demonstrated that PHAs can be produced from bacteria that utilize lignocellulosic woody biomass (Bertrand et al., 1990). The reason many bacteria utilize the lignocellulosic material is because certain bacteria have the ability to exploit many of the sugars present. The sugars that are most utilized are the pentose sugars, for example, xylose and arabinose (Bertrand et al., 1990). It has also been demonstrated that hexoses, such as glucose, can also be utilized (Bertrand et al., 1990). It should be noted that the utilization of sugar cannot be generalized for all bacteria; only the ones that most likely will grow on lignocellulosic biomass. The current project will attempt to utilize the sugars present in the lignocellulosic biomass in order to produce PHAs such as those seen in literature (Madison and Huisman, 1999).

Furthermore the overall goal will be to maximize both PHA production amounts as well as the sugar quantities present in the lignocellulosic biomass (Avérous and Le Digabel, 2006). This may be done with a varying degree of results as sugar quantity will vary depending on the woody biomass used, for example, switch grass, birch, or aspen (Bertrand et al., 1990). The reason this research is being pursued is based upon the fact that in the 1980s, there were 375 million tons of biomass that could be utilized to make PHAs (Jeffries et al, 1983; Madison and Huisman, 1999). Moreover, the process has many areas still not well understood which could be modified for better PHA production (Madison and Huisman, 1999).

## **1.2 Lignocellulosic woody biomass project Michigan Tech**

Michigan Technological University (Michigan Tech) is currently pursuing a project in order to utilize the sugars present in lignocellulosic woody biomass in order to produce novel bioplastics from bacteria. The project involves several departments: Biological Sciences, Chemical Engineering, and Chemistry. Each department has a specific task that must be completed in order for the project to be finished successfully. The project involves developing and utilizing techniques in order to convert a variety of different woods into a sugar solution using both a dilute sulfuric acid treatment and an enzymatic treatment (Figure 1). This is done using a hydrolysis reaction which releases sugars present in the cellulosic woody biomass into a usable form. The finished product is referred to as hydrolysate; depending on the treatment different sugars will be released. Dilute acid hydrolysate will release five sugars (arabinose, glucose, xylose, mannose and galactose.) The enzymatic hydrolysate will have only glucose present (Le Digabel and Avérous, 2006).

The Biological Sciences department was responsible for screening potential bacteria that can produce bioplastics. After the screening was finished the goal was to maximize the production of the bio-plastics. A variety of methods were used in order to proficiently accomplish this task. Once maximization has been accomplished it was necessary to extract the bioplastic from the bacteria, this was attempted using a technique in involving Room Temperature Ionic Liquid (RTILs) (Cull et al., 2000). RTILs will allow for an environmentally friendly technique to separate out the plastic and hopefully the peptidoglycan from rest of the cell material. This would allow for peptidoglycan and the PHA to be used together in order to make a stronger plastic than the PHA itself

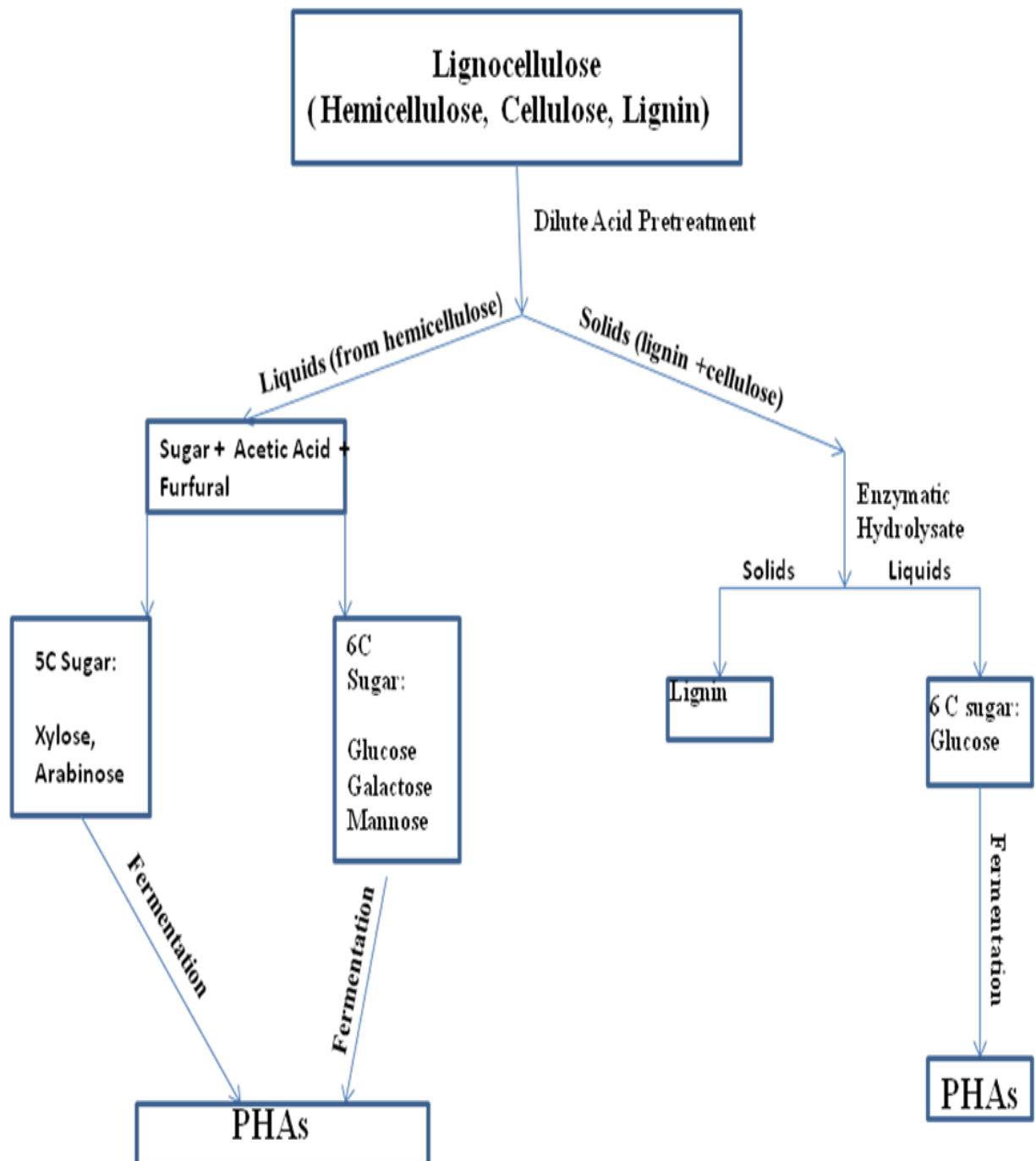


Figure 1. Hydrolysis of lignocellulose in process used at Michigan Tech.



(personal communication with Dr. P. Heiden., Michigan Tech Chemistry). The RTILs are being designed with the goal of targeting organic compounds; RTILs have high concentrations of ions which cause the disruption of the compounds which get exposed to the RTIL. The RTIL currently being used is 1-butyl-3-methylimidazolium chloride (BMIMCl), which is essentially a salt solution with the ability to dissolve organic materials (Liying et al., 2006). It is predicted that the RTIL will be able to separate the cell components of the bacteria, and peptidoglycan as well as they are both organic materials. After separation peptidoglycan can be combined with PHBs in order to form a co-polymer (personal communication with Dr. P. Heiden., Michigan Tech Chemistry).

### **1.3 Lignocellulosic biomass hydrolysate information**

As shown in Figure 1, there are two types of hydrolysate that will be produced. The first, dilute sulfuric acid treatment; and the second, enzymatic hydrolysate. Each of the hydrolysates has different characteristics associated with it. For example, the dilute acid hydrolysate will have five sugars present (arabinose, glucose, xylose, mannose and galactose); it will have a pH of 1.7 and furfural concentrations ranging from 0.5 g/L - 2 g/L (Nilevbrant et al., 2001). The dilute acid treatment will also contain acetic acid levels ranging from 2 g/L - 10 g/L. This combination, although good to prevent bacterial growth during development, may have inhibitory affects on bacteria after developmental stage. (Taherzadeh et al., 1997). Enzymatic hydrolysate is produced using a cellulase enzyme which over time breaks down the pretreated cellulose present in the woody biomass producing only the sugar glucose (Liying et al., 2006).

#### **1.4 Bacteria that produce PHAs**

PHAs are produced from a wide variety of bacteria; the most common type of PHA produced is polyhydroxybutyrate (PHB). In 1925 the French scientist Lemoigne first observed the production of tiny granules within the bacterium *Bacillus megaterium* (Prieto et al., 2007). These granules were found to contain hydroxybutyrate monomers; these monomers eventually came to be referred to as PHB. It was not until roughly 60 years later that *Pseudomonas putida* GPO1, previously known as *Pseudomonas oleovorans* GPO1, was discovered to produce not only PHBs, but PHBs with different chemical composition (modifications to types of hydroxybutyrate monomers). Depending on the composition, the bacterial-produced plastic had different strengths (Steinbüchel et al., 2001). Selection of bacteria For this project the goal was to find a bacterium capable of utilizing lignocellulosic woody biomass and the sugars present from within the biomass to produce PHAs. Preliminary research was done on cultures and strains that were found in literature most capable of producing PHAs from utilizing the sugars present in the woody lignocellulosic biomass. (See Table 1.)

#### **1.5 Criteria for selecting bacteria**

The bacteria selected must be able to produce PHAs using the sugars present in the woody biomass. The bacteria must be able to grow in a pH not necessarily optimal and must be able to be put under limiting conditions such as lack of nitrogen or oxygen in order to produce the PHA. The bacteria must also adapt themselves in a way that they are able to maximize the length of their stationary phase, as PHAs are secondary metabolites produced during the stationary phase when nitrogen or oxygen is limited.

The reason the above criteria were established is that the bacteria selected must be

able to grow on the hydrolysate, meaning that they must be able to sufficiently utilize any amount from 4 - 40 g/L of glucose found in the enzymatic hydrolysate or other varying amount of sugars found in the dilute acid hydrolysate. The bacteria must be able to be adapted to utilize a pH not equal to that of their optimal as the hydrolysate is highly acidic and to buffer it is very expensive. The bacteria must be able to deal with certain toxic compounds found in the hydrolysate such as furfural and acetic acid in the dilute acid hydrolysate and tetracycline found in the enzymatic hydrolysate, both of which have the ability to and the initial intention to kill bacteria. Lastly, the bacterium that is selected must be able not only to pass all these requirements but must be able to produce PHAs and do so in relatively large amounts.

**Table 1. Polyhydroxyalkanoates (PHA)- producing bacteria of potential interest**

<b>Bacterial Type</b>	<b>Pros for Use</b>	<b>Cons for Use</b>	<b>Reference</b>
<i>Pseudomonas lubricans</i> strain RS1 <sup>a,b,f</sup> ATCC BAA-1494	Newly discovered species; able to utilize arabinose	Uses mainly amino acids and not sugars	Saha., 2006
<i>P. oleovorans</i> <sup>a, b, d, e</sup> ATCC 29347	Yields high PHA amounts	Multiple strains; some may yield PHAs and others may not grows on several sugars.	Sheu et al., 2000
<i>P..pseudoalcaligenes</i> <sup>a, b, e,</sup> ATCC 17440	Versatile metabolism and ability to utilize certain sugars	Multiple strains; some may not yield PHAs in as large quantities as others	Braunegg et al., 1998
<i>P. putida</i> <sup>a, b</sup> ATCC 17453	First strain discovered as PHA-producer	Multiple strains; some may yield PHAs and others may not; use a only few sugars	Luengo et al., 2003
<i>Bacillus megaterium</i> <sup>a, b, d f</sup> ATCC 11561	Possible for genetic manipulation Sugar utilization (glucose, xylose, fructose),peptidoglycan cell wall	May produce endospores ; not as high of PHB yield compared to Gram-negatives	Tomohiro et al., 2002
<i>Cupriavidus necator</i> <sup>b, d, e</sup> ATCC 17697	(PHB, PHV <sup>d</sup> ) producer*~converts sugars to various PHAs.	Some strains do not produce PHAs; some high-producing strains are greatly genetically modified	Lee et al., 1999
<i>Saccharophagus degradans</i> <sup>a, c, d, e</sup> ATCC 43961	Versatile metabolism; able to use cellulose	PHAs only form under conditions in which oxygen levels are lowered to put stress on cells.	Ekborg et al., 2005

<sup>a</sup> Grows optimally at pH 7.0.

<sup>b</sup> Used in this study.

<sup>c</sup>PHB – polyhydroxybutyrate; PHV – polyhydroxyvalerate.

<sup>d</sup>Can utilize sugar to make PHAs

<sup>e</sup>Gram-negative

<sup>f</sup>Gram-positive

## **1.6 Toxicity associated with Hydrolysate**

In order to have the best understanding of the maximization of growth parameters, several studies must be conducted in order to monitor how certain chemicals placed in the hydrolysate during the acid pre-treatment will affect bacterial growth after the treatment. Tetracycline, furfural, and acetic acid levels as well as pH, oxygen, and nitrogen conditions must be monitored in order to truly select bacteria that will be able to grow on the hydrolysate for reasons explained earlier.

### **1.6.1 Tetracycline**

Tetracycline is broad spectrum antibiotic. It is used to inhibit 30S ribosomal function by binding the 16S rRNA thereby blocking the aminoacyl-tRNA. Tetracycline is added to the enzymatic hydrolysate in order to prevent growth of bacteria (Anderson and Dawes., 1990).

### **1.6.2 Furfural**

Furfural is a liquid aldehyde that is present in the dilute acid pretreatment hydrolysate. Furfural forms from the breakdown of pentoses present in the woody biomass. The reason why furfural is present is that it is produced as a toxic byproduct from the dehydration of xylose another pentose sugars present in the lignocellulosic biomass (Nilevbrant et al., 2001).

### **1.6.3 Acetic Acid**

Acetic acid is found in the dilute acid pretreated hydrolysate. The levels of acetic acid can range from 2 g/L to as high as 10 g/L (Taherzadeh et al., 1997). The concern is that acetic acid may drop the pH of the solution below the bacterium's optimal pH, inhibiting its growth. However, it has been shown that PHA production can actually be

increased with higher acetic acid levels assuming that pH is buffered to 5.5-7.0 (López-Cortés et al., 2008). The reason is that acetic acid, if utilized correctly by the bacteria, can help form acetyl-CoA, which as described below is necessary for PHA production (Figure 2), so a balancing act may need to be performed to get a good pH as well as keep some acetic acid present (López-Cortés et al., 2008).

## **1.7 Monitoring major parameters: pH, oxygen, and nitrogen**

### **1.7.1 Oxygen monitoring**

When growing any aerobic bacteria, it is crucial to make sure there is enough oxygen present. Large amounts of O<sub>2</sub> are needed when growing the bacteria for proper metabolism to occur. When synthesizing PHBs using aerobic bacteria, the role of oxygen becomes crucial. The role oxygen serves changes depending on whether the bacteria are Gram-negative or Gram-positive. In both Gram-positive and Gram-negative bacteria oxygen regulation is important to proper cell function. It has been shown in a study that when dissolved oxygen (DO) is limited to a certain degree (30%-60%), the PHB production quantity changes. 30% is the best DO level for optimal PHB production (Nath et al., 2008). The mechanism behind this is that, under DO conditions that are limited, an influx of acetyl-coA will move towards PHB production and away from the TCA cycle (Nath et al., 2008). From Figure 2 it can be seen that PHB formation and the TCA cycle share the same precursor, acetyl-coA (Nath et al., 2008). Interestingly, even though the 30%-60% oxygen deficit may help facilitate PHB production initially, it is important to monitor length of time and duration of oxygen limitation, as some Gram-positive bacteria such as *B. megaterium* will produce endospores when the nutrient or oxygen levels become insufficient with time levels lower than 1% (Anderson and Dawes., 1990).

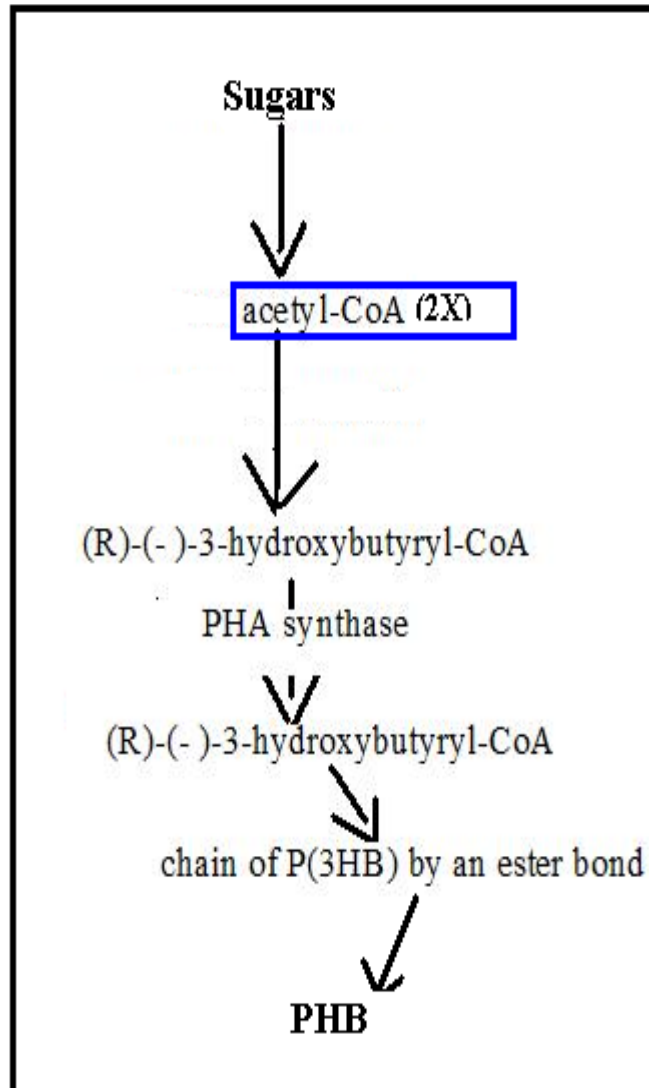


Figure 2. PHA production pathway based on pathway in (Welton et al., 1998).  
2X depicts 2 acetyl-CoA molecules

### 1.7.2 Nitrogen and Carbon monitoring

Nitrogen and carbon are essential in order to produce PHBs. Nitrogen and carbon monitoring has been shown to have an association that if high enough amounts of carbon and low enough amounts of nitrogen; it may stimulate the bacteria to produce maximum amount of PHBs as products (Anderson and Dawes., 1990; Madison and Huisman.,1999). In fact, it has been shown that when nitrogen conditions are limited and the carbon source

is abundant, some Gram-negative bacteria can accumulate up to 60-80% of their weight as PHB (Anderson and Dawes., 1990; Lefebvre et al., 1997). The mechanism behind the production is that when bacteria are growing they need many nutrients (carbon, nitrogen, phosphorus, etc.). When one major nutrient such as a carbon source becomes excess in the cell solution and other macronutrients nutrients such as nitrogen or phosphorus are limited, the bacterium prepares itself for survival. This means the bacterial cell builds up the excess carbon in the form of PHB and waits for nitrogen or other limiting nutrients to possibly return back to the system to prolong its growth. This would, of course, hold true only for Gram-negative bacteria. When working with certain Gram-positive bacteria, such as *Bacillus megaterium*, there is the potential that the bacteria will produce endospores if nutrient conditions are limited for too long. Therefore, the PHBs must be produced before this occurs. Therefore nitrogen limitation, although necessary for PHB production, must be monitored very closely as to not be detrimental to bacterial cell formation (Lefebvre et al., 1997).

#### 1.7.3 pH monitoring and concerns

It has been documented in recent studies by López-Cortés et al. (2008) that, if the pH of the enzymatic hydrolysate is adjusted slightly using NaOH or a phosphate buffer, growth of *B. megaterium* is possible as the pH is brought from the starting level of 4.7 to a final pH level of 5.0 – 5.5. (This pH is below its optimal pH for growth, 6.0 – 8.) The final enzymatic hydrolysate (at Michigan Tech) following the treatment stages can be at about pH 4.7. Most bacteria producing PHAs cannot tolerate such pH levels. *B. megaterium* and *Cupriavidus necator*, which both require pH 6.0-8, also will not be able to tolerate such levels so adaptation may be required.



A literature review has been conducted to determine if there are any microorganisms that will produce PHAs under acidic conditions and utilize the sugars present in the woody biomass. Microorganisms such as *Saccharophagus degradans* have been considered for study. However, due to the bacteria's need to grow at a pH much less than 4, and the need for a complex medium *Saccharophagus degradans* is not being pursued (Esteban et al., 2008). Therefore, for this study the best route will be to use base buffering system.

### **1.8 Chemical Composition of PHAs**

PHAs are polyesters or polymers that contain functional ester R groups. The functional R groups present on the PHAs have the ability to switch designation depending on the carbon/energy source used when growing the bacteria (Figure 3). The most common PHA, PHB, is often accompanied by a copolymer called polyhydroxybutyrate-valerate (PHBV) (Steinbüchelet al., 2001). Often these combinations are responsible for giving more desirable products. For example, the ethyl group that is part of polyhydroxy-valerate, PHV, or PHBV has been shown to give a more flexible and desirable plastic. Therefore, this is often a justification to have a copolymer with the PHB (Braunegg et al., 1998; Steinbüchel et al., 2001).

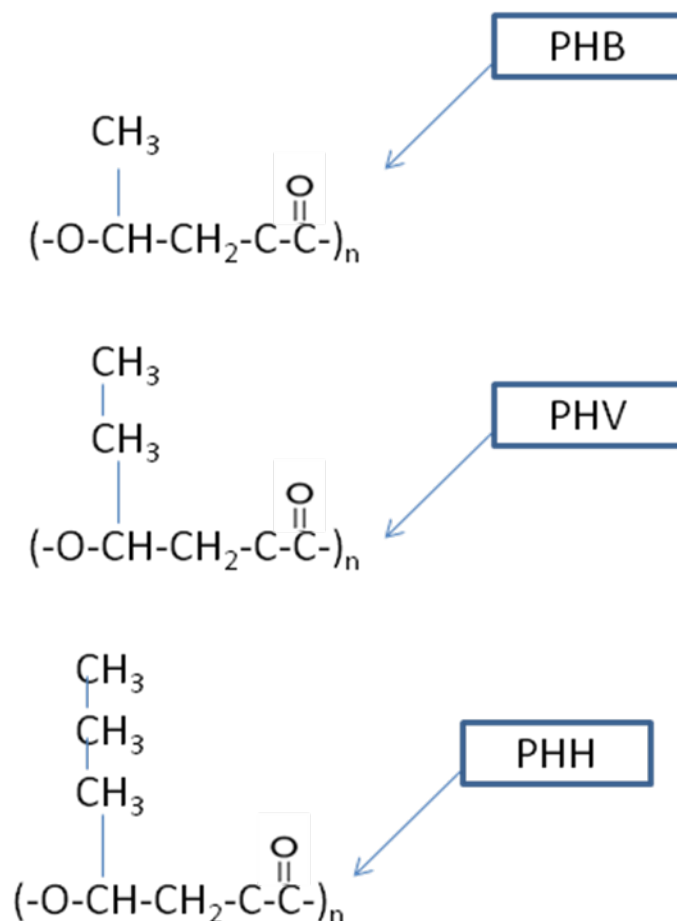


Figure 3. Chemical structure of PHAs (modified from Steinbüchel, 2001). R groups (shaded boxes) Structures in this figure are PHB (R=methyl), PHV (R=ethyl), and PHH (R=propyl)

### 1.9 Pathways involved in production of PHAs

There are several key pathways involved with PHA production. The first pathway can be seen in Figure 2 (Welton., 1998). Figure 3 depicts the structures following PHA formation. It can be seen that acetyl-CoA is crucial for the process of PHA production. Acetyl-coA is always found in bacteria that produce PHAs as it is produced from the utilization of glucose or other sugars which means that the more sugars the higher the production of acetyl-coA.

## **1.10 PHA detection methods**

PHAs have several methods of detection (which will be explained in more detail in the methods sections). However, in order to give a brief overview on the methods, they will be defined now. When screening bacteria for PHAs two main methods are commonly used (Koning et. al., 1999). The first is qualitative, used for quick results in order to know whether the PHAs are present in the bacteria or not. Common examples of these types of tests are Nile Blue A and Sudan Black B stains (Ostle et al., 1982). There is also a variety of molecular techniques to find the genes responsible for producing the PHAs for rapid screening of hundreds of samples (Sheu et al., 2004). Qualitative techniques are rapid; however, they only provide initial information on PHA presence. In order to gain full understanding of PHA presence the researcher must use a quantitative method. The purpose in using a quantitative test method is because it will confirm not only PHA presence but also indicate amount and type present.

### **1.10.1 Staining**

The reason for using Nile Blue A stain in place of Sudan Black B is because of its ability to bind to excess fatty deposits and energy deposits such as PHAs and fluoresce orange after binding, whereas Sudan Black B simply stains deposits black. Therefore, Nile Blue A stain is also preferred due to the stain's ability to fluoresce bright orange at 360/460nm, as compared to Sudan Black that does not fluoresce. The Nile Blue A technique allows for fast preliminary screening to occur, giving a rough insight to whether PHAs are present in the cell. (Ostle et al., 1982). (See appendix A for procedure on cell Nile Blue A staining.)

### 1.10.2 Molecular approach

The molecular approach from Sheu et al. (2004) has been shown to be specific for a variety of bacteria. The thought is that, with proper techniques and samples, the gene of interest (*phaC*) can be located both quickly and cost effectively. The significance pertaining to the *phaC* gene is that the gene is only active in bacteria that produce PHAs. This gene will therefore allow for rapid screening to take place, and increase the chances of finding a bacterium of interest more quickly.

### **1.11 Quantitative Analysis using Gas Chromatography**

Gas chromatography (GC) has been used to a great extent by other laboratories in order to confirm PHA production from bacterial cells. The principle behind GC is that each compound will leave the column at a different rate. Therefore, a different amount of time is taken for each compound to leave the column. This concept is called retention time (Oehmen et al., 2005). It is using this logic that different PHBs and copolymers can be measured (Figure 4).

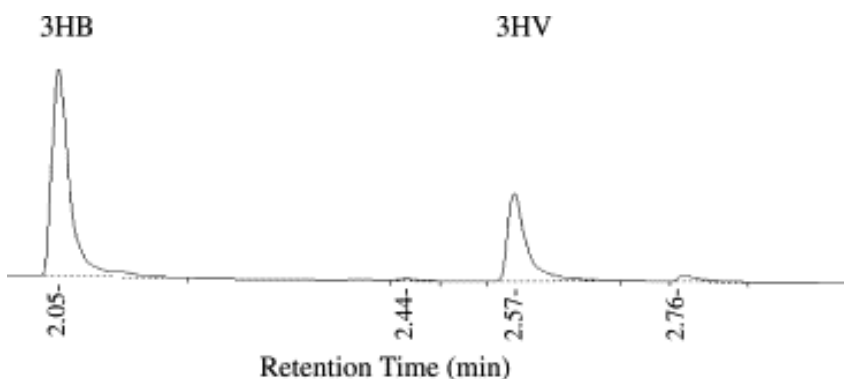


Figure 4. Separation of poly-β-hydroxybutyrate (3HB) and poly-β-hydroxyvalerate (3HV) using gas chromatography techniques (Oehmen et al., 2005).

### **1.12 Extraction, Recovery, and Quantification of PHAs and Peptidoglycan**

A comparison was made between the characteristics for PHA and peptidoglycan levels and potential extraction and quantification techniques for *B. megaterium* and *C. necator*. In the enzyme extraction method, a combination of enzymes (alcalase, a protease) with sodium dodecyl sulfate (SDS) and ethylenediaminetetraacetic Acid (EDTA) were used for the extraction of PHAs from the cell. This method was gentle and selective and did not involve the use of harmful chemicals. This process can provide 95% purity of PHA. The granules are recovered using membrane filtration. (Yasotha et al., 2006, Jiang et al., 2006, de Koning et al., 1997).

In the solvent extraction method, Soxhlet extraction using a combination of chloroform and methanol can be used for extracting PHAs. The process is reported to produce 24% purity (Jiang et al., 2006).

### **1.13 Peptidoglycan and PHBs**

The reason for choosing a Gram-positive bacterium in contrast to a Gram-negative bacterium is partially due to the potential ability to combine the Gram-positive cells' abundant amount of peptidoglycan with the modest amount of PHB (as compared to Gram-negatives' 70-80% PHB) . It has been reported within the literature that Gram-positive bacteria will have as much as 50% of the cells dry weight composed of peptidoglycan and 40% of weight PHB (Colwell and Grigorova., 1987). This combination leads to new ideas not previously pursued. The significance is that these two materials normally present in certain Gram-positive bacterium often are not of interest to industry because the PHB alone is often very weak and fragile, and

peptidoglycan is often not very strong once extracted from the bacterial cell (Chen et al., 2001). The thought is that if these two polymers were separated from the cell, and then recombined perhaps one could create an ideal co-polymer that would be stronger than either PHB or peptidoglycan by itself. This idea is novel and information pertaining to structure of PHBs and peptidoglycan will be discussed later in this report.

After PHAs were produced from the bacterium that is chosen for this project, RTILs were used in order to separate out the cell material from the PHA and peptidoglycan. Figure 5 depicts peptidoglycan, a structural polymer found in bacterial cell walls. Currently, the most common method of breaking the wall in Gram-positive bacteria in order to release PHAs involves the use of methanol and chloroform or certain enzymes such as Pronase E, SDS and EDTA to add gentleness to the process (Yasotha et al., 2006). These chemicals first dissolve the lipid outer layer of the cell wall and then break apart the actual inner layer of the rigid peptidoglycan (Yasotha et al., 2006). The concerns with these reagents are that they are expensive (Yasotha et al., 2006). Therefore RTILs should be preferred to be used instead as they are far safer and cost effective (personal communication with Dr. P Heiden Michigan Tech Chemistry). Preliminary examination showed that the RTIL BMIMCl should break the cell wall peptidoglycan (Liyang et al., 2006), but yet still preserve the structural integrity of the PHAs even through the extraction process. (Imamura et al., 2006). (See Figure. 7 for mechanism of reaction.)

The RTILs are ideal for use as they are in a liquid form under room temperature. Thus one does not have to heat them up to dangerous extreme temperatures, such as with other extraction liquids (Swatloski et al., 2002). RTILs have the ability to be reused many

times by performing a simple purification technique. From preliminary assessments, RTILs have been shown to be able to heat up to about 250°C before they break down and degrade. This information is crucial because in some of the process applications RTILs may get heated inadvertently, but only to 100 or 150°C (well below degradation temperatures). Moreover, it further supports use of RTILs in extraction process (Imamura et al., 2006; personal communication with Dr. P. Heiden., Michigan Tech Chemistry).

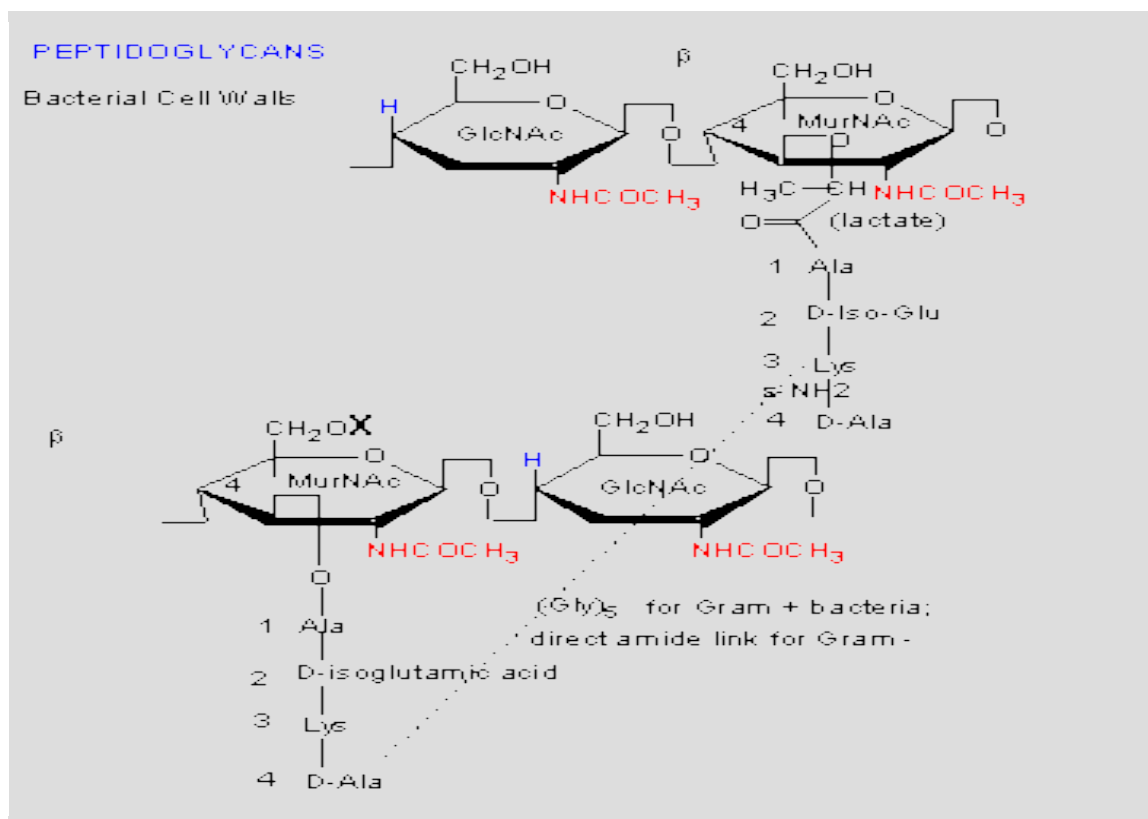


Figure 5.: Chemical structure of peptidoglycan  
( <http://www.daviddarling.info/encyclopedia/P/peptidoglycan.html> )

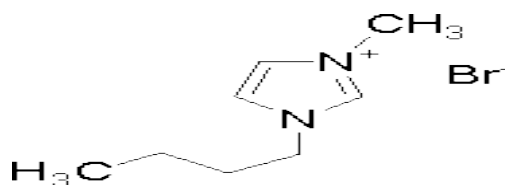


Figure 6. Structure of 1-butyl-3-methylimidazoliumchloride (BMIMCl) (modified from Liying et al., 2006).

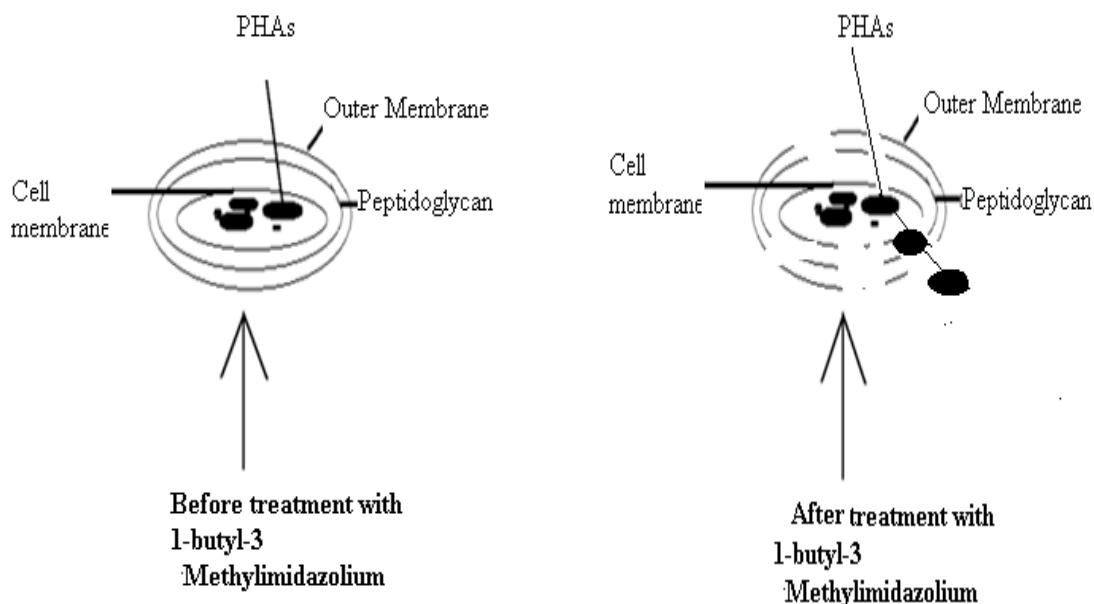


Figure 7. Anticipated results: before and after treatment with RTIL 1-butyl-3 methylimidazolium on Gram-negative bacteria

Figure 7 depicts how BMIMCl will hopefully work to separate both the outer membrane and the peptidoglycan inner layer of the Gram-negative cell wall. The area of interest is that the reagent is able to do so without destroying the PHAs which are produced inside the cell membrane. This reagent therefore allows for a cheap non-toxic method for extracting PHAs from bacterial species of interest. Figure 7 depicts a Gram-negative bacterium exposed to BMIMCl. This idea should transpose onto Gram-positive bacteria as well, because both have organic walls. Therefore, when exposed to the RTILs, both should break apart similarly, resulting in larger amounts of peptidoglycan in Gram-positive bacteria.



## Experimental Methods

### 2.1 Bacterial screening from ATCC cultures

In order to find bacteria that produce both PHAs and utilizes the sugars present in the woody biomass, a literature review was conducted. The first objective was to organize a table of potential organisms found in the literature (Table 1). After construction of this table several cultures were obtained from the American Type Culture Collection (ATCC) and screened for both carbon source utilization and PHB production. Of the 7 cultures listed in table 1, all cultures were screened with the exception of *Saccharophagus degradans*.

#### 2.1.1 Nile A blue staining

Staining techniques found in Ostle et al. (1982), with some modification, in order to see which strains from ATCC produce the PHAs. The microscope used was a Zeiss Axioplan 2 with Acridine Orange filter set and differential interference contrast (DIC); the wavelength was 360-460nm, and UV intensity was 63-95%. This method allowed for quick identification and also allow for elimination of some bacterial species. The method also allowed for other potential species to move forward to the second objective of carbon source utilization and maximization of utilization of the sugars present in the biomass.

#### 2.1.2 Analysis of criteria necessary for growing bacteria

After completion of objective one, it was determined that two bacterial species were suitable to move onto objective two: a Gram-negative bacterium, *C. necator* ATCC #17697, and a Gram-positive bacterium, *B. megaterium* ATCC #11561. These bacteria were selected because of their expected ability to utilize sugars present in the woody

biomass hydrolysate (primarily xylose and glucose) and after utilization produce PHAs (data shown in results section). Objective two focused on maximizing carbon source utilization and PHA production, through a series of growth rate studies, which monitored many parameters, such as pH, temperature, and sugar concentrations. Several protocols were developed for this (listed below).

## **2.2 Carbon source utilization methods**

### 2.2.1 Initial screening

The cells were grown over a 48 hour period at 30°C. PHAs were monitored using the Nile Blue A technique. The positive control was grown on Nitrate-limited minimal medium NRLM with fructose as both cultures were known to grow on this sugar; the negative control was NRLM (in Appendix C). The concentration of sugar was 4g/L, as this was the concentration of sugar found in the hydrolysate at the time (personal communication with Chemical Engineering Michigan Tech). The sugars tested were glucose, xylose, arabinose, galactose, and mannose. If growth was found on any of the substrates under optimal conditions, the next step was to attempt to grow the bacterium on NRLM with xylose (NRLM-X) and NRLM with glucose (NRLM-G) for PHB production (see Appendix C).

### 2.2.2 Temperature and nitrogen effects observed

Temperature study:

Two replicates of each sample were used at each temperature. The two replicates were kept between 20°C - 40°C in order to monitor the bacteria's ability to deal with different sugars. They were checked for growth after 24, 48, and 72 hours. At each time

interval, the flasks were removed and plated for viable counts using NRLM. One major modification that was performed was that the sugar levels were varied. Absorbances of the replicates were measured using a spectrophotometer at  $A_{660}$ . Nephlo flasks were used for monitoring the study, and temperature conditions being monitored were below and above those suggested by ATCC. Tryptic Soy broth (TSB) was used in order to help promote maximum growth (Collins, 1989).

### 2.2.3 Maximization of carbon source utilization

Once PHBs were produced, the bacterium was selected to grow on a medium that was representative of the amount of sugar produced from the lignocellulosic hydrolysate after a buffering for pH was done. If growth occurred on representative medium under non-limiting conditions, further tests on the representative medium under nitrogen limiting conditions were conducted. The final analysis was to adjust pH, oxygen, and nitrogen levels to allow for maximal PHB production. pH conditions ranged from 4-8.

## **2.3 Toxicity studies**

### 2.3.1 Tetracycline

An experimental study was conducted in order to see if the 0.04mg/ml and 0.08 mg/ml of added tetracycline had inhibitory effects on organisms grown in the hydrolysate. Tryptic soy broth (TSB) was inoculated with *B. megaterium* with tetracycline and grown at 30°C for 20 hours; readings were taken every hour after an initial 4 hour lag phase. TSB was used in order to provide the optimal amount of nutrients to the bacteria. TSB was also used because *B. megaterium* has been reported in literature to do best in TSB (Collins., 1987).

### 2.3.2 Furfural

A toxicity study was conducted using furfural; the levels being studied were those found in the acid pretreatment, which can range from 0.5 - 2 g/L (Nilevbrant et al., 2001). Based off this information, growth studies were conducted in order to gain the best understanding of how furfural impacts *B. megaterium*. The protocol used involved two replicates each of 0.5 g/L, 1 g/L, and 2 g/L furfural plus one blank (no bacteria, no furfural) and one control (no furfural). The medium used for growth of the bacteria was TSB. The temperature at which the bacteria were grown was 30°C. The duration of the *B. megaterium* growth study was 20 hours and the pH level was adjusted to be 6.

### 2.3.3 Acetic acid

Studies were conducted to see the effect of acetic acid on the growth of *B. megaterium*. Levels after treatment range from 2 g/L to 10 g/L (Taherzadeh et al., 1997). The protocol used involved two replicates each of 0.5 g/L, 1 g/L, and 2 g/L acetic acid plus one blank (no bacteria, no acetic acid) and one control (no acetic acid). Acetic acid used was glacial acetic acid, normality of 17.4 (99.7%) and density of 1.05. The medium used was TSB. Temperature was 30°C; the study was run over a time-period of 48 hours and NaOH buffer had to be used to raise the pH to range of 5.6-6.5.

## **2.4 Procedure for using RTILs with cells to get PHB**

One gram BMIMCl was added into 4 ml of culture broth with cells, vortexed and placed in an incubator at 50°C for 10 minutes. The RTIL sample was then removed from the incubator and observed microscopically, using DIC and Zeiss Axioplan 2 microscope. The different solutions of BMIMCl were kept at different temperatures and for different

periods of time. Samples were kept in temperature range from 25°C - 60°C for 5 - 30 minutes to test for effects of temperature and time. One sample was kept at each temperature for 5 min, the sample was then observed under Zeiss Axioplan2 microscope to see if the solution worked. It was then kept at temperature for 10 minutes, observed, kept 15 minutes observed, kept 20 minutes observed, kept 25 minutes observed, and kept for 30 minutes. This was done using 8 different temperatures of 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C and 60 °C. Each temperature and time had 2 replicates, therefore n=80 observations.

## **2.5 Peptidoglycan separation procedure using *B megaterium*:**

The peptidoglycan was extracted from the cell and separated out; see appendix D for the detailed procedure. The cells were grown in TSB at 30°C for 48 hours in order to maximize cell growth rates and biomass production. The cell solution was divided into two one-liter flasks containing 500 ml of TSB each, so as to fit in the incubator. The samples were observed microscopically using Zeiss Axioplan2 microscope and DIC filter set before the separation procedure began in order to make sure a pure culture of *B. megaterium* was being used. (See appendix D).

## Results and Discussion

### 3.1 Selection of PHB- producing/ sugar- utilizing bacteria

As part of objective one, two bacteria out of the six tested were selected to move forward. (The only bacterium listed in Table 1 not tested was *Saccharophagus degradans*). The reason the other four bacteria were not selected was because they were either not able to grow on the sugars present in the hydrolysate or were not PHB-producing strains (see Table 1). *B. megaterium* and *C. necator* were selected. *B. megaterium* was chosen to be studied because of its potential ability to produce PHAs when grown on the enzymatic hydrolysate that contains only glucose and the dilute acid hydrolysate that contains the sugars xylose and glucose, which *B. megaterium* can also use for growth (see Figure 8). The Gram-negative *C. necator*, unfortunately, was not able to grow on any sugar except fructose, which is not found in the lignocellulosic biomass. *C. necator* will be considered a possible strain for this project in the future if an epimerase is used to convert glucose to fructose.

The reason *C. necator* was originally selected for this project was because it was previously known as *Ralstonia eutropha*, which was reported to utilize glucose and produce PHBs (Du et al., 2001). It seems that, when the name was changed, some confusion apparently arose as to the characteristics of *R. eutropha* vs. *C. necator*. It was discovered that the *C. necator* ATCC strain #17697, referred to as *R. eutropha* (Du et al., 2001) is not able to utilize glucose but instead mainly uses fructose according to Bergey's Manual of Determinative Bacteriology (Garrrity, 2005). It can be concluded that perhaps some confusion still exists between the old classifications of *R. eutropha* and the new *C. necator* as strain #17699 from ATCC is different from Bergey's Manual of

Determinative Bacteriology as it has the ability to utilize glucose (Wang and Yu, 2007). The overall conclusion is that strain #17697 does not have the ability to utilize glucose, but perhaps in the future if strain # 17699 is used it may have the ability to utilize the glucose present in the hydrolysate. Therefore if strain # 17699 was used it may have yielded different results.

### **3.2 PHA production on different sugars found in lignocelluloses**

The Gram-positive *B. megaterium* was found to grow and produce PHAs using several of the sugars found in lignocelluloses, i.e., glucose and xylose, at levels that have been detected in the acid pretreatment and enzymatic hydrolysates produced at Michigan Tech (4 g/L; see Figure 8). Growth and biomass were very similar for both sugars. (The concentrations of the sugars were later raised towards much higher levels.) PHA production was detected microscopically during stationary phase with both glucose and xylose (Figure 9 and Appendix F). Before stationary phase was reached, it was seen that no PHB production was occurring (Figure 9). Furthermore, it can be seen that endospores were not being produced until 61 hours after inoculation. This endospore production was due to the decrease in nutrient availability and occurred late in the growth curve due to the lack of nitrogen. Figure 9, E depicts a cell sample with endospores. There is also a change in overall fluorescence because of the endospores, because the amount of viable cells producing PHBs decreases by the time the endospores are produced.

PHA production appeared to be about the same with both sugars at the 4g/L concentration based on microscopic staining procedures. Thus, *B. megaterium* appeared to be a good candidate for production of PHAs from both of the main 6- and 5-carbon sugars found in woody biomass.

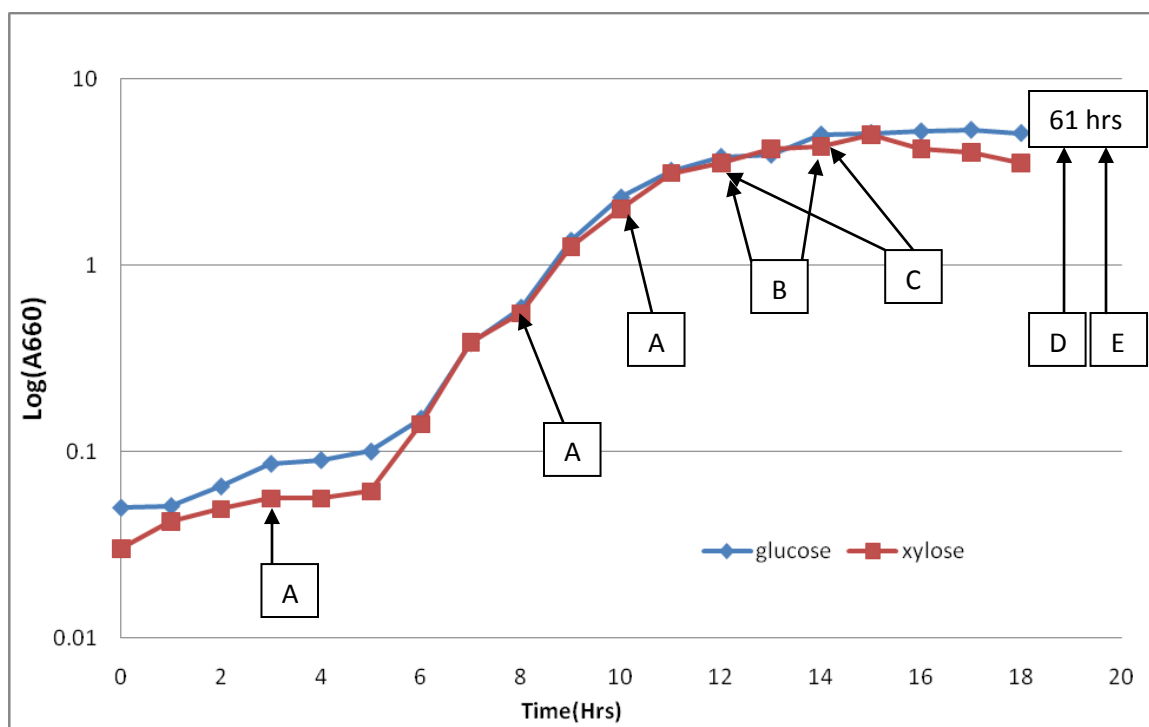


Figure 8. Growth of *B. megaterium* with NRML and different sugars as carbon/energy (4 g/L) at pH 6 and 30°C.

### 3.3 Toxicity and initial sugar screening studies

#### 3.3.1 Toxicity studies

In this section toxicity as an important growth-affecting parameter is examined. Shown in Figures 10-12 are *B. megaterium* growth curves when exposed to tetracycline, furfural and acetic acid respectively.

Figure 10 and Table2 show that *B. megaterium* is able to grow virtually uninhibited when exposed to twice the amount of tetracycline as found in the enzymatic hydrolysate (0.08 mg/ml). This is important as it means the hydrolysate should remain contaminant free until *B. megaterium* is placed into the hydrolysate (Lying et al., 2006), as most bacteria will be affected by these levels of tetracycline. The reason why *B. megaterium* is most likely not affected by the tetracycline is because it is a “hardy” strain,



and seems to possess a mechanism to lessen the affect of tetracycline on in its protein synthesis (Hitchins and Slepecky, 1969).

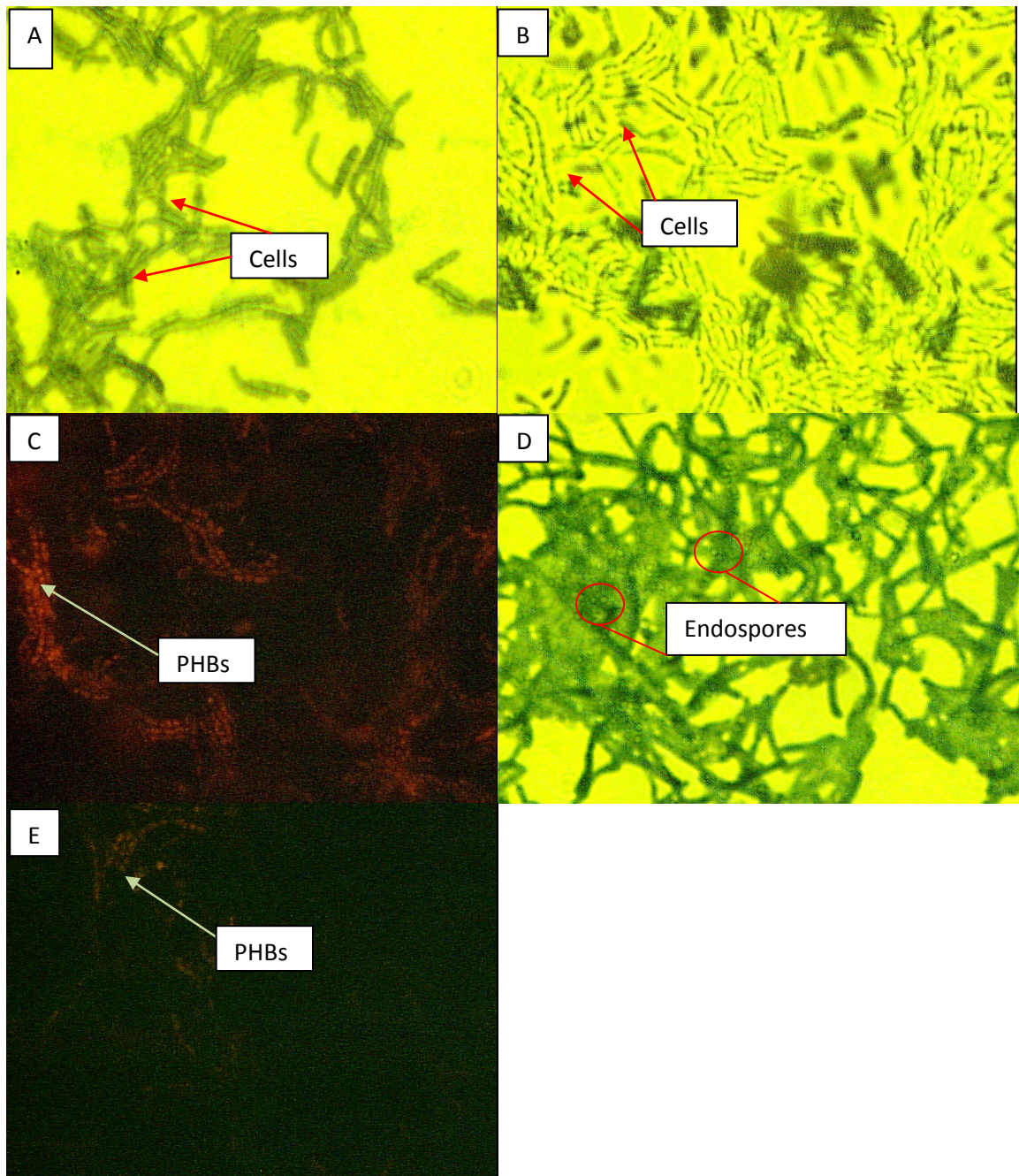


Figure 9. PHA production by *B. megaterium* grown in a NRML-G and NRML-X at pH 6 and 30°C observed at 1000X. Cells were observed with Nile Blue A from inoculation (time 0) until 61 hours after inoculation (A) using DIC microscopy (cells appear green and no PHB production is occurring), (B) using DIC microscopy (cells appear green), (C) epifluorescence microscopy (PHAs fluoresce orange), (D) using DIC microscopy (cells

begin endospore production), and (E) epifluorescence microscopy (PHAs fluoresce orange, endospores do not).

Figure 11 and Table 3 show that *B. megaterium* is able to grow in the presence of furfural at lower levels. The levels of furfural tested are indicative of those found in the hydrolysate. When no furfural is present percent biomass is best; when larger amounts of furfural are introduced, a negative effect in growth rates and percent biomass is observed. It was found that at 4g/L and above no growth occurred due to the toxicity associated with the furfural. However, at 2 g/L or less, which is the expected amount of furfural to be present in the hydrolysate, growth does occur.

Figure 12 and Table 4 show that, if acetic acid is properly buffered, then the higher the concentration the better the biomass production. This is most likely due to the excess acetyl-CoA produced from the acetic acid. As shown earlier (Figure 2), acetyl - CoA is a precursor not only to proper cell development but also PHB production. This information is very important because it demonstrates that *B. megaterium* has the ability to use the acetate present as a source to get better PHB production and growth with little added expense, as it must be buffered because without buffering the cells are not able to grow due to the low pH (Nath et al., 2008).

### 3.3.2 Initial sugar utilization screening

After initial analysis of the woody hydrolysate it was determined that the minimal amount of glucose and xylose found in the hydrolysate is enough for growth and PHB production to occur within the cells. It was seen that the higher the sugar levels the better the overall growth and biomass percent. (Figures 13, 14 and Table G-1).

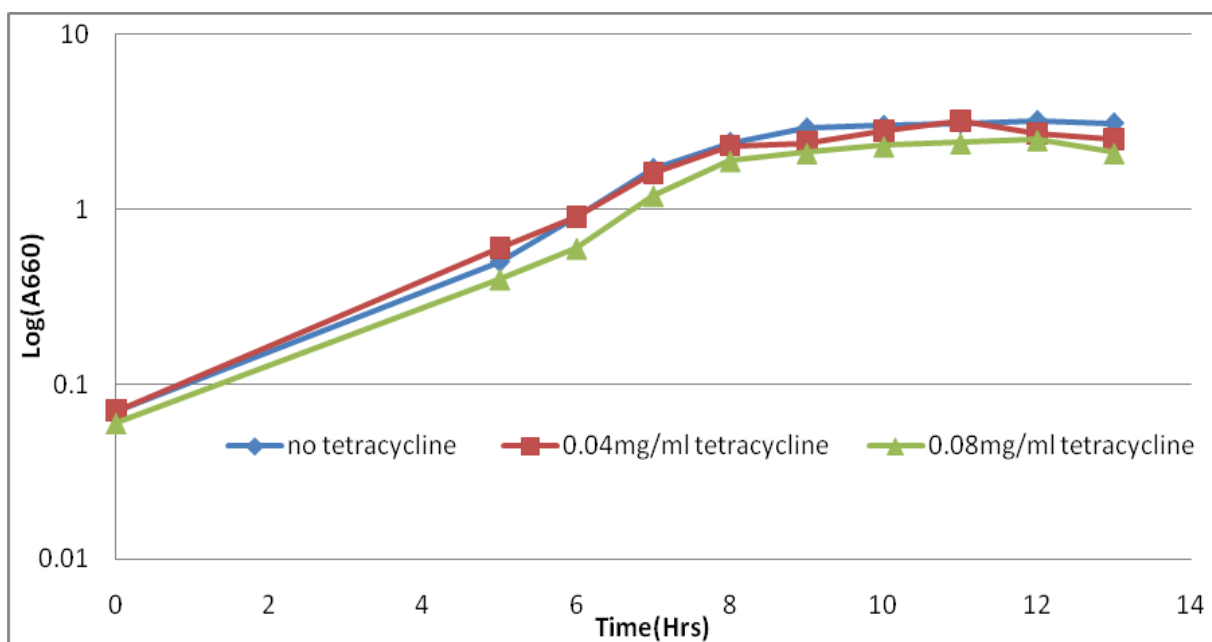


Figure 10. Effects of tetracycline on growth of *B. megaterium* grown on TSB at 30 °C, pH 6.0. (See Figure G-1 for replicate data). Not depicted is a 3.5 hour lag phase.

Table2. Growth rate (k) and percent biomass of *B. megaterium* grown at different levels of tetracycline.

Tetracycline (mg/ml)	k (hr <sup>-1</sup> ) <sup>a</sup>	Percent biomass <sup>b</sup> (8 hrs)
0	2.1±0.03	100
0.04	1.9±0.01	90
0.08	1.9±0.00	85

<sup>a</sup> mean (n=2) ±SD

<sup>b</sup>  $\frac{T0(TI_8-T0)-TI}{T0}$  T0=control  
TI=Time final

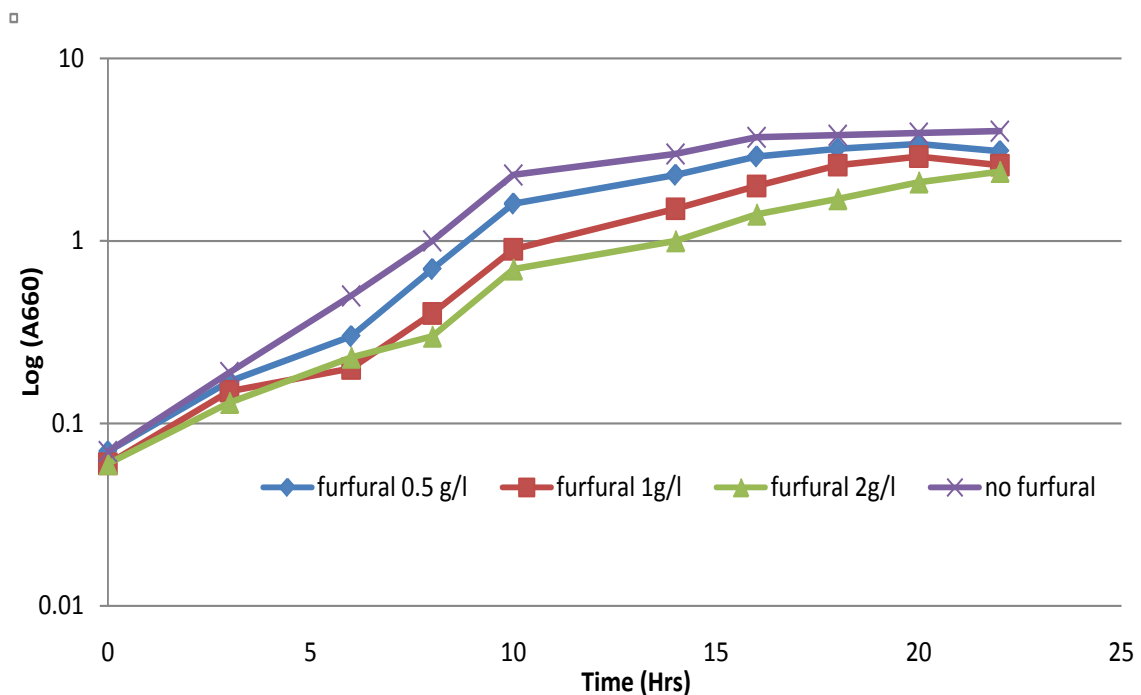


Figure 11. Effects of furfural on growth of *B. megaterium* grown on TSB at 30 °C, pH 6.0. (See Figure G-2 for replicate data). Not depicted is a 5 hour lag phase, also not depicted on graph is that at 4g/L furfural was unable to grow.

Table 3. Growth rate ( $k$ ), and percent biomass of *B. megaterium* when grown in presence of furfural

Levels of furfural (g/L)	$k$ ( $\text{hr}^{-1}$ ) <sup>a</sup>	Percent biomass <sup>b</sup> (9 hrs)
0	$2.0 \pm 0.07$	100
0.5	$1.8 \pm 0.02$	93
1	$1.72 \pm 0.02$	87
2	$1.6 \pm 0.01$	45

<sup>a</sup> mean ( $n=2$ )  $\pm$ SD

<sup>b</sup>  $\frac{T_0(TI_9 - T_0) - TI}{T_0}$   $\frac{T_0 = \text{control}}{TI = \text{Time final}}$

□

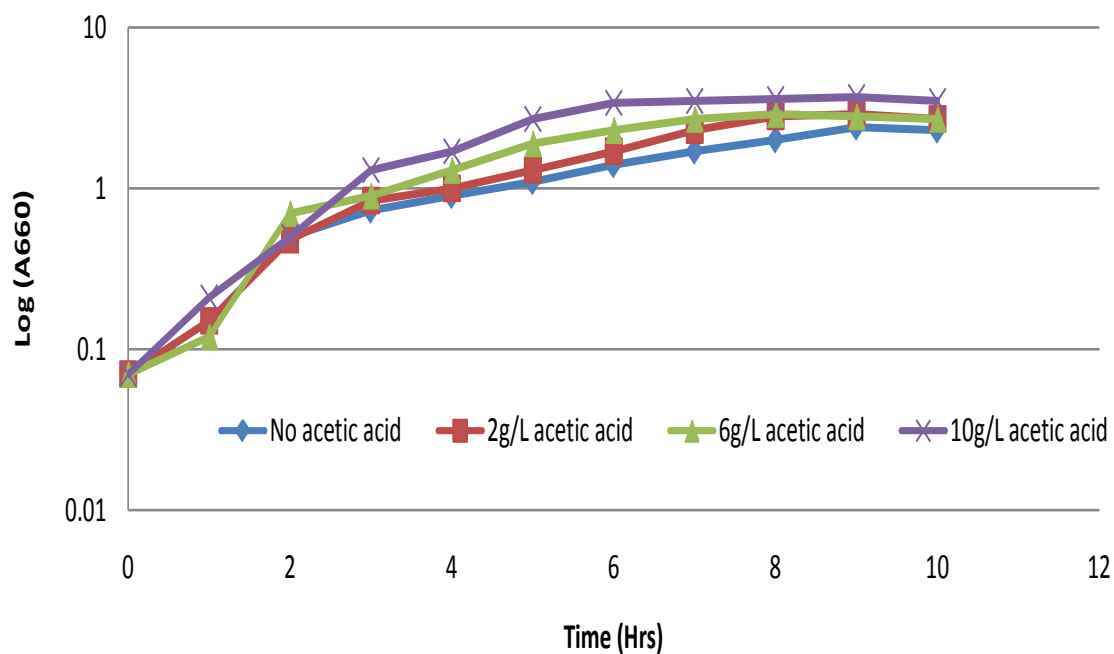


Figure 12 Effects of acetic acid on growth of *B. megaterium* grown on TSB at 30 °C, pH 6.0. Not depicted is a 4 hour lag phase.

Table 4. Growth rate (k), and percent biomass of *B. megaterium* when grown in presence of acetic acid.

Acetic Acid (g/L)	k (hr <sup>-1</sup> ) <sup>a</sup>	Percent biomass <sup>b</sup> (7 hrs)
0	1.9±0.02	100
2	2.0±0.01	110
6	2.1±0.01	130
10	2.2±0.04	150

<sup>a</sup> mean (n=1) ±SD

<sup>b</sup>  $\frac{T0 (TI_7 - T0) - TI}{T0}$  T0=control  
TI=Time final

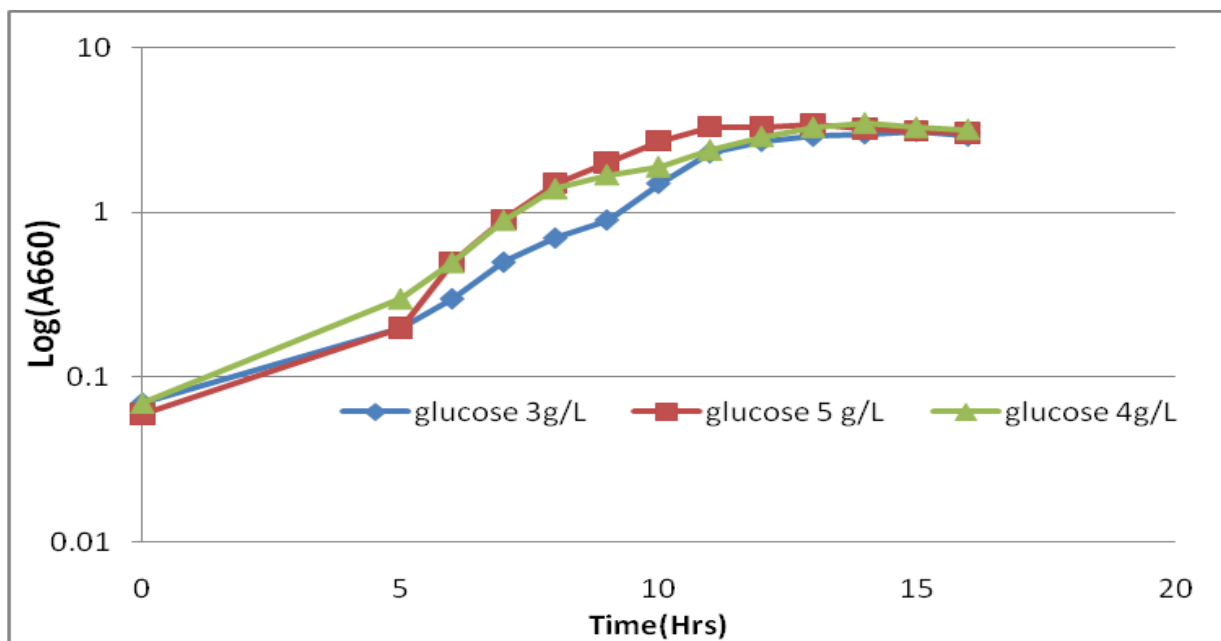


Figure 13. Growth of *B. megaterium* with NRLM-G (3, 4, 5 g/L) at pH 6 and 30°C. (See Figure G-3 for replicate data.) Not depicted is a 5 hour lag phase.

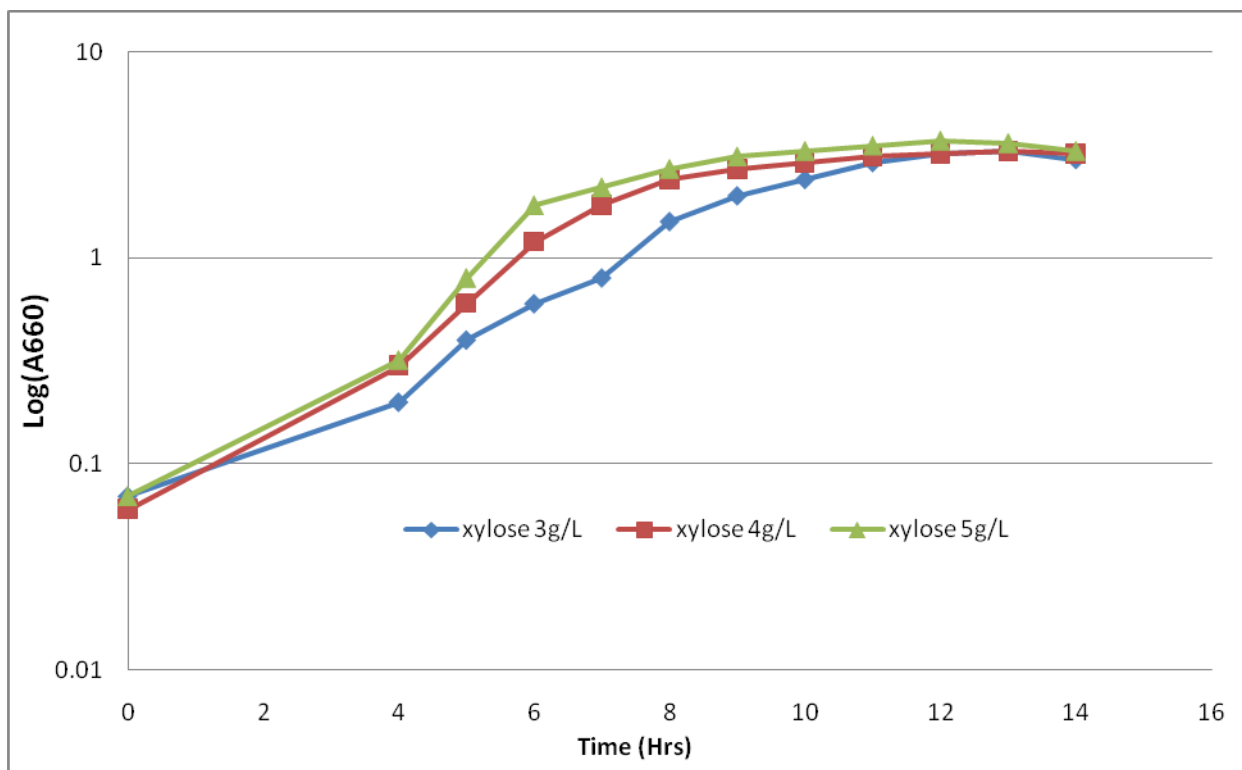


Figure 14. Growth of *B. megaterium* with NRLM-X (3, 4, 5 g/L) at pH 6 and 30°C. (See Figure G-4 for replicate data.) Not depicted is a 5 hour lag phase.

### 3.4 Determination of optimal growth and PHB production of *B. megaterium*

Detailed studies were conducted to determine the optimal growth conditions for *B. megaterium* and for PHB production on xylose and glucose. The parameters that varied were: temperature, pH, nitrogen levels, and sugar concentration (Figure 15). DO is not depicted in Figure 15, because effects it would have on *B. megaterium* cells were not studied.

To determine the optimal growth with respect to pH and temperature, *B. megaterium* was grown in TSB as this medium is reported in literature to be favored by *B. megaterium*. Absorbance readings were taken at set time intervals, and examination was made for PHB deposits during stationary phase. Optimal growth (as biomass) was found at 30°C (Figure 16; Table 5) this was as expected from previous studies. The optimal pH was found to be around 6.0 similar to that found in literature (Anderson and Dawes., 1990). (Figure 17; Table 6), although *B. megaterium* can grow and produce PHBs quite well over a large pH range. No growth was detected at pH 4 or 5, which is similar to what is found in the literature (Anderson and Dawes., 1990).

Previous studies have shown that the enzymatic hydrolysate contains an average amount of 4 g/L of carbon/energy source. Although *B. megaterium* can grow well and produce PHBs with these sugar levels, these levels are not optimal for either biomass or PHB synthesis. Growth studies were conducted recently in order to find the optimal carbon source level for PHB production. Values of 20, 30, and 40 g/L were used, as these are levels that could be obtained from some of the pre-treatments (~20 g/L) or from concentrating the hydrolysates (30 and 40 g/L) (personal communication with Chemical Engineering at Michigan Tech). As shown in Figures 18 and 19 and in the growth data in Table 7, optimal growth/biomass production was obtained at 30 g/L with both xylose and glucose. (The 4 g/L data are included for comparison purposes.)

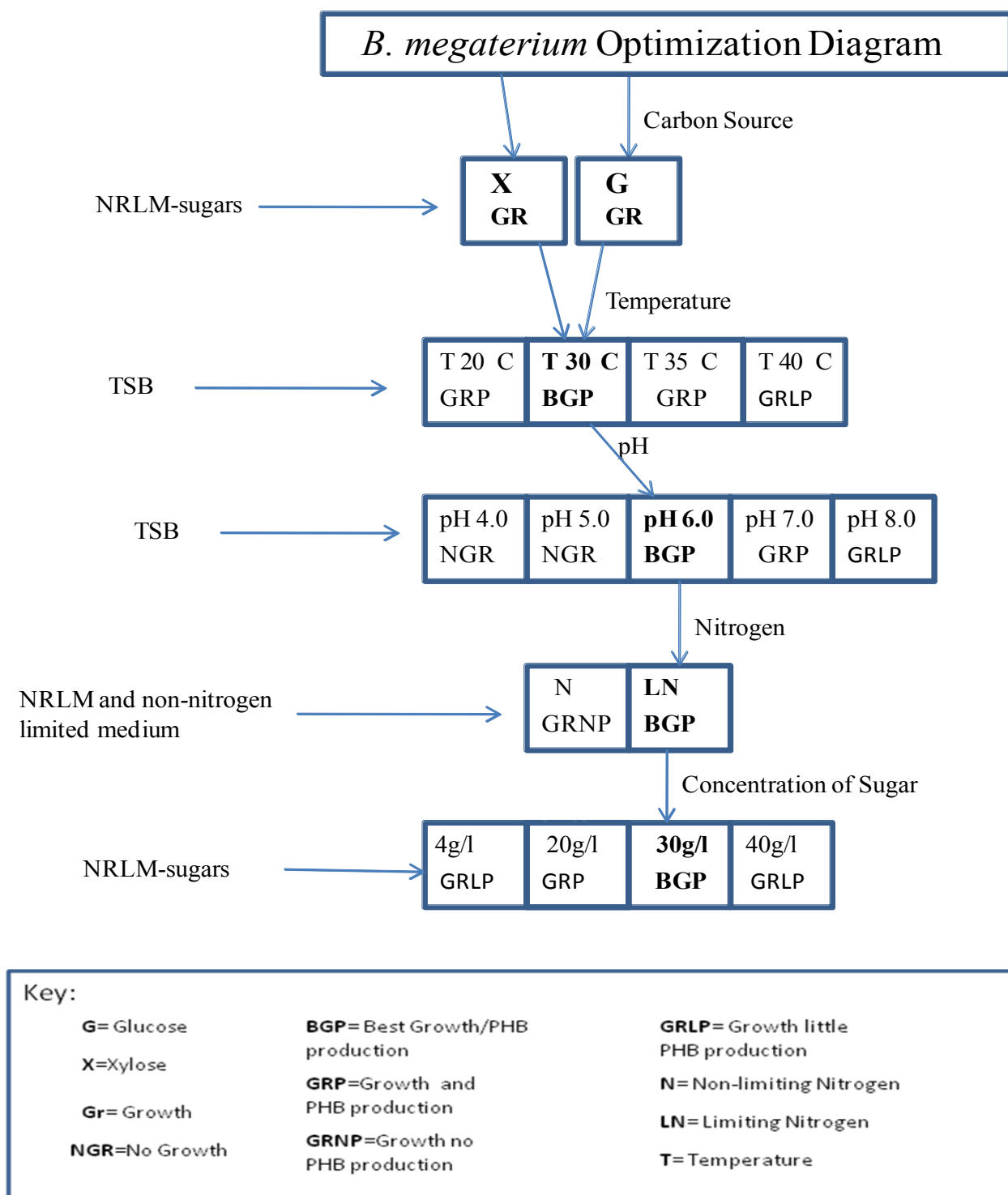


Figure 15 Project plan for optimization of PHB production by *B. megaterium* when grown on xylose (X), and glucose (G). Maximum growth/PHB production conditions are in boldface; these conditions were used in subsequent studies. Results for glucose and xylose were the same for temperature, pH, nitrogen and sugar concentrations.



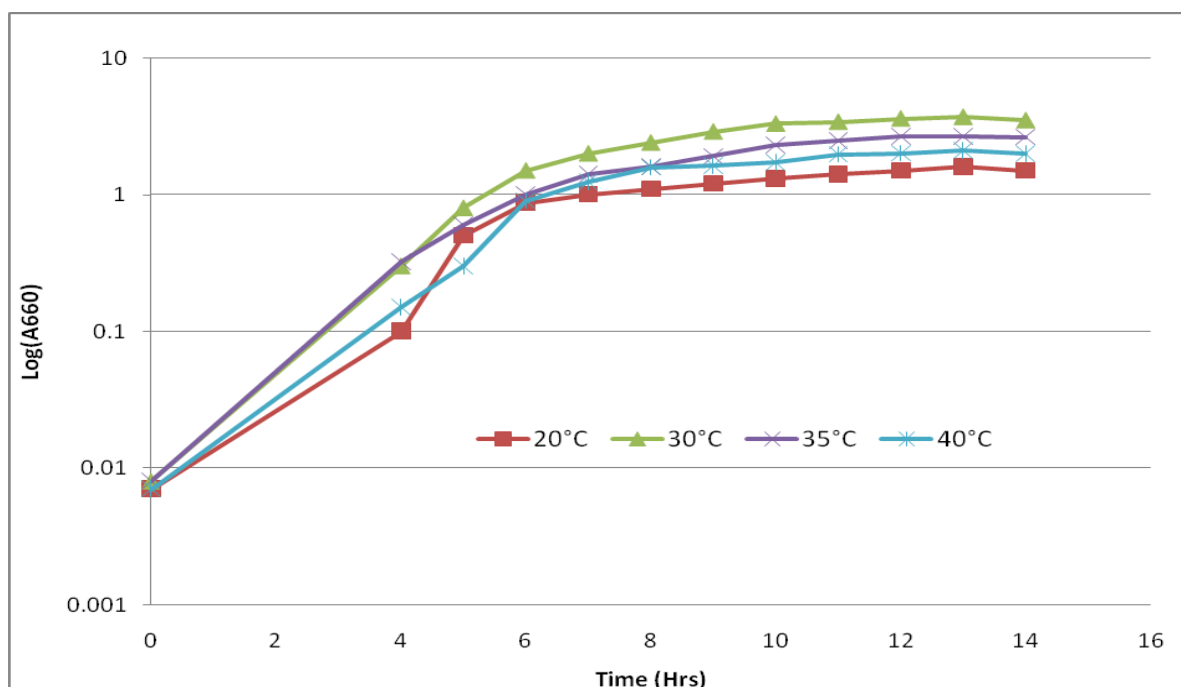


Figure 16. The effect of temperature on the growth of *B. megaterium* when grown on TSB and pH 6.0. (See Figure G-5 for replicate data.) Not depicted is a 4.5 hour lag phase

Table 5. Growth rate (k) and percent biomass of *B. megaterium* grown at different temperatures.

Temperature (°C)	k (hr <sup>-1</sup> ) <sup>a</sup>	Percent biomass <sup>b</sup> (9 hrs)
20	0.93±0.02	40
30	2.1±0.01	100
35	1.9±0.01	96
40	1.6±0.04	83

<sup>a</sup> mean (n=2) ±SD

<sup>b</sup>  $\frac{T_0(TI_9 - T_0) - TI}{T_0}$  T0=control  
TI=Time final

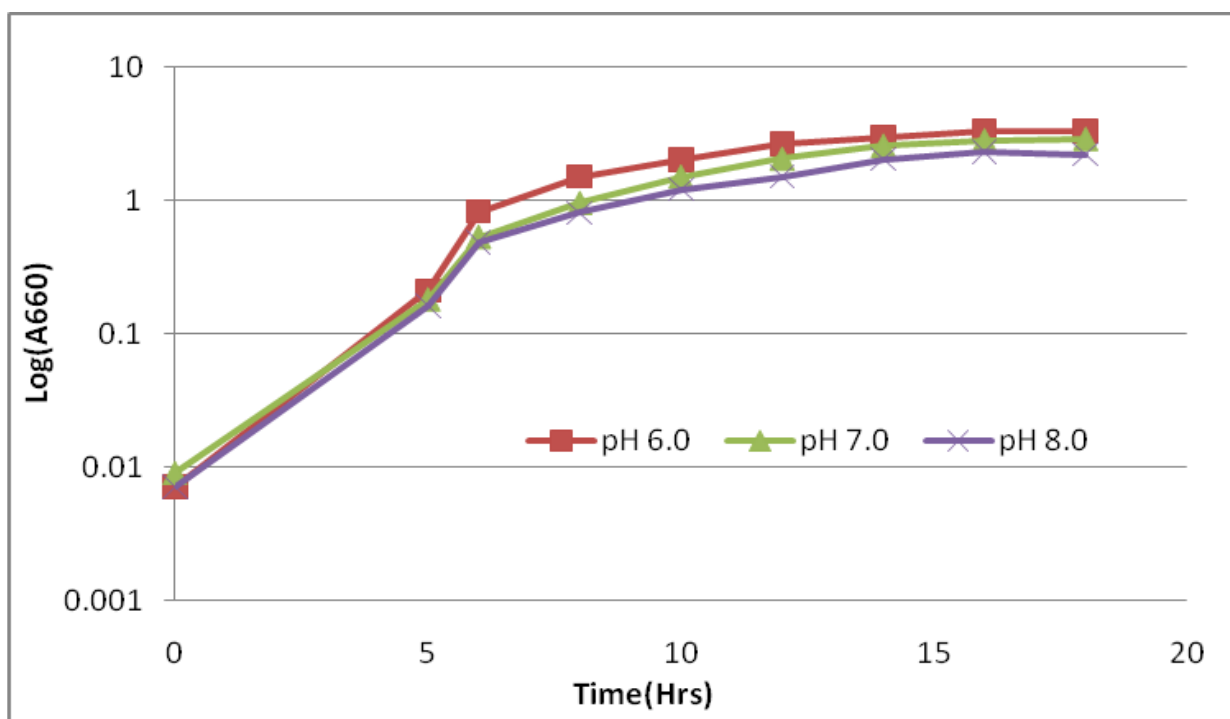


Figure 17. The effect of pH on the growth of *B. megaterium* when grown on TSB at 30°C. (See Figure G-6 for replicate data.) Not depicted is a 3 hour lag phase.

Table 6. Growth rate (k) and percent biomass of *B. megaterium* grown at different pHs.

pH	k (hr <sup>-1</sup> ) <sup>a</sup>	Percent biomass <sup>b</sup> (7 hrs)
4.0	No growth	No growth
5.0	No growth	No growth
6.0	2.1±0.01	100
7.0	2.0±0.02	90
8.0	1.9±0.01	87

<sup>a</sup> mean (n=2) ±SD

<sup>b</sup>  $\frac{T0 (TI_7 - T0) - TI}{T0}$  T0=control  
TI=Time final

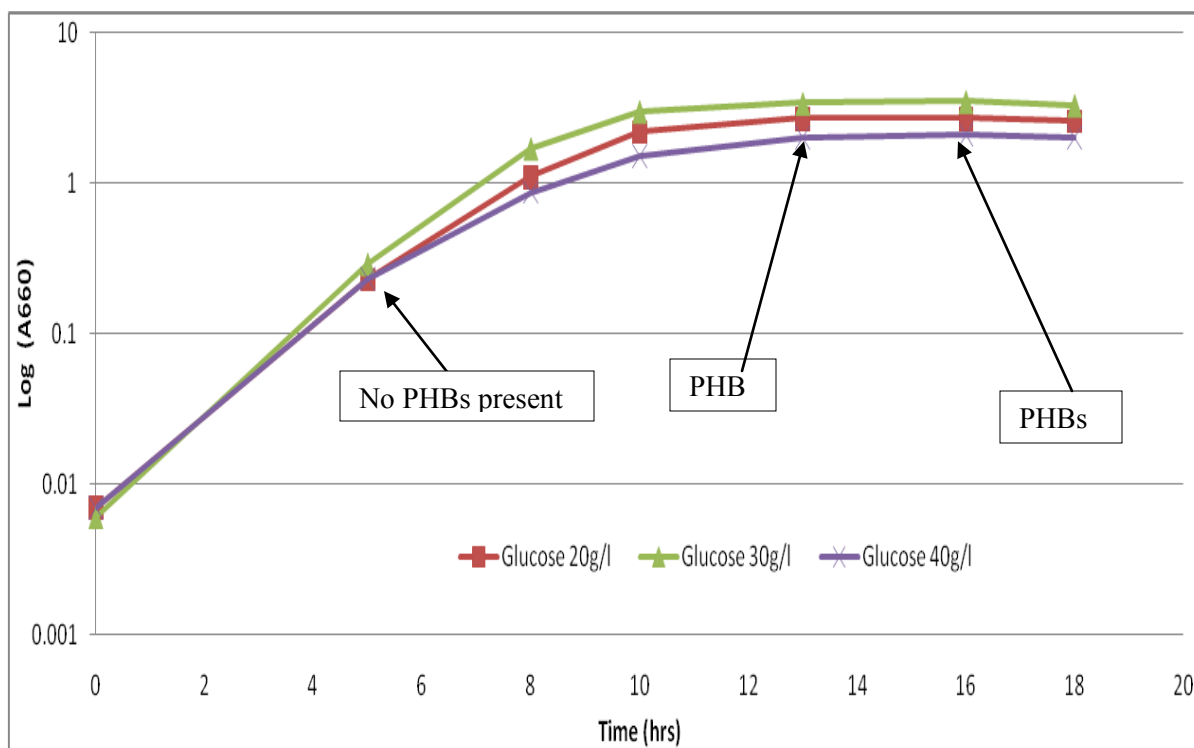


Figure 18. Growth of *B. megaterium* with NRLM-G (20, 30, 40 g/L) at pH 6 and 30°C. Two replicates of each sugar were run. (See Figure G-7 for replicate data.) Not depicted is a 4 hour lag phase.

Very good PHB production was observed (using Nile Blue A staining technique) at the two monitoring times (shown in Figures 18 and 19) at 20 and 30 g/L with both sugars. (See Figure 20 for the photomicrograph results with glucose.) However, little PHB synthesis was detected with either sugar at 40 g/L. (See Figure 21 for the photomicrograph results with glucose and Appendix F for others). This agrees with literature information indicating that 3% w/v (30 g/L) of a carbon source is preferred by *B. megaterium* when producing PHBs (Gouda et al., 2001).

Table 7. Growth rate (k) and percent biomass of *B. megaterium* grown on varying levels of glucose or xylose.

Sugar Level (g/L)	glucose <sup>a</sup>	glucose <sup>b</sup> percent biomass (9 hrs)	xylose <sup>a</sup>	xylose <sup>b</sup> percent biomass (9 hrs)
4	1.6±0.01	46	1.5±0.03	44
20	1.9±0.01	92	1.9±0.01	90
30	2.1 ±0.01	100	2.15±0.21	100
40	1.7±0.02	78	1.7±0.0	78

<sup>a</sup> mean (n=2) ±SD

<sup>b</sup>  $\frac{T0(TI_9-T0)-TI}{T0}$  T0=control  
TI=Time final

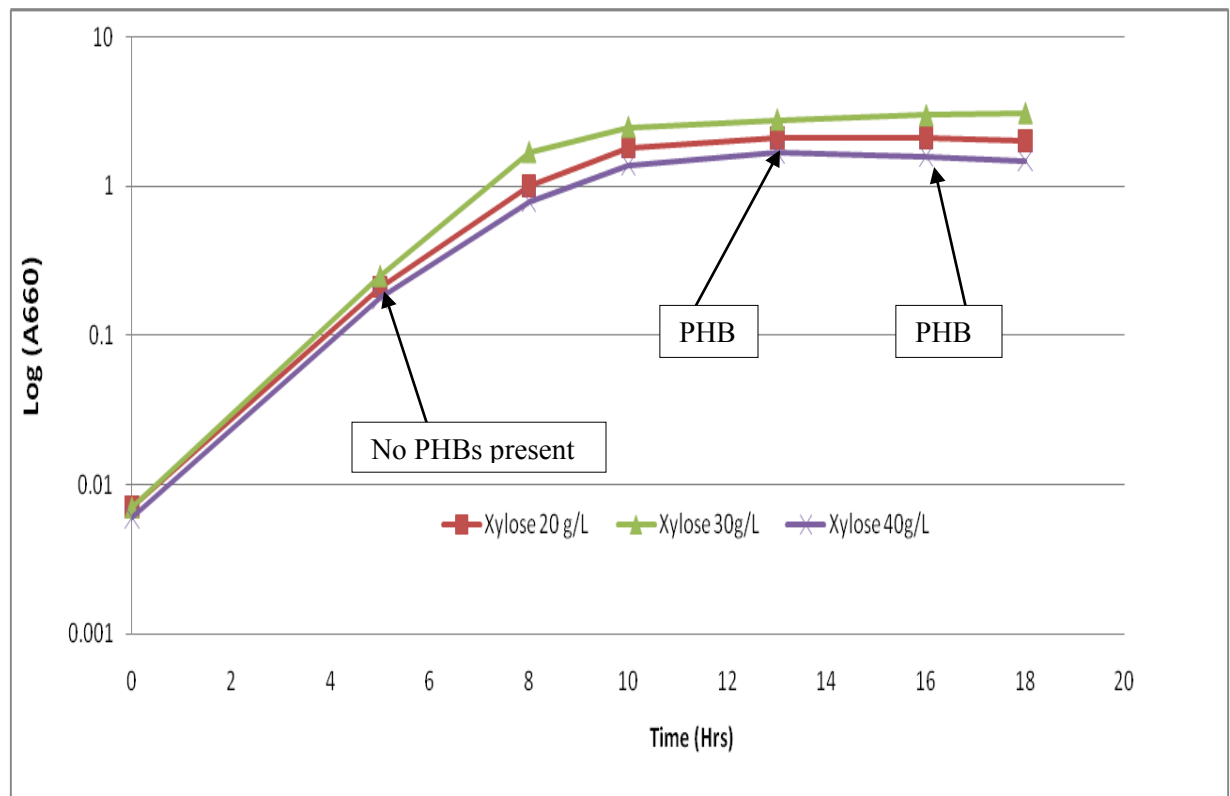


Figure 19. Growth of *B. megaterium* with NRLM-X (20, 30, 40 g/L) at pH 6 and 30°C. Two replicates of each sugar were run. (See Figure G-8 for replicate data). Not depicted in figure is 4 hour lag phase.

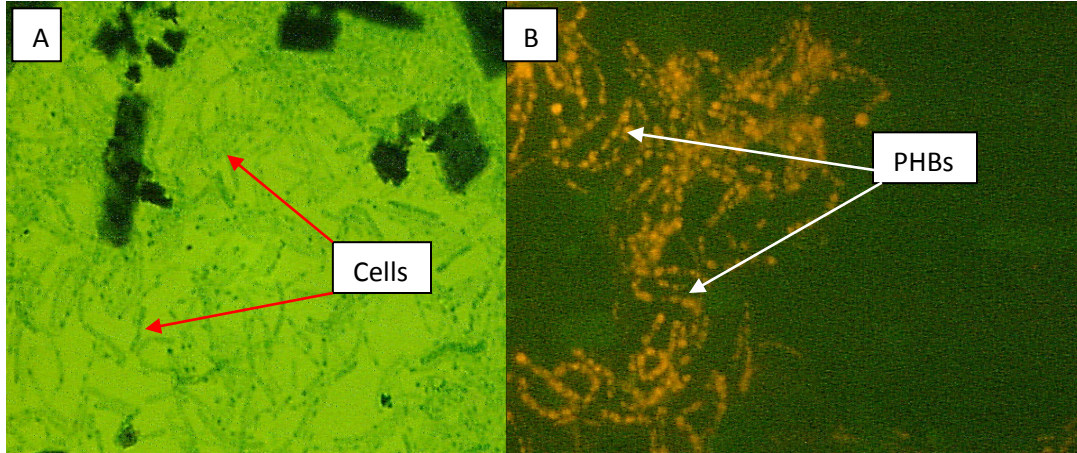


Figure 20. PHA production by *B. megaterium* grown in a NRLM-G at pH 6 and 30°C. Cells were observed during stationary phase with Nile Blue A: (A) using differential interference contrast microscopy (400X cells appear green) and (B) epifluorescence microscopy 1000X.

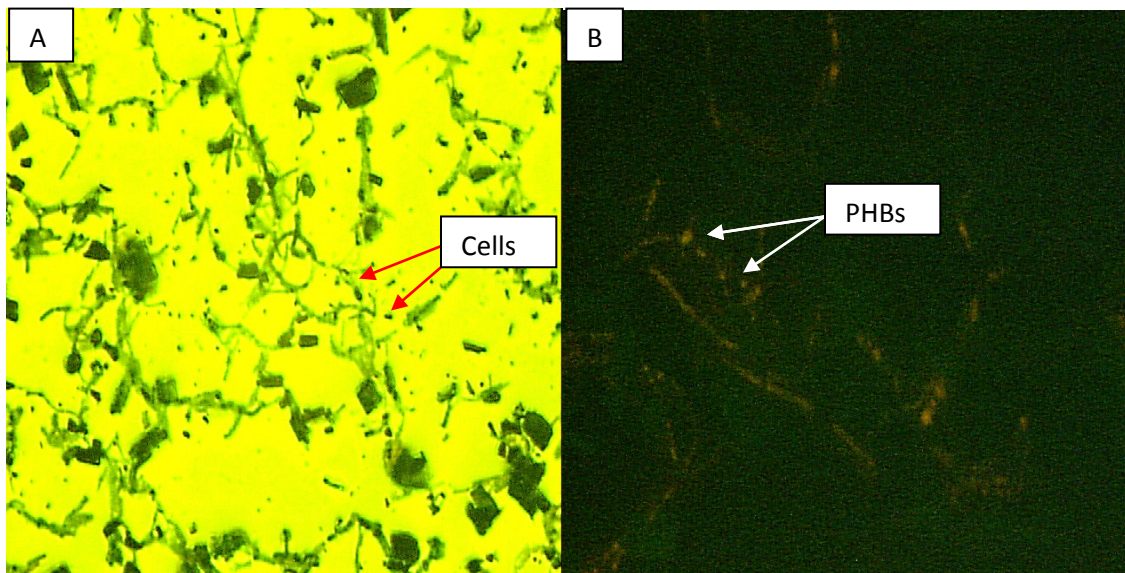


Figure 21. PHA production by *B. megaterium* grown in a NRLM-G 40g/L at pH 6 and 30°C. Cells were observed during stationary phase with Nile Blue A: (A) using differential interference contrast microscopy (400X cells appear green) and (B) epifluorescence microscopy 1000X.

### 3.5 Extraction of PHBs and peptidoglycan from *B. megaterium* using traditional methods and RTILs.

#### 3.5.1 RTILs

After many attempts were made using the RTIL, BMIMCl, it is clear that BMIMCl does not have the ability to break the cell wall of the Gram-positive *B. megaterium*. It was initially thought that perhaps BMIMCl would break the cell wall of *B. megaterium* as the wall is organic and the BMIMCl dissolves organic materials. However, following many screenings and temperature measurements, it seems as if BMIMCl cannot break the cell wall. Shown in figure 22 is *B. megaterium* before and after treatment with BMIMCl at 50% concentration and 60°C. (Note: a wet mount was performed because BMIMCl did not allow for a heat-fixed smear to be made due to its oil-like properties.) Therefore, for purposes of time, traditional extraction methods were used in order to break apart the cell wall of *B. megaterium* and separate out the peptidoglycan in the cell wall and PHB found inside the cytoplasm.

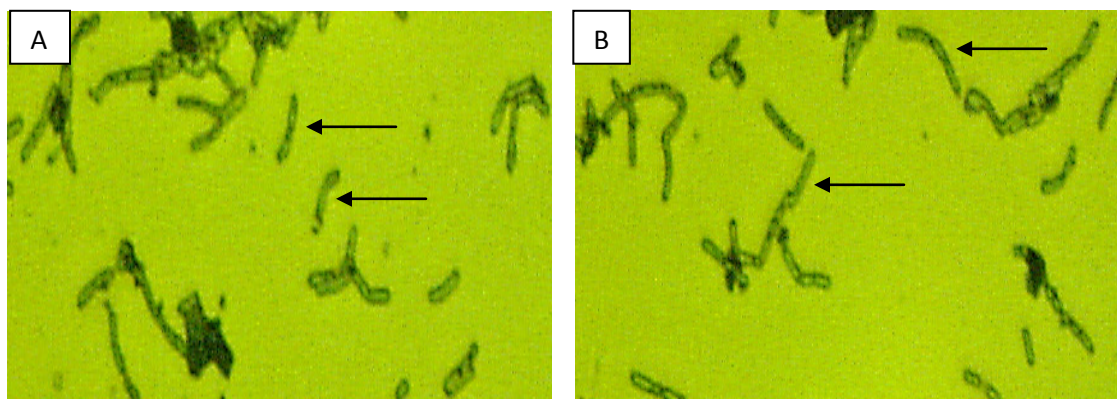


Figure 22. Depiction of *B. megaterium* before and after exposure to BMIMCl 50% concentration. (A) Before exposure to BMIMCl; (B) After exposure to BMIMCl. Cells were kept at 60°C for 20 minutes in order to enhance effects of BMIMCl. (The arrows indicate cells before and after treatment of BMIMCl at 1000X). See Appendix F for more RTIL photos.

#### 3.5.2 Extraction of peptidoglycan

After following the protocol shown in appendix D, a successful extraction of peptidoglycan was achieved. After extraction, a total of 312 mg dry weight was attained. This was done after a 13.5 hour-long procedure and the use of 8 grams of wet cell mass or 2 grams dry weight. The cell culture did not possess any endospores and was confirmed to be *B. megaterium* using microscopic examination. The percentage of peptidoglycan recovered was 16% of the cell mass. This amount of peptidoglycan recovered is low when compared to the potential amount of peptidoglycan that could be found in literature for a *Bacillus species*. Literature cites the amount for a bacillus is closer to 45-50% of the cells mass to be peptidoglycan as PHBs and other components of the cell would make up the remaining material. (Colwell and Grigorova., 1987).



## Conclusions

- 1) The Nile Blue A stain used for objective one was successful. The stain allowed for fast and presumably accurate screening to take place. The stain also allowed for proper selection of two bacterial species for use in these studies..
- 2) *B. megaterium* is the optimal bacterium for this project. It is able to grow in the presence of tetracycline, furfural, and dilute acetic acid; this is very important information as all these substances are potential toxins present within the two forms of the hydrolysate. Also *B. megaterium* seems to have an ability to use the acetic acid present in the hydrolysate to increase biomass. Furthermore. *B. megaterium* is also able to utilize two of the sugars found in the hydrolysate this has many advantages for future study. Moreover *B. megaterium* also has the ability to produce large amounts of peptidoglycan, which can be used as a co-polymer when combined with the PHBs.
- 3) The RTIL BMIMCl seemed not to have the previously desired effect on the bacterial cells. This was most probably due to the non-specificity of BMIMCl with the Gram-positive *B. megaterium*. It was because of BMIMCl's inability work and purposes of time that more traditional methods such as sonication and enzyme treatments were used for PHB and peptidoglycan extraction.



## Recommendations

- 1.) Run *B. megaterium* in the Bio Flow 3000 bioreactor. This would allow for large-scale production of PHAs to take place, also would have allowed for other parameters to be more closely monitored. These parameters are pH fluctuation, DO levels, and agitation rate and glucose and xylose disappearance using HPLC.
- 2.) Run PHB extraction through the GC to get confirmation. This GC information would confirm the purity, and structural integrity of those PHBs extracted.
- 3.) Optimize the peptidoglycan separation procedure; this would allow for higher yields and higher purity samples of peptidoglycan to be attained.
- 4.) Study the effectiveness of extracting PHBs and peptidoglycan together. The reason for this recommendation is due to the fact that if the two processes were to be combined then the yields and production would most likely be higher.
- 5.) Work to get growth of *B. megaterium* on the hydrolysate (dilute acid) so as to demonstrate *B. megaterium* usefulness with the lignocellulosic materials.
- 6.) Work more with RTILs in order to get a better understanding of the mechanisms behind them, and also to get them to work better with *B. megaterium*, in the aspect of disrupting the cell wall.
- 7.) Look at the effect of combining acetic acid with furfural.
- 8.) Use the limited nitrogen medium with acetic acid to see PHB production.
- 9.) Make synthetic hydrolysate and mix with varying levels of real hydrolysate.

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## **Appendix A:**

### Modified Nile A blue staining procedure (modified from Ostle et al. 1982)

- 1.) Make up new staining solution (add 99% water: 1% Nile Blue A stain) (Keep the stain in the incubator at 55°C right after making stain so as to keep the stain as warm as possible until ready to place slide in jar.)
- 2.) Use cells from plates and add at least three loops of culture, with one drop of water and make a heat-fixed slide
- 3.) Place slides into a Coplin staining jar and leave in incubator for 10-20 minutes at 55°C (20min typically yields better results).
- 4.) Remove slides from jar and place on staining rack. Add 8% acetic acid to slide and let stand for 1 min.
- 5.) Wash slide with water. Let air dry.
- 6.) Observe bacterial cells on the slide under microscope using Acridine Orange Filter set when using epifluorescence.
- 7.) Turn on UV light at 68-95% PHBs should give bright orange fluorescence with Acridine Orange Filter set.



## Appendix B:

### Gas Chromatography protocol (modified from Oehmen et al. 2005)

- 1.) Use the DB-5 Capillary column. (The length of the column is 30 meter length. The internal diameter is 0.25mm. The column is coated with a 0.25micrometer film with split injection ratio of 1:15.)
- 2.) Use Helium as a carrier gas.
- 3.) Operate the flame ionization detector (FID) unit at 300°C with an injection temperature of 250 °C.
- 4.) Set the oven temperature at 80°C for 1min. Increased at 10°C per minute to 120°C. and then to 270°C at 45°C per minute and held for 3minutes.
- 5.) Use external standards 0-3mg of a R-3HB, R-3-HV, copolymer(7:3) obtained from (Fluka) and 0-3mg of 2-hydroxycaproic acid (Sigma)
- 6.) Run the two standards to aid in identification of both the PHB as well as any copolymers that may be present.

### Gas Chromatography

Table B-1: GC Parameters for PHA analysis (Oehmen et al., (2005).

GC Column	Length (meter)	Internal Diameter (mm)	Film Diameter (µm)	Carrier Gas	FID (°C)	Oven Temperature (°C)	Retention time (min.)
DB-5	30	0.25	0.25	Helium	300	80	3

## Appendix C:

NRLM used for *B. megaterium* for PHB production (modified from Anderson and Dawes. 1990).

\*\*Medium is autoclaved for 35 minutes at 121°C and desired sugar amounts are filter-sterilized using 0.2µm filters, and added to solution after cooling. The sugar amount added to the solution varied depending on test being conducted. An example of the solution was made was A+B=C. “A was sugar amount” “B was minerals” and “C was final solution”. The final solution was made using two 500ml flasks, one contained double concentrated sugar. The other flask contained double concentrated minerals, and when mixed made 1 liter normal strength solution, see mineral medium components are below.

- |   |   |
|---|---|
| 1. ) 2 g/L of (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>     | 8.) 30 mg/L of MnCl <sub>2</sub> 4H <sub>2</sub> O    |
| 2.) 6.8 g/L of Na <sub>2</sub> HPO <sub>4</sub> 7H <sub>2</sub> O | 9.) 300 mg/L of H <sub>3</sub> BO <sub>3</sub>        |
| 3.) 1.5 g/L of KH <sub>2</sub> PO <sub>4</sub>                    | 10.) 200 mg/L of CoCl <sub>2</sub> H <sub>2</sub> O   |
| 4.) 100 mg/L of CaCl <sub>2</sub> 2H <sub>2</sub> O               | 11.) 20 mg/L of CuSO <sub>4</sub> - 5H <sub>2</sub> O |
| 5.) 60 mg/L of NH <sub>4</sub> -Fe(III) citrate                   | 12.) 20 mg/L of NiCl <sub>2</sub> - 6H <sub>2</sub> O |
| 6.) 200 mg/L of MgSO <sub>4</sub> 7H <sub>2</sub> O               | 13.) 30 mg/L of NaMoO <sub>4</sub> 2H <sub>2</sub> O  |
| 7.) 100 mg/L of ZnSO <sub>4</sub> 7H <sub>2</sub> O               |   |

## Appendix D:

### Peptidoglycan Separation Procedure ( modified from Colwell and Grigorova. 1987).

- 1) Weigh centrifuge vial, add cells grown for 48 hours on TSB, pH 6.0,
- 2) Centrifuge at 12,000 x g for 10 minutes following centrifugation
- 3) Decant, then weigh vial again.
- 4) Suspend 2 grams wet weight of cells in 6 ml of 0.05M phosphate buffer (pH 7.2) and sonicate on ice until cells are broken using a Misonix Sonicator 4000.
- 5) Observe cells under microscope to confirm disruption. Sonication should take place for 2 min with half second on half second off duty cycle and amplitude of 37%. (observe turbidity and repeat if solution is not turbid)
- 6) Centrifuge at 1800 x g for 10 min. ( removes undisrupted cells) Use supernatant and Centrifuge supernatant at 12000 x g for 1 h
- 7) Resuspend in 5ml 0.05M phosphate buffer.
- 8) Add the cell suspension from step 5 (5ml) to 1 ml 25% sodium dodecyl sulfate (SDS). Heat at 100 °C for 40 min.
- 9) Centrifuge at 12000 x g for 1 hr at 30°C
- 10) Wash pellet twice in phosphate buffer 0.05M pH 7.6. Then centrifuge for 10 min at 12000x g.
- 11) Resuspend pellet in 2ml 0.05M phosphate buffer. (pH 7.6) and
- 12) Add 100µL of Pronase E from Sigma to solution. (The concentration of the enzyme solution is 1mg/ml, and enzyme solution must be filtered before using a pore size of 0.2µm.)
- 13) Incubate the enzyme treatment for 2hr at 37 °C. Centrifuge the solution at 12000 x g for 30min, and wash with same buffer and centrifuge for 10 min at 12000 x g.
- 14) Suspend the pellet in 2 ml 5 % trichloroacetic acid (TCA) and keep at 100 °C for 20 min. in screw cap jar, as TCA vapors are very toxic and transfer work must be done under the hood.

- 15) Cool to room temperature, transfer 2ml suspension to a glass centrifuge tube free of chloroform, so as to not degrade amount of peptidoglycan present. Centrifuge at 12000 x g for 30min.
- 16) Collect pellet and wash three times with phosphate buffer (pH 7.6). Centrifuge after each washing for 10 min. at 12000 x g.
- 17) Wash with 2ml ethanol (95 %) centrifuge for 10 min at 12000x g
- 18) Wash with 2 ml diethyl ether (99 %) with centrifugation, at 12000 x g for 10 mins.
- 19) Dry cells in vacuum oven at 105°C for 20 minutes. The powder is then removed from the test tube. The dry weight was determined by weighing the tube before and after addition of the peptidoglycan.
- 20) Prepare Peptidoglycan sample of peptidoglycan now ready.

**Safety notes:** TCA must be worked with under the hood, as it is a very caustic agent. Please also consult material safety data sheets (MSDS) before working with TCA.

## **Appendix E:**

### PHB separation protocol (modified from Hahn et. al. 1995).

1. Place 1-g portion of dried cells into 5% (vol/vol) sodium hypochlorite
2. Keep cells at 30 °C for 15 minutes in 5% sodium hypochlorite solution.
3. Centrifuge mixture at 4,000 x g for 15mins at 25 °C (three separate phases form)
  - a.) Upper phase is a hypochlorite solution (pipette off)
  - b.) Middle phase contains non-PHB cell material (pipette off)
  - c.) Undisrupted cells and PHBs are in the bottom phase remove pellet from container
4. Resuspend the pellet and wash with hot acetone (60°C) for 20 min
5. Recover clear polymer solution by centrifugation at 12000 x g for 10 min.
6. Keep pellet at 100 ° C for 20 minutes in order to dry.
7. Obtain dry powder.

## Appendix F:

Photos of PHBs taken using Zeiss Axioplan2 microscope.

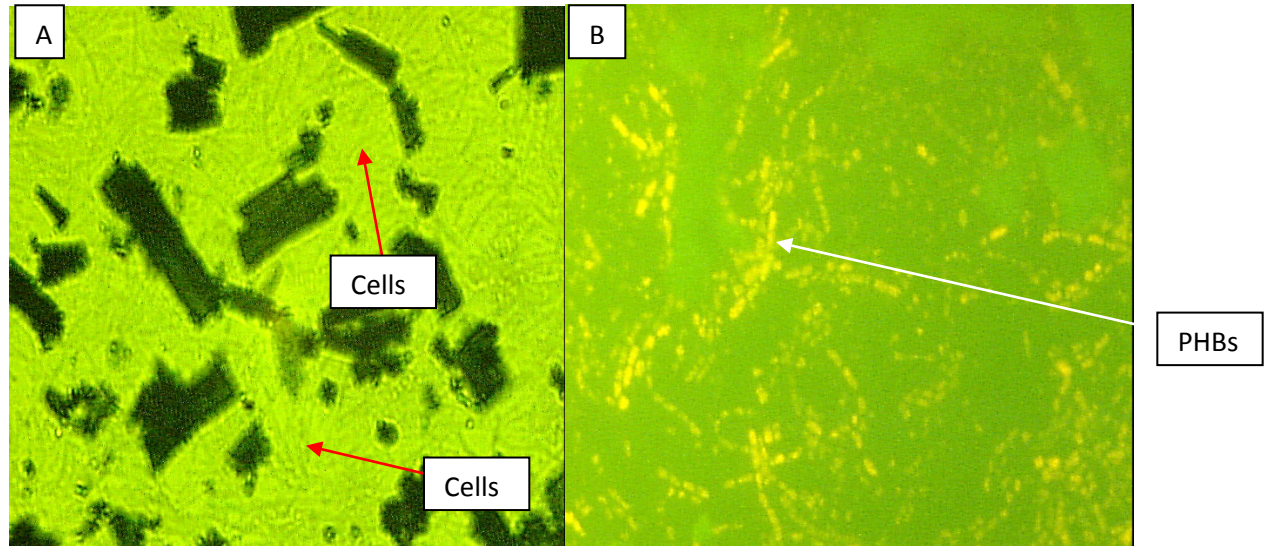


Figure F-1. PHA production by *B. megaterium* grown in a NRLM-X 30g/L at pH 6 and 30°C. Cells were observed during stationary phase with Nile Blue A: (A) using differential interference contrast microscopy (400X cells appear green) and (B) epifluorescence microscopy 1000X.

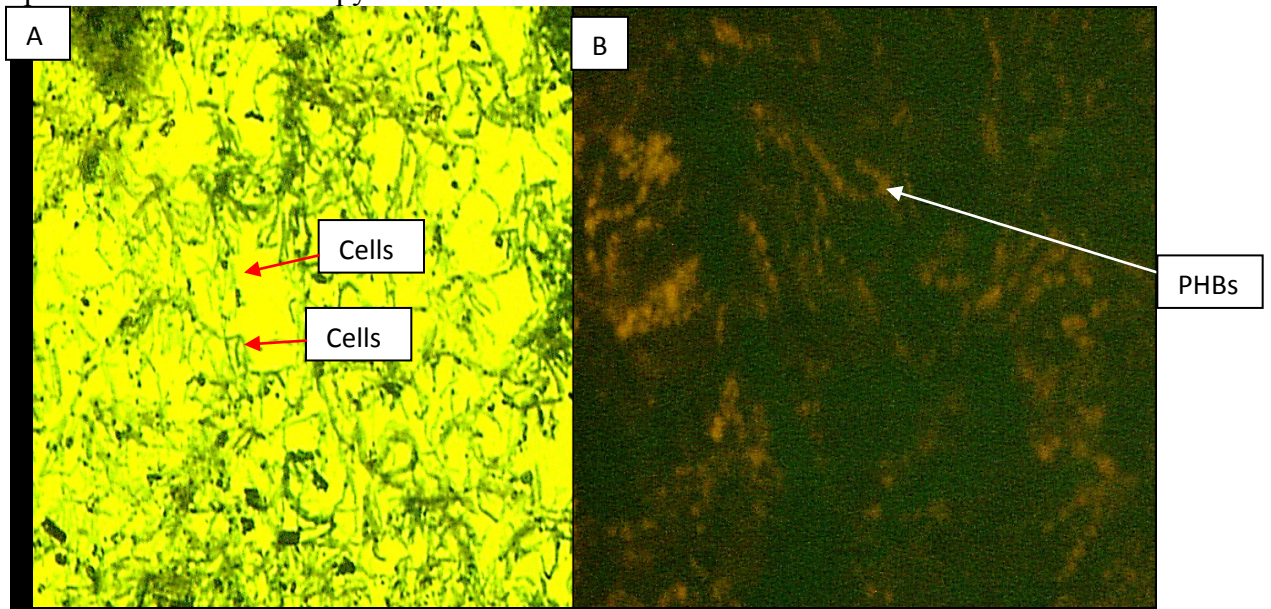


Figure F-2. PHA production by *B. megaterium* grown in a NRLM-G 20g/L at pH 6 and 30°C. Cells were observed during stationary phase with Nile Blue A: (A) using differential interference contrast microscopy (400X cells appear green) and (B) epifluorescence microscopy 1000X.



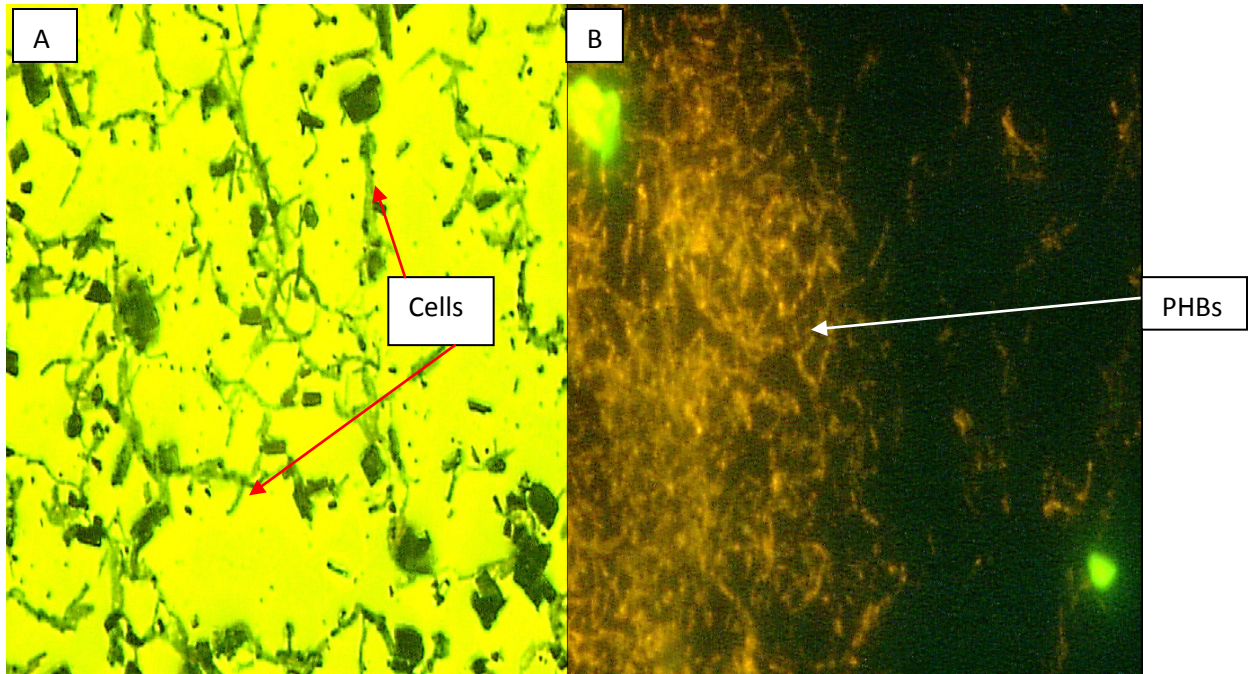


Figure F-3. PHA production by *B. megaterium* grown in a NRLM-G 30g/L at pH 6 and 30°C. Cells were observed during stationary phase with Nile Blue A: (A) using differential interference contrast microscopy (400X cells appear green) and (B) epifluorescence microscopy 1000X

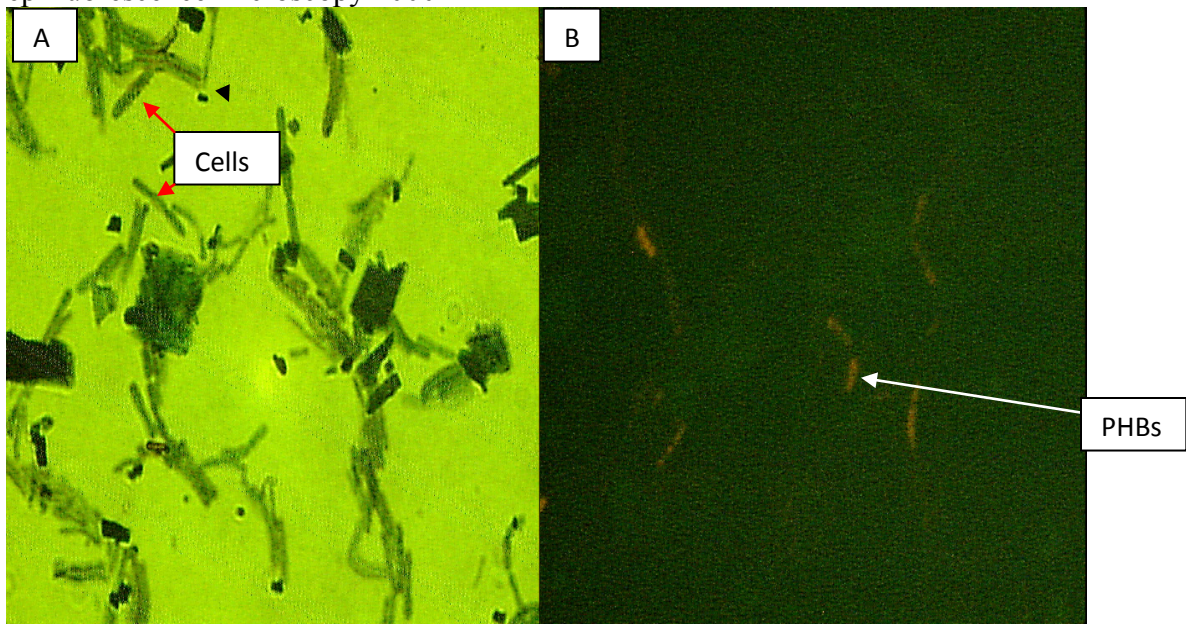


Figure F-4. PHA production by *B. megaterium* grown in a NRLM-X 40g/L at pH 6 and 30°C. Cells were observed during stationary phase with Nile Blue A: (A) using differential interference contrast microscopy (400X cells appear green) and (B) epifluorescence microscopy 1000X.

## Appendix G:

Replicate Data for Toxicity and Growth studies:

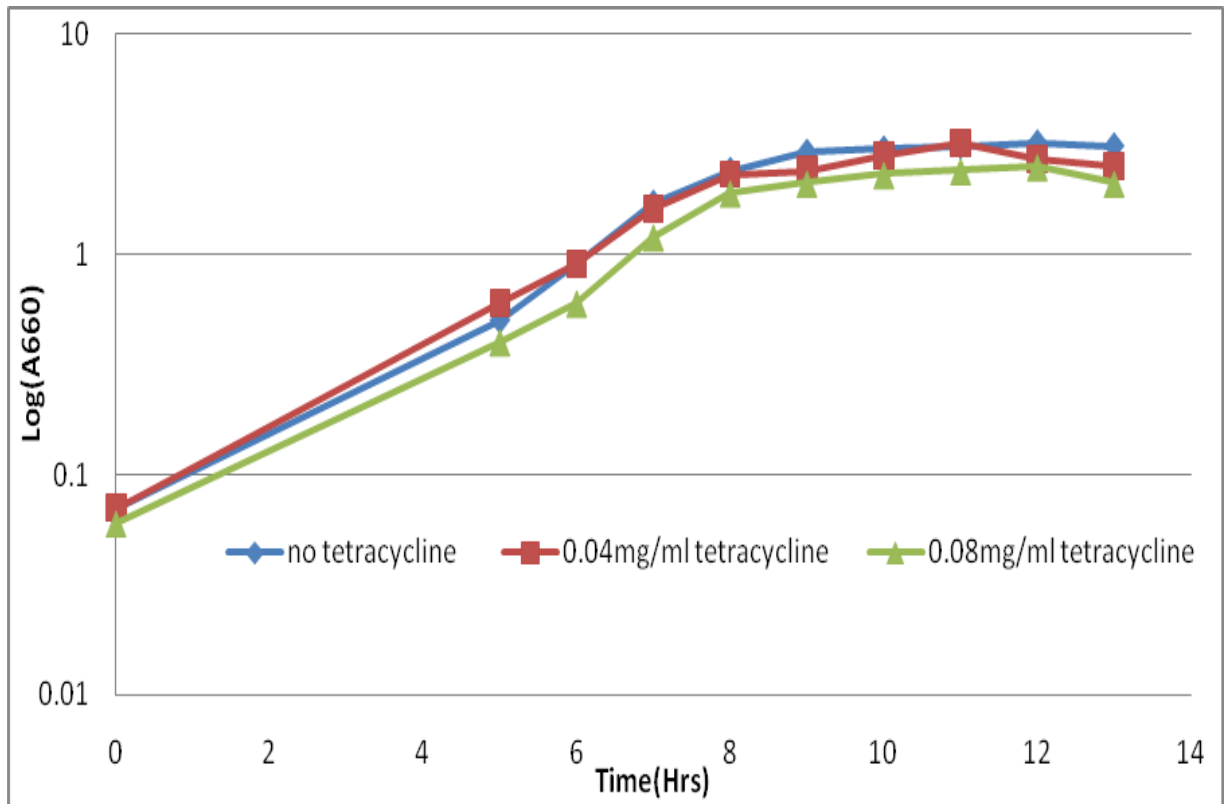


Figure G-1. Effects of tetracycline on growth of *B. megaterium* grown on TSB at 30 °C, pH 6.0. Graph depicts replicate data; not depicted is a 3.5 hour lag phase.



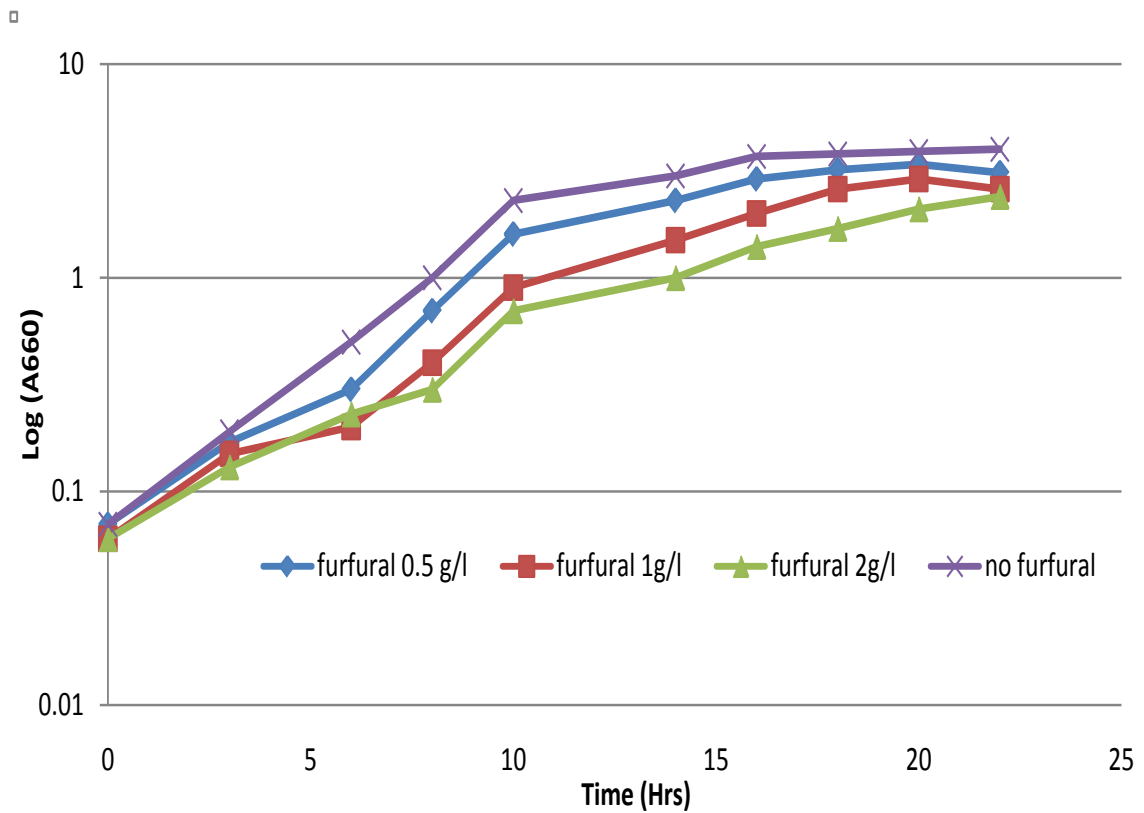


Figure G-2. Effects of furfural on growth of *B. megaterium* grown on TSB at 30 °C, pH 6.0. Graph depicts replicate data; not depicted is a 5 hour lag phase. Not depicted in figure is no growth at 4g/L.

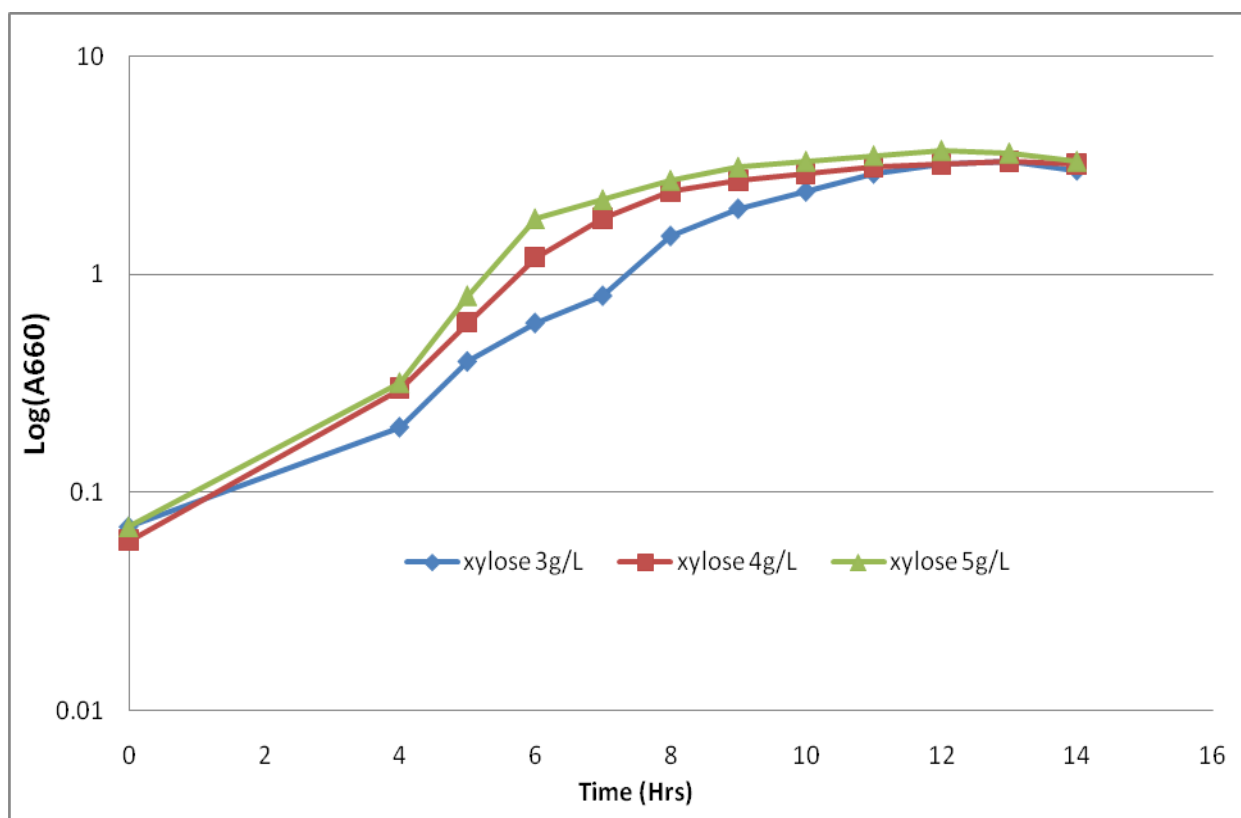


Figure G-3. Growth of *B. megaterium* with NRLM-X (3, 4, 5 g/L) at pH 6 and 30°C. Graph depicts replicate data; not depicted is a 5 hour lag phase.

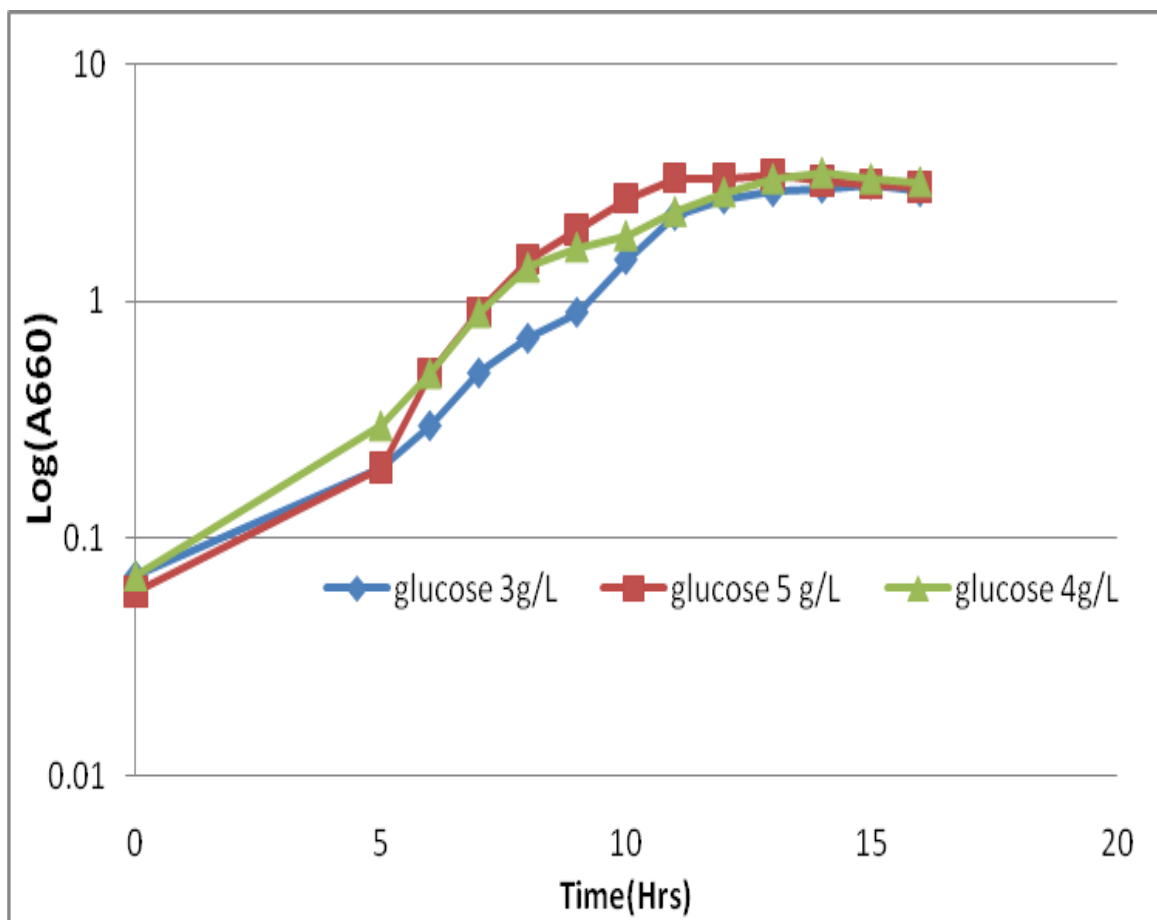


Figure G- 4. Growth of *B. megaterium* with NRLM-G (3, 4, 5 g/L) at pH 6 and 30°C. Graph depicts replicate data; not depicted is a 5 hour lag phase.

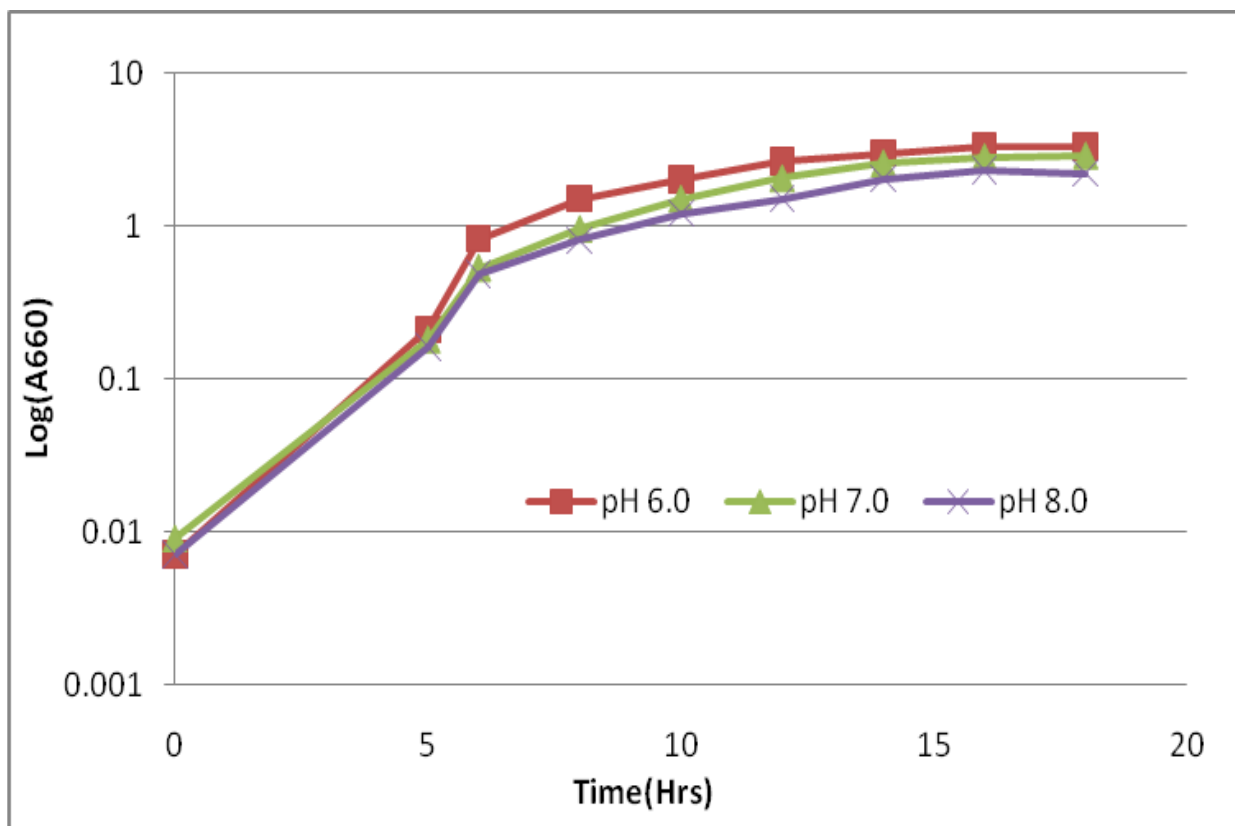


Figure G-5 The effect of pH on the growth of *B. megaterium* when grown on TSB at 30°C pH 6.0. Graph depicts replicate data; not depicted is a 4.5 hour lag phase.

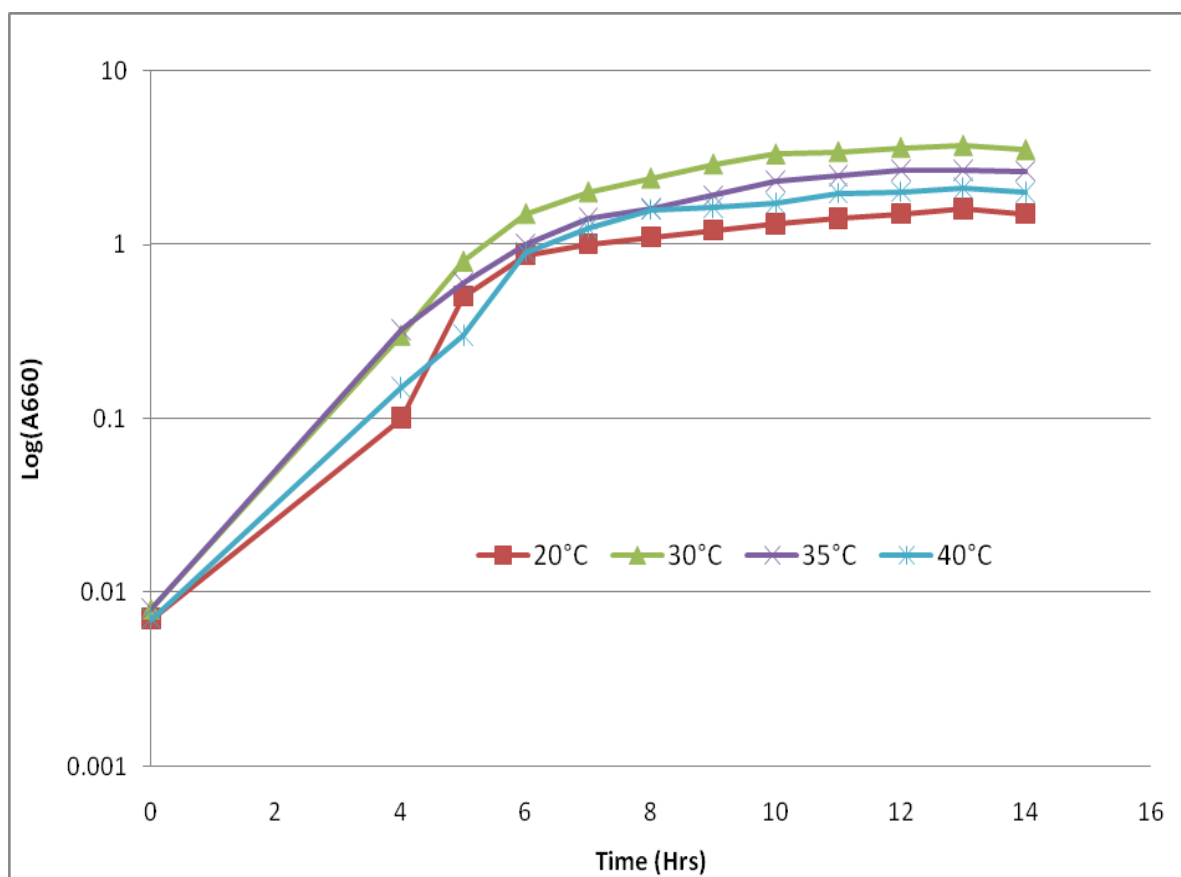


Figure G-6. The effect of temperature on the growth of *B. megaterium* when grown on TSB, at 30°C and pH 6.0. Graph depicts replicate data; not depicted is a 3 hour lag phase.

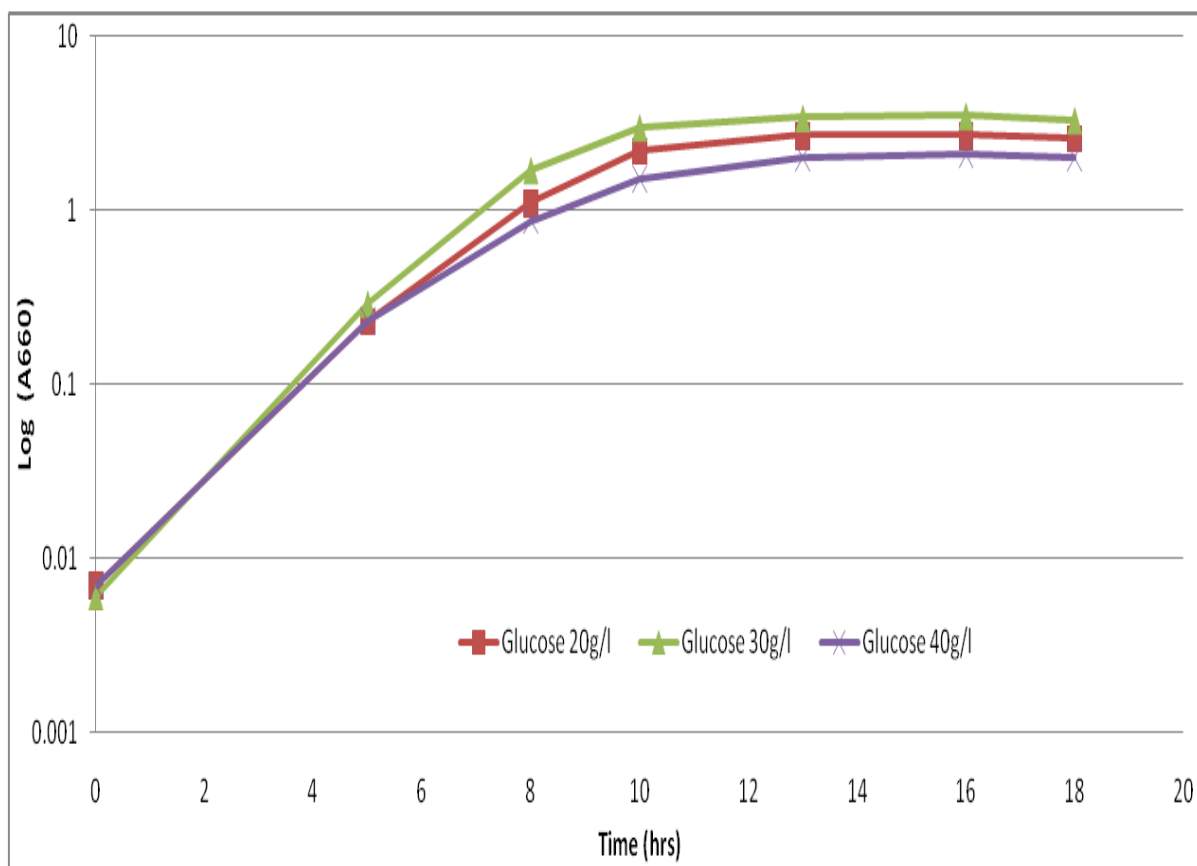


Figure G-7. Growth of *B. megaterium* with NRLM-G (20, 30, 40 g/L) at pH 6 and 30°C. Two replicates of each sugar were run. Graph depicts replicate data; not depicted is a 4 hour lag phase.

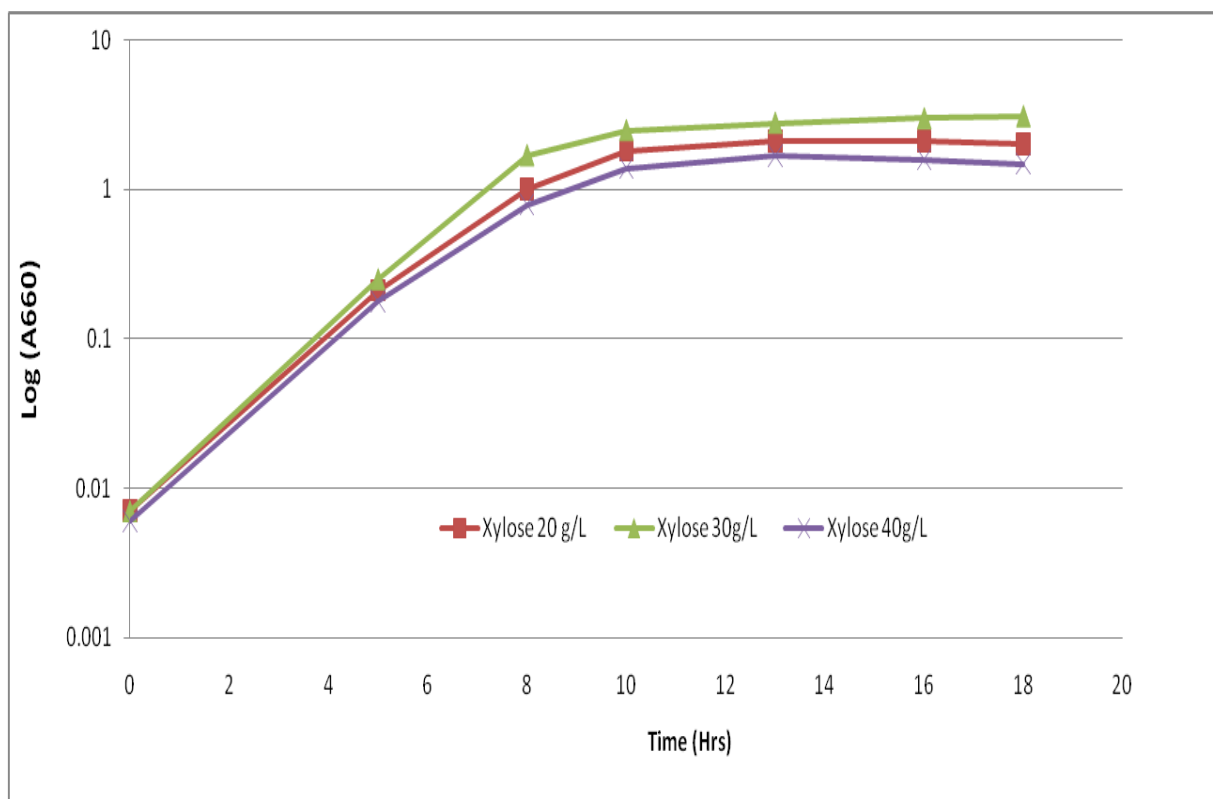


Figure G-8. Growth of *B. megaterium* with NRLM-X (20, 30, 40 g/L) at pH 6 and 30°C. Two replicates of each sugar were run. Graph depicts replicate data, not depicted is 4 hour phase.

Table G-1. Percentage of biomass and growth rate (k) of *B. megaterium* grown on 3,4,5 g/L glucose and xylose.

Sugar Level (g/L)	glucose <sup>a</sup>	Percentage of biomass <sup>b</sup> (9 hrs)	xylose <sup>a</sup>	Percentage of biomass <sup>b</sup> 9 (hrs)
3	1.5±0.01	75	1.4±0.03	74
4	1.6±0.01	79	1.5±0.01	76
5	1.7 ±0.01	84	1.7±0.21	86

<sup>a</sup> mean (n=2) ±SD

<sup>b</sup>  $\frac{T_0(TI_9-T_0)-TI}{T_0 TI}$  T0=control  
TI=Time final