Production of polyhydroxyalkanoates (PHA) from paper industry wastewater

The effect of the adaptation of bacteria on their ability to accumulate PHA

Jeanne Le Guern  –  Abdulahi Bere

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Course coordinator : Maria Sandberg
ABSTRACT

Nowadays, the environmental issue is stronger than ever. Almost everywhere in the world, actions are being taken to limit global warming. To reach the objective of not exceeding a 2°C increase, the use of fossil fuels must be greatly reduced or even stopped and thus alternatives must be found. Plastic is a material with great negative impacts on the environment because its manufacture requires oil and leads to pollution of the oceans and land. To overcome this, researchers are turning to bioplastics. These new materials with the same properties as plastic are either bio-based or biodegradable. While some of these bioplastics are not necessarily ecologically good, others are both bio-based and biodegradable, making them the perfect solution. This is for example the case of PHAs. This type of polymer can be synthesized by bacteria from wastewater from paper mill. This is fully in line with Sweden's environmental approach: reducing the carbon footprint and using waste from the country's most widespread sector. The University of Karlstad has conducted a lot of different research on these bacteria coming from the forest industry called Gruvön in order to find the best conditions. Most of the time, the higher percentage of polymers contained in bacteria was as high as 20%, which is quite a low concentration. Our aim was to find out if adapting the bacteria and reusing them could increase their ability to accumulate PHA in order to reach 40% or even 60%. Indeed, if this percentage could be up to 50%, large-scale production of this type of polymer could be simpler and more economical to achieve.

In this project, 3 accumulation experiments were conducted on the same bacteria. During 1 month, we alternated 3 periods of famine and feast. For 25 to 30 hours, the bacteria were overfed, so they stored carbon and synthesized the polymers. Afterwards, for a week or more, the bacteria were fed once a day, which corresponds to a famine. Our research hypothesis was that in the first experiment, 20% would be reached and then in the 2 following experiments, bacteria would reach a percentage of 40% or 60% PHA.

The results of our experiments show that we were wrong, we could not reach more than 23%. The first experience was the most fruitful with the 23%. In the second experiment, 16% was reached and after the third one, only 15%. So it happened the opposite of what we had assumed. However, the bacteria did not die and endured 3 periods of famine/feast, which was not guaranteed. As the experiments went on, the accumulation speed decreased and the maximum PHA content decreased also. The yield was found to be similar for all 3 experiments. Based on these results, it was assumed that the bacteria were weaker at the end in both accumulating PHA and cell division. It was concluded that the bacteria cannot adapt in this way and perform better just after coming out of the treatment plant.
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I- INTRODUCTION

The current global environmental objective is to significantly reduce carbon emissions in the coming years in order to limit the increase in average temperature. To achieve this, we must greatly reduce the use of fossil fuels, or even abandon it altogether. Solutions exist such as the use of renewable energies and the recovery of organic waste. We have in our possession many kinds of waste that have energy potential, we should research them to find alternatives.

Among our many consumption, plastic is an important factor. In 2018, 359 million tons were produced worldwide. [1] Due to its properties, plastic is an extremely interesting material for industries. It is very shock-resistant, malleable, can be transparent, adapts to any shape and is impermeable. However, if we look at its ecological impact, we realize that this material is a real scourge for the environment. On the one hand, its production requires the use of fossil fuels. On the other hand, it takes hundreds of years to disappear, causing pollution of oceans and land. With 8 million tons of plastic thrown into the ocean each year [2], marine life is seriously threatened. Beyond having caused entanglement on more than 270 different species, the greatest scourge are the micro plastics. For instance, an average person could ingest approximately 5 grams of plastic every week [3]. The big problem with plastic is that it takes 100 to 1000 years to decompose. Today, we can find microplastics everywhere: into our plates, into the sea, into the soil, into animals or even us. To overcome this problem and since we are not ready to stop the production and consumption of plastics, we need to find other bases to make plastic and reduce its lifespan. The solution is found in bioplastics. The term bioplastic can be confusing because it encompasses different types of materials. [4] It can mean either biobased, i.e. made from organic waste or biodegradable meaning a plastic that degrades naturally but that can be made from petroleum. Otherwise, it can also mean both biobased and biodegradable. Currently, they represent approximately 0,75% of the global polymers market. [5] Most are made from sugar cane, corn, soybeans or molasses. These are not necessarily biodegradable and require agricultural land.

The best solution is to ensure that the material does not leave dirty waste when it disappears. This is particularly the case with polyhydroxyalkanoates (PHAs). PHA are polyesters produced by microorganisms. They are thermoplastic polymers, which can be ductile and more or less elastic depending on their manufacture. They have a fairly good resistance to UV light and low water permeability. Due to these properties, these polymers are biodegradable and can be used as food packaging materials. [6] This is a very good thing because single-use food packaging is the most common type of plastic discarded in nature. Generally, PHA production contains several steps including fermentation, separation of biomass from the sludge, biomass drying, PHA extraction and PHA drying [7] as shown in the diagram below.

![Diagram of the PHA manufacturing process in our case](chart1.png)

**Chart 1 : Diagram of the PHA manufacturing process in our case**
Bacteria are cultivated in bioreactors in large quantities. They are kept active with oxygen. Then, a carbon-containing substrate is added and that leads to a "stress" for the bacteria, which transform the base molecules already present into PHA. Indeed, PHA is used for the energy requirement of the bacteria. Afterwards, the PHA is extracted from dried biomass using environmentally friendly solvents such as acetone. [8]

After carrying out these experiments in the laboratory, the aspiration is to develop this on a large scale and make it possible to manufacture bioplastics from PHA created by forest industry bacteria. Today, industrial production of PHA by more than 20 companies has been established worldwide. [9] However, this production is not economically interesting and therefore does not compete with fuel-based plastics. The percentage of polymers often reached at Karlstads University is about 20%. The problem is that these bacteria require living space and chemicals such as acid acetic acid to feed them, acid sulfuric acid to kill them and acetone to extract the polymers. Therefore, it would be more optimized if the percentage of polymer inside bacteria could be higher. That way, we would have a better yield and large-scale production would be more plausible.

In this project, the production of PHA from forest sludge was examined. The goal was to find out if the bacteria could be adapted and forced to accumulate more polymers. For this purpose, we carried out 3 experiments of at least 24 hours each by reusing the same bacteria. We alternated periods of famine and feasting to see if it increases their ability to accumulate PHA. Accumulation experiments were performed in cylindrical tanks and then analysed by FT-IR, bacterial concentration and extraction of polymers. The nutrient used was diluted acetic acid. Bacteria use it as a source of carbon to build polymers. Our hypothesis was that by the end of the first experiment we would have achieved at least 20% PHA and that in the following experiments we could achieve 40% or even 60%.
II- METHODS

1. Preamble

During this project, three accumulation tests were carried out with the same sludge throughout the project. The description of the experiments and its conditions can be found in table 1.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date</td>
<td>23-24 sept</td>
<td>7-8 oct</td>
<td>19-20 oct</td>
</tr>
<tr>
<td>Duration (hours)</td>
<td>29</td>
<td>31</td>
<td>26</td>
</tr>
<tr>
<td>Volum of sludge (L)</td>
<td>51</td>
<td>32</td>
<td>21</td>
</tr>
</tbody>
</table>

*Table 1: Description of the experiments*

In all experiments, the pressure was 10 NL/min bar and the temperature was 35°C.

Bacteria were taken from the Gruvön paper mill and treatment plant. This plant belongs to the group BillerudKorsnäs. It is one of the bigger one in Europe and is located near Karlstad. It produces strong paper, formable paper and some liquid packaging such as Tetra pac for milk cartons for example. We are going to use an activated sludge treating wastewater.

![Diagram representing the several step of the treatment process at Gruvön.]

They use 25 000 m³ of water every day and discharge 5 tons of bio sludge. Afterwards, they burn this organic waste. This sludge contains different kind of sugar and cellulose. The activated sludge treating wastewater used in this project come from their excess sludge.

2. Feeding process

The global aim of that experiment and of this project is to feed bacteria as much as possible. In our case, the food provided is acetic acid. To do this, we have to give them enough food to make them producing polymers but not too much either, otherwise they could die. Just “Lagom”.

2.1. Structure of reactors

The reactors used consisted of a large cylindrical plastic pipe measuring 2.5 meters with a diameter of 20 cm. They each had a pressure and oxygen regulation system, a thermometer immersed in the sludge, a pH electrode and an oxygen one, both connected to a measuring
instrument that stored the data on a USB key. The pH and dissolved oxygen (DO) data were retrieved every 30 seconds during the experiments and every 5 minutes between experiments.

A food pump which provide 4.2 L/h at 100% of its capacity

6%HAc

Flowmeter
Pressure regulator
Compressed air

Air diffuser
Valve
Sampling tube

*Chart 3: Diagram of a reactor (Source: [10])*

2.2. Acetic acid dosage

Bacteria will be fed according to a process of alternating periods of feast / famine. Bacteria consume oxygen when they use acetic acid, so the level of DO contained will decrease. When the bacteria have consumed all the acid, the dissolved oxygen content will increase again: a dose of acid must be given back. Indeed, the bacteria must not be in a period of famine. Concerning the pH, this one decreases when a dose of acetic acid is given because the acid has a very low pH. As the bacteria eat the acid, the pH will gradually increase. Oxygen and pH are constantly monitored: it is our way of communication with the bacteria. Regarding the pH value, it must always be above 5.8 and below 9 because beyond these values, bacteria are inhibited. A 6% diluted acetic acid solution was used.

2.3. At the beginning

Basically, we try to understand how they eat so that we can anticipate their resting time afterwards. We look for the right dosage so that they don’t wait between each “meal”. We start by giving them a small dose to "wake them up". Then we can increase it little by little, trying to find a dosage that is convenient for us. It is important to watch oxygen and pH levels at the beginning because we do not yet know how they will react. The resting time is proportional to the dose of food. If we have difficulty estimating the right dose for 1 hour or 2 hours of rest, we can try several values and make a graph.
Adaptation of bacteria

For instance, these are values from the beginning of the first experiment of the 23\textsuperscript{th} of September.

<table>
<thead>
<tr>
<th>Feeding time</th>
<th>Percentage</th>
<th>Volume</th>
<th>Rest time</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 min</td>
<td>10%</td>
<td>35 mL</td>
<td>20 min</td>
</tr>
<tr>
<td>7 min</td>
<td>10%</td>
<td>49 mL</td>
<td>26 min</td>
</tr>
<tr>
<td>10 min</td>
<td>20%</td>
<td>140 mL</td>
<td>80 min</td>
</tr>
</tbody>
</table>

*Table 2: Values concerning the way of feeding for the 1st exp.*

With this graph, it can be assumed that to reach 2 hours of rest, we should give 210 mL. By a quick calculation, we find that 20 minutes at 15% corresponds to 210 mL. This is made to find the optimal dosage, i.e. the one where the dose lasts 2 hours. Once found, the pump providing the acetic acid is automatically activated by a timer.

Moreover, at the very beginning, it is interesting to take a sample to know the parameters at time 0 for analysis of HAc (cf II-3.1) and concentration of bacteria (cf. II-3.2).

\textbf{2.4. During the process}

Once we've found the right dosage, all we have to do is set a timer to control the feed. Throughout the entire process, it is important to steadily take samples (cf. II-3.1)

\textbf{2.5. At the end}

At the very end, a sample should be taken again for bacterial concentration (cf II-3.2). Moreover, we can take a large sample so the polymers can be extracted from it (cf II-3.3). We can also place a few drops of sludge in a mini tube and then observe under the microscope. (cf II-3.4)

\textbf{3. Analysis}

\textbf{3.1. Sampling and analysis of HAc}

To take a sample:
We pour sludges into the jug and we retain it back to the tank. We pour it again and we consider it as a sample.
Then we put it into the vassel to reduce PHA less than using H2SO4.
We measure the PHA. If it’s less than 2, we pour into a centrifugal vessel to separate the water from the biomask. Then we start the centrifuge machine (4000 c / 5 minutes).

When the water got separated from the biomask. And then we put the biomask into the heater at 100°C;

Before analysis samples, we had to grind the dry biomass.

To analyse the samples:

We used a software called Microlab and the machine connected to it. This machine have a crystal that analyse the absorbance of a powder. We extracted the data from the machine and put everything in an excel file. Then we were able to make the FTIR curves from these data.

3.2. Determination of the bacterial concentration of the sludge

We want to know the concentration of bacteria in the sludge.

Materials:
- 1 scale with an accuracy to the 10,000th
- 2 aluminium cups
- A 50mL-graduated cylinder
- Sludge
- Oven

Procedure:

We are going to do the same thing twice to have 2 samples. At the end, we will take the average of the 2 results. Indeed, since we are interested in one concentration, it is more prudent and rigorous to have several results to compare.

First step: Weigh the empty cups and write down their mass.

We can called them DS1 and DS2 (DS= dry solid).

Second step: Remove some sludge from the large container into a pitcher. Then, take the graduated cylinder and pour 30 mL. It doesn't matter if it's not accurate as long as the exact volume is known. Write down this volume for both samples ($V_1$ & $V_2$). Afterwards, pour the contents of the cylinder into its corresponding cup. Put both cups on the oven at 100°C to dry them out and wait until the next day.
Third step: Once dry, remove them from the oven and weigh them with the same scale.

Fourth step: Calculate the concentration.

\[
m_1 = DS_{1\text{ (full)}} - DS_{1\text{ (empty)}} \ [g]
\]
\[
m_2 = DS_{2\text{ (full)}} - DS_{2\text{ (empty)}} \ [g]
\]
\[
Cm_1 = \frac{m_1}{V_1} \ [g.L^{-1}]
\]
\[
Cm_2 = \frac{m_2}{V_2} \ [g.L^{-1}]
\]
\[
\bar{Cm}_{\text{end}} = \frac{Cm_1 + Cm_2}{2} \ [g.L^{-1}]
\]

3.3. Extraction and crystallisation

Material:
- 1 grinded sample of biomass
- Acetone
- 1 graduated cylinder
- A crystallizer
- 2 test tubes
- A heater
- A centrifuge

Procedure:
This experiment is divided into 2 distinct parts: extraction and crystallisation. Its aim is to separate the polymers from the biomass and then to crystallize them in order to locate and extract them.

First step: With the scale, put about 0.5g of biomass in each test tube. Write down these exact masses. \(S_1 \ & S_2\)

PS: The maximum is 0.5g of biomass. We try to put 0.5g but if our grinded sample is too small, we put less than that.

Second step: Measure approximately 10 mL of acetone with the graduated cylinder and then pour it on a tube. Do it again for the other tube. It doesn’t have to be very accurate.

Third step: Use the heater with the program called POLY which boil at 125°C during 120 minutes. It is a good thing to stir them twice an hour.

Then, this first part is done. What happens next depends on the polymer concentration. Normally, methanol is used but in our case with very little polymer, we can do it without. It is better because methanol is very toxic.

Fourth step: Let the tubes cool for a few minutes. At the same time, weigh the empty crystallizer and note its weight. \(m_{\text{empty}}\) If biomass and polymers are not well separate, we can use the centrifuge.

Fifth step: Gently open both tubes, paying attention to outgoing gas and pour them into the container.
Sixth step: Wait few minutes and polymers will appear. We are supposed to see something looking like a jelly. Incubate the crystallizer until next day to get all acetone away.

Seventh step: On the next day, weight the full container \( (m_{\text{full}}) \) and calculate the percentage of PHA.

\[
m_{\text{bacteria}} = s_1 + s_2 \ [g] \quad m_{\text{polymers}} = m_{\text{full}} - m_{\text{empty}} \ [g] \\
\Delta = \frac{m_{\text{polymers}}}{m_{\text{bacteria}}} \ [%]
\]

3.4. Microscopic observation of polymers inside bacteria

The aim is to be able to see the polymers inside bacterias thanks to the Nile blue.

Material:
- A sample of sludge
- An incubator
- A microscope with light
- A plastic pipette
- Nile blue
- A mini tube

Procedure:

First step: Fill the mini tube with sludge using the pipette.

Second step: Put 1 or 2 drops of Nile blue. Shake it and incubate it for a few tens of minutes. Incubation is at 50°C.
Third step: Turn on the microscope. Place one drop of the mixture on the ... and observe it. We can see some fluorescent parts: these are the polymers.

III- RESULTS

In this section, the results of the analyses are presented. Below is a table of parameter values presenting the conditions of the experiments.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volum of sludge</td>
<td>L</td>
<td>51</td>
<td>32</td>
</tr>
<tr>
<td>Bacterial concentration start</td>
<td>g/L</td>
<td>10,435</td>
<td>6,24</td>
</tr>
<tr>
<td>B concentration end day</td>
<td>g/L</td>
<td>/</td>
<td>7,39</td>
</tr>
<tr>
<td>B concentration end end</td>
<td>g/L</td>
<td>11,02</td>
<td>5,36</td>
</tr>
<tr>
<td>Maximum of % PHA</td>
<td>%</td>
<td>23</td>
<td>16,22</td>
</tr>
<tr>
<td>Acetic acid (6%)</td>
<td>mL</td>
<td>3514</td>
<td>1029</td>
</tr>
</tbody>
</table>

*Table 3: Conditions of the three experiments*

The first experiment required more acetic acid because the volume was larger than in the following experiments. The highest rate of PHA was achieved in the first experiment.

1. **Experiment n°1**

1.1 **Evolution of HAc**
Globally, the pH curve follows a coherent evolution as well as that of oxygen. In the beginning, there were short and close feeding periods and then they became more regular and spaced out. Between 3 and 13 hours, oxygen peaks are indicative of a good way to feed. Bacteria remained without food for a very short period of time. Around 15 hours, the pump did not start because the pH remained the same for 4 hours and the oxygen peak was large. Bacteria were in a short famine period but afterwards, they would have been full at the end.

1.2. FT-IR graph
This FT-IR curve is not very good because the peaks around 1500-1550 and 1600-1675 should overlap. Indeed, they represent the carbons and nitrogen present in the biomass: it will always be the same values, the accumulation experience does not influence this. The peak representing the polymer concentration is approximately 1725. We can observe that the absorbance has increased significantly over time. At the very beginning, the bacteria contained 10% polymer and at the end of the experiment, they contained more than 20%. According to the extraction, they contained exactly 23% of polymers. Roughly, we can make the approximation that the normalized absorbance corresponds to the percentage of polymer in our entire project.

### 1.3. Concentration of bacteria

<table>
<thead>
<tr>
<th>Time</th>
<th>Mass of dry solid (g)</th>
<th>Volume of sludge (mL)</th>
<th>Mass concentration (g. L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Start</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>23/09 – 9:00</strong></td>
<td>0.3597 0.3546</td>
<td>33.5 35</td>
<td>10.74 10.13</td>
</tr>
<tr>
<td><strong>End</strong></td>
<td>0.2993 0.3519</td>
<td>28 31</td>
<td>10.69 11.35</td>
</tr>
<tr>
<td><strong>“New” sludge</strong></td>
<td>0.3147 0.3218</td>
<td>30 31</td>
<td>10.46 10.38</td>
</tr>
</tbody>
</table>

Table 4: Test results about bacterial concentration of the sludge for the 1st exp.

During the experiment, the bacteria multiplied a little bit among themselves: from 10.435 to 11.02. Once the experiment was completed, the sludge was moved to another container and mixed with unused sludge, resulting in a slight decrease in concentration. This "new" sludge will be used for experiment 2.

2. **Experiment n°2**

2.1 **Evolution of HAc**

Chart 7: Evolution of HAc for the 2nd exp.
For the second experiment, the evolution of pH indicates a regularity in the process and that the bacteria were doing well. At the very beginning, there were several short "meals" to "wake them up" and then it quickly settled down. According to the oxygen curve, the bacteria were never waiting for food and were probably full by the end.

2.2. FT-IR graph

Contrary to the graph of the first experiment, here the curves all overlap perfectly around the peak for the polymers. A precise and regular increase in absorbance is observed, hour by hour. At instant zero, the absorbance is 10% and at its maximum, it is 16%.

2.3. Concentration of bacteria

<table>
<thead>
<tr>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>7/10 – 8:15</td>
<td>7/10 – 16:30</td>
<td>8/10 – 11:45</td>
</tr>
<tr>
<td>m₁</td>
<td>m₂</td>
<td>V₁</td>
</tr>
<tr>
<td>0,191</td>
<td>0,205</td>
<td>31</td>
</tr>
<tr>
<td>0,228</td>
<td>0,2262</td>
<td>31</td>
</tr>
<tr>
<td>0,1651</td>
<td>0,0917</td>
<td>31</td>
</tr>
</tbody>
</table>

Table 5: Test results about bacterial concentration of the sludge for the 2nd exp.
3. **Experiment n°3**

### 3.1. Evolution of HAc

**Chart 9: Evolution of HAc for the 3rd exp.**

In this third experiment, the bacteria were regularly fed for 25 hours. There are no particular peaks in the oxygen curve but thanks to the pH one, we know that the bacteria have been receptive to HAc. At 3.15 hours, there is an abrupt decrease in oxygen because the oxygen pump initially at 10NL/min was lowered to 5NL/min in the hope of better perceiving the oxygen fluctuations. According to the oxygen curve, it seems that the bacteria were full at the end.

### 3.2. FT-IR graph
Chart 10: FT-IR curves for the 3rd exp.

This graph seems good. Absorbance, initially at 11%, gradually increases up to 15%.

3.3. Concentration of bacteria

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mass of dry solid (g)</th>
<th>Volume of sludge (mL)</th>
<th>Mass concentration (g L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>0.2521</td>
<td>32</td>
<td>7,878</td>
</tr>
<tr>
<td>19/10 – 9:00</td>
<td></td>
<td>30</td>
<td>9,88</td>
</tr>
<tr>
<td>Sample 2</td>
<td>0.2289</td>
<td>35</td>
<td>6,54</td>
</tr>
<tr>
<td>19/10 – 16:30</td>
<td></td>
<td>31</td>
<td>8,51</td>
</tr>
<tr>
<td>Sample 3</td>
<td>0.2751</td>
<td>31</td>
<td>8,87</td>
</tr>
<tr>
<td>20/10 – 11:45</td>
<td></td>
<td>33</td>
<td>8,95</td>
</tr>
</tbody>
</table>

These results are rather disjointed. It seems unlikely that the concentration decreased for the first few hours and then returned to the same level at the end. But overall, the concentration of bacteria in the sludge is just under 9 g/L.

IV- DISCUSSION

1. Discussion about results

As a reminder, we wanted to reach 20% of polymers on the first experiment. The objective of the second and third one is to reach a percentage of at least 30% of polymers in the bacteria. Indeed, we would like to reach this percentage because from this one, it is worth extracting the polymers.

First, all three experiments went well. In all cases, the FTIR curves are normal and represent a steady increase in absorbance during accumulation. The absorbance is directly proportional to the percentage of polymer. Concerning the oxygen and pH curves, it can be observed that the bacteria never ran out of food and were always full at the end of each experiment. Regarding the bacterial concentration in the sludge, the results are basically consistent. It is observed that the bacteria multiply during the accumulation experiment. Above all, the bacteria did not die and this is a real success. Especially in the third experiment, after one month of treatment, it was not sure that they could withstand a third day of accumulation. So, overall the experiments were successful.

Secondly, to compare the 3 experiments, we calculated the speed at which the bacteria produced PHA as a function of time using the FTIR data. In every cases, it seems that we have reached a maximum in the accumulation of PHA because after 24 hours, the rates stabilize and are almost horizontal. This means that the experiments proceeded normally and did not need to be continued further, bacteria were full and contained their maximum amount of polymer.
In the first experiment, the polymer accumulation rate of the bacteria, which was 23%, was higher than in the second or third experiment. According to other research projects conducted at the university, it was logical to reach about 20% during the 1st accumulation.

Nevertheless, the results of the second and third experiment are strange. Firstly, the initial polymer concentration of the second experiment is the same as first one even though we had reached more than 20% after the first one. Surprisingly, it seems that the bacteria have lost their PHA during the inter-experience period. This is probably the most unexpected result of the project. The hypothesis was that after the 23% reached in the first experiment, the bacteria would continue to increase their PHA percentage during the 2nd accumulation session starting at 23%. Perhaps when the bacteria are in a long period of starvation after a high accumulation (a stressful episode for the bacteria), they would begin to consume their accumulated polymer. In facts, their polymer manufacturing process can be reversed and they are able to feed on PHA.

Moreover, the rate of accumulation is lower. Because of these results, we hypothesized that experiment 2 might have been altered because of the high pH. Indeed, since bacteria have difficulty evolving above 9 of pH because they are inhibited, this might have been the reason why our PHA level was much lower than expected. To test this hypothesis, we decided to carry out a third experiment by lowering the pH.

During the third experiment, we basically observed the same results as the second one. Despite an initial polymer concentration value slightly higher than in experiments 1 and 2, the maximum reached was only 15%. Indeed, the rate of accumulation was even lower than before. Based on these results, it appears that the high pH had no impact on the second experiment. A logic emerges as the experiments progress: the longer the bacteria are kept, the slower their rate of accumulation and their capacity to accumulate decreases. This is the opposite of our initial hypothesis. We can perhaps deduce that the bacteria were more likely to multiply among themselves rather than to store PHA. Indeed, during each experiment, the concentration of bacteria in the sludge increased more or less considerably.
To understand a little more about what happened, the yield based on grams of PHA related to grams of acetic acid is a meaningful indicator. Using the values of the bacterial concentration and the given doses of acid acetic (Table 3), the yield (gPHA/gHAc) can be calculated.

For the first experiment, the volume of sludge was 51 liters and the mass concentration of bacteria was 11,02g/L. This resulted in 562,02 grams of dried biomass in the reactor. The percentage of PHA reached was 23% of the weight of a bacterium, so there are 129,26 grams of PHA in the reactor. For this experiment, 3514 mL of a 6% acid acetic acid solution was used. This is equal to 129,26gPHA/210,8gHAc, or 0,61gPHA/gHAc. Hence, the yield obtained is 61%, which is rather high. Thus, 61% of the acid acetic used allows the bacteria to accumulate PHA and the remaining 39% is used by the bacteria to create new biomass. For the second experiment, the values result in 37,16 gPHA. With 61,74 gPHAc pure used, a yield of 0,6019 gPHA/gPHAc is obtained. Finally, for the last experiment, there was 28,1 gPHA for 46,41 gHAc which corresponds to a yield of 0,6055 gPHA/gHAc. Thus, in all three cases, the yield was about the same despite a marked decrease in the capacity to accumulate. That means that bacteria are as weak at accumulating PHA as they are at cell division. The deduction exposed just above is therefore false: bacteria were not more likely to multiply among themselves rather than to store PHA. As a result, they are generally weaker but have not changed their behaviour or nature.

In summary, the bacteria may not tolerate being adapted and reused after being fully fed. In this case, they would perform better when they just come out of the treatment plant. We wanted to figure out if the bacteria can be adapted to contain 40% or more of polymers. According to our results, this does not seem possible by this way. However, we can't use a single project as a basis to say that it is impossible to achieve this goal because there are a lot of factors influencing the results.

**How many bioplastics could be created from Gruvön's organic waste every day?**

In the Gruvön factory, in the biological treatment, they discharge 5 tons of excess bio sludge every day which are intended to be burned. Based on the results of this project, it is interesting to calculate how many bioplastics could be created from this organic waste. If the percentage of PHA reached is 23%, then 1 150 000 gPHA could be created per day. According to the yield found, these polymers would require 1 875,445 kg/HAc. Acid acetic is often sold diluted at 80% so that corresponds to 2 344 L/80%HAc. The price often found is 2,05€/kg excluding tax so for one day, it would cost 4 805€ just in acetic acid, without acetone. However, if the price is not considered, 1,15 tons of PHA per day is a really good result knowing that it is currently burnt. The cost found reinforces the idea that the ability of bacteria to accumulate PHA would have to be increased for this solution to be feasible.

2. Discussion about methods

2.1. Sampling

Inconsistencies appeared in the bacterial concentration values in experiments 2 and 3 (Table 3). This may be due to the fact that the sludge having already been used, there was some residue at
the bottom of the container that was not there at the beginning of the project. The composition of the mud changed visually during the experiments and in the third experiment, black lumps floated while the liquid was much more transparent than at the very beginning. Even if the sludge was kept active with the oxygen pump, it is likely that the mixture was no longer homogeneous. The uncertainties are probably due to the design of the reactor and the sampling tube located at the bottom of the reactor. Depending on the agitation, the biomass could end up sticking to the bottom or even in the pipe, resulting in distorted samples. As a result, when the sample was taken, it was almost impossible to have the same sample twice. Thus, the abrupt changes in bacterial concentration found in experiment 2 and 3 are not really reliable.

2.2. FT-IR analysis

Concerning the FTIR curves, most of the results are consistent, but it happened that one or two values were totally different from the rest. In this case, it is preferable not to rely on them. This can happen either because of the sampling or because of the FT-IR analysis. In one case, the sample may not be representative of its environment or the solid biomass has not been sufficiently grinded. Because of that, the crystal of the analysis machine has difficulty in providing the right absorbance value. In the other case, the crystal might not be completely clean, hence the importance of using ethanol to clean, or a foreign element may have been inadvertently mixed with the biomass powder, distorting the results.

V- CONCLUSION

Bacteria can't really tolerate to be adapted. The highest PHA level accumulated is 23% during the first experiment. The more accumulation experiment are carried out, the faster the rate of accumulation decreases and the more the maximum PHA content decreases. The yield was found to be similar for all 3 experiments, meaning that bacteria are as weak at accumulating PHA as they are at cell division. Based on these results, it is concluded that the bacteria do not adapt in this way and perform better immediately after leaving the treatment plant. It can be said that the bacteria were slowly killed as they accumulated. However, all biomasses react differently and many small parameters influence the data. This project would be a basis for further research. The adaptation of bacteria would be a real solution for the production of PHA and there is surely a way to make them increase their capacity to accumulate polymers.
VI- REFERENCES


[2] Every year, 8 million tons of plastic end up in the oceans : an article from the French newspaper La Libération
https://www.liberation.fr/planete/2019/11/14/chaque-annee-8-millions-de-tonnes-de-plastique-finissent-dans-les-oceans_1763117

[3] No plastic in nature : An analysis for WWF by Dalberg and University of Newcastle

https://storymaps.arcgis.com/stories/9d8b03c72bc140e0be2e2ddec96b94


[6] Polyhydroxyalkanoates – Wikipédia
https://en.wikipedia.org/wiki/Polyhydroxyalkanoates

[7] Industrial production of PHA : a paper on a research project written by Guo-Qiang Chen
https://www.researchgate.net/publication/225759875_Industrial_production_of_PHA

https://emballagesbiodegradables.school.blog/2016/12/17/pha/

[9] Production of Polyhydroxyalkanoates : An abstract of a research project about Current Developments in Biotechnology and Bioengineering

[10] Produktion av polyhydroxyalkanoater (PHA) från massa- och pappersindustriellt avloppsvatten : Thesis carried out at Karlstad University by Hanna Wänglöv in March, 2020