LAB University of Applied Sciences

THE EFFECT OF PH ON POLYHYDROXYALKANOATE ACCUMULATION IN ACTIVATED SLUDGE UNDER CELLULOSE, HEMICELLULOSE AND LIGNIN FEED: HYDROLYSIS STAGE

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Raul Ballester Valls

Abstract

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The effect of pH on polyhydroxyalkanoate accumulation in activated sludge under cellulose, hemicellulose, and lignin feed: hydrolysis stage

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Abstract

This thesis is part of a larger project where the main objective is to promote the bio-based circular economy through the development of low-carbon solutions and new sustainable businesses. The project carries out pilot projects and develops circular economy business opportunities for sludge and bioplastic solutions. The new solutions are related to the production of polyhydroxyalkanoates (PHA) from activated sludge carbon and the development and use of plastics together with companies.

This thesis focused more specifically on evaluating how pH affects the hydrolysis of activated sludge carbon and accumulation of polyhydroxyalkanoates under the feed of cellulose, hemicellulose, and lignin to adjust the carbon to nitrogen ratio. This was done by calculating the organic matter and ash content of the activated sludge samples from the Lahti Aqua wastewater treatment plant and starting up different bioreactors with samples of the same sludge, together with the nutrients needed to keep microbes alive by making them to hydrolyze complex carbon sources and accumulate PHAs.

Every day, over a period of approximately 15 days, two pH adjustments were made to the bioreactors, with samples taken from each bioreactor at the end of each adjustment. The samples were analyzed for the PHA that had accumulated in them, obtaining results using gas chromatography and mass spectrometry (GC-MS) equipment. Finally, the results obtained were evaluated and it was assessed whether the established objectives were met, drawing some conclusions such as the concentration of PHA is higher at a pH 7 than at a basic pH between 9 and 10.

Keywords

polyhydroxyalkanoates, sludge, pH, bioreactor

Resumen

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Título de la publicación

El efecto del pH en la acumulación de polihidroxialcanoato en el lodo activado bajo la alimentación de celulosa, hemicelulosa y lignina: etapa de hidrólisis

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Resumen

Esta tesis forma parte de un proyecto más amplio cuyo objetivo principal es promover la economía circular de base biológica mediante el desarrollo de soluciones de bajo carbono y nuevos negocios sostenibles. El proyecto lleva a cabo proyectos piloto y desarrolla oportunidades de negocio de economía circular para soluciones de lodo y bioplástico. Las nuevas soluciones están relacionadas con la producción de polihidroxialcanoatos (PHA) a partir de carbono de lodos activados y el desarrollo y la utilización de plásticos junto con las empresas.

Esta tesis se centra más específicamente en evaluar cómo el pH afecta a la hidrólisis del carbón de lodo activado y la acumulación de polihidroxialcanoatos bajo la alimentación de celulosa, hemicelulosa y lignina para ajustar la relación entre el carbono y el nitrógeno. Para ello se calcula el contenido de materia orgánica y cenizas de las muestras de lodo activado de la planta de tratamiento de aguas residuales de Lahti Aqua y se ponen en marcha diferentes biorreactores con muestras del mismo lodo, junto con los nutrientes necesarios para mantener vivos a los microbios haciéndolos hidrolizar fuentes complejas de carbono y acumular PHAs.

Cada día, durante un período de aproximadamente 15 días, se hicieron dos ajustes de pH en los biorreactores, con muestras tomadas de cada biorreactor al final de cada ajuste. Las muestras fueron analizadas para el PHA que se ha acumulado en ellas, obteniendo resultados mediante cromatografía de gases y espectrometría de masas (GC-MS). Finalmente, se evaluaron los resultados obtenidos y se valoró si se cumplieron los objetivos establecidos, sacando algunas conclusiones como que la concentración de PHA es mayor en un pH 7 que en un pH básico comprendido entre 9 y 10.

Palabras clave

polihidroxialcanoatos, fango, pH, biorreactor

CONTENTS

LIST OF ABREVIATIONS

ASTM	American Society of Testing Materials
$bioCO_2$	Biogenic carbon dioxide
CO_2	Carbon dioxide
Dw	Dry weight
FS	Fixed solids or Ash content
GC-MS	Gas Chromatography and Mass Spectrometry
HDPE	High Density Polyethylene
H_2SO_4	Sulfuric acid
IS	Internal standard
LDPE	Low Density Polyethylene
N_2	Nitrogen
Na	Sodium
Na_2SO_4	Sodium sulfate
PBAT	Polybutylene terephthalate adipate
PBS	Polybutylene succinate
PCL	Polycaprolactone
PES	Polyethersulfone
PET	Polyethylene Terephthalate
pH	Hydrogen potential
PHA	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrate
PHBV	Polyhydroxybutyrate-valerate
PHHx	Polyhydroxyhexanoate

PHV	Polyhydroxivalerate
PLA	Polylactic acid
PP	Polypropylene
PS	Polystyrene
PVC	Polyvinyl chloride
SPI	Society of the Plastics Industry
SRT	Sludge retention time
SSF	Fixed suspended solids
TPS	Thermoplastic starch
UV	Ultraviolet
VFA	Volatile fatty acids
VS	Volatile solids
VSS	Volatile suspended solids
Ww	Wet weight
WWTP	Wastewater treatment plant

1 INTRODUCTION

Today, the environment is threatened by various threats that put not only ecosystems but all human beings at risk. The problems caused by pollution are much more serious than we think. Not only is the air we breathe less healthy or natural resources, such as water, are scarce. The consequences and harmful effects on both our health and the planetary balance are already evident. Numerous reports produced by observatories and specialised institutions continually point to the need to do something to curb this great challenge, which fuels other phenomena such as global warming or climate change. (ACNUR Comité Español 2017.)

Today many of the products we consume and the industries that produce them generate pollution, either directly from their production or indirectly from their degradation in the environment. We find pollutants in the air we breathe, in our food, in our clothes and cosmetics. In addition, the plastic pollutant has literally flooded our lives. The relationship between the current economic and production model and the impacts on health and the environment are becoming increasingly evident. We live with air pollution daily, due to traffic and emissions from industries. Air pollution is responsible for a high number of premature deaths and respiratory diseases in our cities. The predominant food production model prevents us from controlling both food production and consumption. One of the consequences of this model is the use of toxic pesticides in industrial agriculture, which has harmful effects on our health and on other important living beings such as bees. (Greenpeace 2020.)

Knowing the environmental problems is the first step to become aware of their importance and to claim and participate in actions for the protection and recovery of nature. The main environmental problems that we currently face are climate change, pollution, deforestation, soil degradation, energy, water scarcity, species extinction and loss of biodiversity, invasion and illegal trafficking of species, waste and overfishing. (Eroski Consumer 2014.)

Focusing on pollution, it is important to emphasize the pollution caused by plastic, which is undoubtedly one of the greatest concerns today. At present, plastic is used to manufacture and wrap many of the products we buy and consume. The problem comes when we do not want it anymore. This happens mostly with disposable plastic for packaging and wrapping. Plastic is used because it is easy and cheap to make and because it lasts a long time. Unfortunately, these same advantages make it the number one ally of pollution. Its low price means that you can get rid of it quickly, and its long existence means that it remains in the environment for long periods of time, where it can cause great damage. Since it cannot be

broken down and requires high energy ultraviolet rays to kill it, the amount of plastic being wasted in the oceans is increasing considerably. (Nu2 mar y arte 2020.)

Recent research shows that 8 million tons of garbage per year reach the seas and oceans, which means that every second more than 200 kilos of garbage ends up in the oceans. Of this total amount, 80 % comes from the land, and once deposited, 70 % remains on the seabed, 15% in the water column and 15 % on the surface, so we can say that what we see is only the tip of the iceberg. (Estévez 2019.)

The aim is to create materials that have characteristics such as biodegradability or multi-functionality, which, together with reuse and recycling, completely or largely stop the use of conventional plastics or recycle them, thus helping to reduce pollution and take greater care of the environment around us. One of the biodegradable plastic raw material of biological origin that has been discovered and with which work is currently underway to further develop it is polyhydroxyalkanoate (PHA), which comes from the bacterial fermentation of plant raw materials and is used in the production of caps and bags. (RAJAPACK 2019.)

The main objective of this thesis was to evaluate the influence of pH on the hydrolysis and accumulation of PHA in activated sludge from the Lahti Aqua wastewater treatment plant. Cellulose, hemicellulose, and lignin were fed to adjust carbon to nitrogen ratio appropiate for the hydrolysis and PHA accumulation. In order to reach the proposed objective, several bioreactors were started up with the sludge samples and the necessary feed to assist the hydrolysis of this activated sludge. Thus, daily analyses of the PHA content and pH adjustments of the bioreactors were carried out to see the pH influence on biomass hydrolysis seen in VFA production and related pH reduction, and the simultaneous biosynthesis of the polyhydroxyalkanoates in the activated sludge was followed.

This thesis is part of a project called BIOSYKLI, which is coordinated by LAB University of Applied Sciences, and the partners are LUT University, Helsinki University, Lahti Region Development LADEC, Päijät-Häme Waste Management Ltd and the Finnish Plastic Association. The main objective of the project is to promote bio-based circular economy thorough developing low-carbon solutions and new sustainable business in the Päijät-Häme region. The project focuses on four main topics, which are the development of effective biowaste collection, the exploitation of organic waste as a raw material for biodegradable products, the development of the use of biobased plastics, as well as the development of carbon dioxide cycles and promoting the use of biogenic carbon dioxide (bioCO_2). The project promotes environmental, economic and social aspects of sustainable development in line with circular economy principles, as well as equal possibilities for all genders.

The BIOSYKLI project pilots and develops circular economy business opportunities for sludge and bioplastic solutions. The new solutions are related to producing polyhydroxyalkanoates from sludge and developing utilization of bio-based plastics together with companies. As a result of the project, the expertise of SMEs in the Päijät-Häme region and the region's role as a strong contributor to future circular economy and bio-based circular economy solutions will be strengthened. Overall, the project's results will promote environmental, economic and social sustainability, in line with circular economy principles.

The thesis is divided into two main sections, one practical and the other more theoretical. In the first part, the necessary information has been gathered to carry out the experimental project in the laboratory where various tasks, analyses and adjustments have been carried out, obtaining data with which it is possible to evaluate to what extent the objective of the work has been fulfilled. The second phase has consisted of setting out the main objectives of the work and putting it into context by writing down all the steps carried out in the practical phase, structuring them in parts and explaining the purpose for which each process was done, how it was done and the means that were needed to do it. It also presents the results and conclusions that have been reached.

The written thesis is structured in three different sections. To begin with, there is the introduction where the reader is shown a general idea of the thesis. The purpose of the thesis is put into context by describing the current situation, the main objectives are written, and the structure of the project is also described in general terms. The second section deals with the methods that have been carried out in practice and lists the materials and equipment that have been needed for this. In each sub-section of this point, there is a theoretical explanation with definitions and the purpose with which each method is carried out, another part where the materials that have been worked with are classified and finally the description of the method step by step. There are a total of four different methods or processes. Within the same section, the origin of the samples with which the work was carried out has also been described. Finally, the last phase of the written thesis includes the results obtained shown in tables and graphs for a better understanding, an explanation or discussion about these results and, finally, the conclusions obtained from them, evaluating the development of the project as a whole and considering if and to what extent the objectives proposed previously have been met. All the information provided will be duly referenced, showing on which sources the foundation of the whole thesis has been based. This list of references will be shown at the end of the document.

2 PLASTICS

2.1 Plastics in general

Definition

Plastics are organic materials formed by macromolecules. These molecules are formed by reactions in which many units of small molecules (monomers) are joined together to form long polymer chains. These reactions are called polymerization reactions. (Mareca López 2007.) The atoms that form these chains mainly contain carbon, although they may also contain elements such as oxygen, nitrogen, hydrogen and sulphur. (Tecnología 2020.)

The term "plastic" comes from the Greek "plastikos", which means that it can be molded. It refers to the malleability, or plasticity, of the material during manufacture, which allows it to be melted, pressed or extruded into different shapes, such as sheets, fibres, plates, tubes, bottles, boxes, etc. (PlasticsEurope 2020.) Depending on their origin, plastics can be natural, if they are obtained directly from vegetable raw materials such as cellulose, or synthetic (artificial), which are made from compounds derived from crude oil, natural gas or coal. Currently, most plastic raw materials on the market are derived from the distillation of crude oil. The plastics industry uses 6 % of the crude oil that passes through the refineries, to convert it into plastic. (Tecnología 2020.)

Plastics can also be divided into two distinct categories based on their chemical composition. One category is polymer composite plastics that have only aliphatic (linear) carbon atoms in their main chains. The structure of polypropylene can serve as an example, as shown in Figure 1. (Rodriguez 2020.)

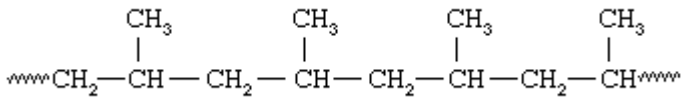

Figure 1. Polypropylene structure (Rodriguez 2020)

The other category of plastics is made up of heterogeneous polymers. These compounds contain atoms such as oxygen, nitrogen or sulphur in their main chains, as well as carbon.

An example would be polycarbonate, whose molecules contain two aromatic rings (benzene). The structure can be seen in Figure 2. (Rodriguez 2020.)

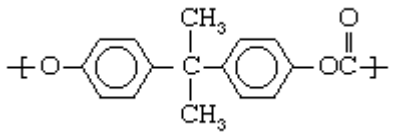

Figure 2. Polycarbonate structure (Rodriguez 2020)

Properties

It is difficult to generalize about the properties of plastics because of the great variety that exists. In general, they have poor electrical conductivity, so they can be used as electrical insulators, and low thermal conductivity, so they can be used as insulating materials that transmit heat very slowly. Considering how lightweight plastics are, they are very resistant, which explains why they are used together with metal alloys to build airplanes. They have good chemical resistance. Most plastics burn easily because they are made of carbon and hydrogen. Many plastics soften with heat and, without being melted, are easily mouldable, which means that they can be used to make parts with complicated shapes. This condition is known as plasticity. (Dept. Tecnología 2020, 1.)

Plastic is an immensely versatile material, ideal for a wide range of industrial and consumer applications. The relatively low density of almost all types of plastics gives plastic products the benefit of lightness. Also, while most plastics have excellent thermal and electrical insulation properties, electrically conductive plastics can be manufactured if required. They are resistant to corrosion by many substances that attack other materials, making them durable and suitable for use in very demanding applications. Some are transparent, so they serve as optical devices. They can be easily moulded into complex shapes and allow the integration of other materials to form ideal products for a wide range of functions. In addition, if the physical properties of a given plastic do not fully meet the requirements, its balance of properties can be modified for example with fillers, colours, foaming agents, flame retardants, and plasticizers, to meet the demand of a specific application. (PlasticsEurope 2020.)

The low cost of manufacture, their resistance to deterioration, waterproofing and the possibility of colouring them in different shades are some of the reasons why plastics are so popular. However, they also have a number of drawbacks: many of them are not recyclable and can therefore contribute to pollution; moreover, plastics often do not resist excessive heat, melting and sometimes they are releasing toxic substances. (Pérez Porto & Gardey 2013.)

Types of plastic

Depending on their structure and behaviour, there are three different types of plastics. Firstly, there are thermoplastics, which soften with heat and acquire the desired shape, which is preserved when cooling. This heating and cooling process can be repeated as many times as required without the plastic being damaged, which is why they are easy to recycle. Some examples of thermoplastics are PVC (for pipes, gloves, waterproof suits, etc.), polystyrene (for packaging and insulation), or methacrylate (for car headlights, windows, tables, etc.). Secondly, thermosets are plastics that become rigid when heated, so they can only be heated once to shape them. If they are heated again, they are no longer useful. This makes them difficult to recycle. This type of plastic includes polyurethane (for foam mattresses, seats, helmets, varnishes, lighters, etc.) and melamine (for kitchen worktops). Finally, elastomers are highly elastic plastics that recover their shape and dimensions when a force is no longer acting on them. They are obtained by vulcanisation, invented by Charles Goodyear by mixing sulphur and rubber at 160 ºC. Examples of this type of plastic are natural rubber (for tyres, hoses, rubber bands, etc.) or neoprene synthetic rubber (for immersion suits). (Tecnología 2020.)

Manufacturing

The plastic materials that are obtained industrially are presented in different forms such as powder, granules, resins, sheets, plates, tubes and threads. These materials are then subjected to a variety of forming techniques depending on the applications for which they are intended and the shape they are to be given. (Dept. Tecnología 2020, 2-3.)

These shaping techniques are injection moulding, where the plastic is melted inside a cylinder and pushed by a piston being injected into a mould, controlling temperature and pressure values. Extrusion, where the melted granules are pushed by a rotating screw and forced to pass through an outlet head whose shape will give rise to the profile. Blowing, where starting from a hot plastic hollow cylinder, air is introduced under pressure until it adapts to the walls of the mould. Vacuum moulding, where thin hot sheets are adapted to the mould when the vacuum is made by sucking the air. Compression moulding, where plastic granules are introduced into moulds where they are heated and compressed

producing the final shape and cure. Resin impregnation moulding, where thin layers of liquid unsaturated polyester or epoxy resin are spread over a mould, to which fibre reinforcements are usually added. All these techniques have in common that they start by heating the granules to soften them, they need some kind of mould and they end with a cooling process to make the plastic solidify, i.e. harden. (Dept. Tecnología 2020, 3.)

Plastic recycling

Plastics are differentiated according to the Plastic Identification Code, which is a system used internationally in the industrial sector to distinguish the composition of resins in packaging and other plastic products. This was done by the Society of the Plastics Industry (SPI) in 1988, in order to promote recycling and to make it more. The different types of plastic are identified with a number from 1 to 7 located inside the classic recycling sign (triangle of arrows following). (Pascual 2019.)

Number 1 is PET (Polyethylene Terephthalate), which is used mainly in the production of beverage bottles. Number 2 is HDPE (High Density Polyethylene), which is normally used in packaging for milk, detergent, motor oil, etc. As number 3, there is PVC (polyvinyl chloride), which is used in shampoo bottles, kitchen oil containers, fast food service items, etc. LDPE (Low Density Polyethylene) is number 4, and is found in supermarket bags, bread bags, and plastic wrap. Number 5 is PP (Polypropylene), which is used in most containers for yogurt, sherbet, bottle caps, etc. PS (Polystyrene) is number 6 and is found in disposable hot drink cups and meat trays. Finally, number 7 usually indicates that it is a mixture of several plastics. Some of the products made from this type of plastic are ketchup bottles for squeezing, microwave dishes, etc. (Pascual 2019.)

2.2 Biodegradable plastics

Definition and properties

Biodegradable plastics are those made from renewable raw materials such as wheat, corn or cornstarch, soybean oil, potatoes, bananas or cassava (Manjón 2019). They differ from other plastics because, under certain temperature and humidity conditions, they are consumed by microorganisms and enter into an oxidation process that favors their conversion into water, carbon dioxide and biomass. In this way, they are reintegrated into the carbon cycle without leaving residues and with a much shorter degradation time than conventional plastics. On the other hand, conventional plastics can also be made from renewable raw materials. They are not biodegradable, but are made from renewable materials, recycling carbon instead of extracting additional carbon from the atmosphere from fossil fuels. These conventional plastics made from renewable raw materials must be

recycled to avoid environmental problems because, unlike biodegradable plastics, conventional plastics are only degraded by the action of the sun's ultraviolet rays. Over time, they break down into small particles, microplastics, which do not undergo changes in their composition and therefore cannot be degenerated by living beings. (RAJAPACK 2019.)

There is currently a great deal of interest in making biomaterial-based plastics. This is because most of the plastics available today come from crude oil. Although research began in the 1980s, it is still being studied and the need for this new alternative is becoming more apparent. Therefore, we need to create other alternatives to these conventional plastics because it would reduce the environmental impact and persistence in the environment. (National Geographic 2019.)

Difference between bioplastics, biodegradable plastics and compostable plastics

The great variety of terms and products related to this topic has made it necessary to generate a classification. However, the boundaries between the concepts of bioplastics, biodegradable plastics and compostable plastics can be blurred by the possible combination of characteristics caused by the mixing of one material with another. On the one hand, we have bio-based bioplastics, which come from renewable sources. In other words, they are manufactured with raw materials that are renewed in two or fewer growth cycles, such as corn, wheat, grass and bacteria. Bio-based plastics are not necessarily biodegradable and compostable. (Tecnologia del plástico 2009.)

The terms biodegradable and compostable refer to the form of decomposition. The main difference between the two terms lies in the time and conditions of degradation. According to the ASTM (American Society of Testing Materials), biodegradable polymers degrade by the action of microorganisms such as bacteria, fungi and algae, while compostable polymers also degrade by the action of microorganisms, but at a rate equal to that of other materials that are compostable such as leaves, paper and wood chips. (Tecnologia del plástico 2009.)

Advantages and disadvantages

Packaging made from renewable organic raw materials saves resources by recycling CO2, eliminates waste and protects the environment. Making responsible use of plastics as well as reducing greenhouse gas emissions is essential, which is why it is also important to analyze the advantages and disadvantages of these biodegradable plastics. The advantages are that they are made from natural materials, can be consumed by micro-organisms and help the life cycle to run its course. Therefore, less energy is needed for their production. The decomposition of plastics from renewable materials also releases

CO_2, but the carbon first binds to the biomaterials in the atmosphere. Therefore, biodegradable plastics from renewable raw materials recycle the carbon instead of increasing the carbon load in the atmosphere. (SP Group 2020.)

The use of biodegradable plastics also has some disadvantages today, which we hope will be solved in the future, such as the fact that very few biodegradable plastics are derived from agricultural waste, so an increase in large-scale manufacturing could have a negative impact on food availability and cause a rise in food prices (SP Group 2020). It has a higher production cost in relation to conventional plastic. However, in recent years the production process has been optimized to reduce costs and increase productivity (EcoInventos 2016). Although the plastics are biodegradable, many times they do not end up in proper composting systems, so they are thrown to garbage dumps, places that do not have the right conditions for their decomposition. This is due to the fact that in Europe there is no collection network for these materials or correct separation of them, so it is very difficult for consumers to see the difference between a normal plastic and a biodegradable one. (SP Group 2020.) Despite this, biodegradable plastics are an ideal option for reducing the use of plastics and for the care of the planet (EcoInventos 2016). Some of the biodegradable plastics being developed can be classified according to whether they are of synthetic or biological origin. (RAJAPACK 2019.)

2.2.1 Synthetic origin

A synthetic element is a chemical element that does not occur naturally on Earth and can only be created artificially. An example of synthetic biodegradable plastics is polybutylene succinate (PBS), which is one of the oldest biodegradable plastics to be invented, made from 100 % renewable sources. It is a plastic that is biodegradable under composting conditions, with excellent processability and thermal resistance. (Universidad San Buenaventura 2019, 2.) Regarding its applications, it is ideal for bottles, trays and different packaging solutions (RAJAPACK 2019).

Polyethersulfone (PES) is a material with good electrical insulation properties and excellent chemical resistance. Due to its amorphous molecular structure, it is translucent and has a yellowish-brown shade. It also has good resistance to hydrolysis. (Ensinger 2020.) As applications, this hydrophilic material is widely used in the food and pharmaceutical industry (RAJAPACK 2019).

Polybutylene terephthalate adipate (PBAT) is an amorphous and biodegradable thermoplastic material. It is very flexible and has good thermal stability, up to 230 ºC. It has low water barrier properties, good heat tolerance and is characterized by its flexibility and

transparency. According to the chemical composition and molecular weight it can be processed into films, sheets, printed products, fibres, elastics or painted articles. It has good processability in blown film extrusion as applications, they are increasingly used as films for food packaging. (Mexpolymers 2020.)

Polycaprolactone (PCL) is a very strong material with a high molecular weight. It is breathable and hydrophobic and is easily pigmented and coloured. It has good resistance to grease and humidity. Its cost is relatively low, and thanks to its compatibility with many materials, it has multiple applications for example adhesives, compatibilizing agents and films, as well as in medicine, but its most common use as an additive is in the manufacture of special polyurethanes. (Mexpolymers 2020.)

Finally, thermoplastic starch (TPS) is formed by a mixture of polyesters with starches from different plants (RAJAPACK 2019). It is partially crystalline, and its density is higher than that of most conventional thermoplastics and also biopolymers. Although it has low resistance to solvents and oils, this can be improved by mixing with other components. It is easy to process, but vulnerable to degradation. Packaging is the main application for modified starch-derived polymers. They also have a great number of applications for paper, cotton and natural fibers. (Textos Científicos 2009b.)

2.2.2 Biological origin

One example of biodegradable plastic of biological origin is polylactic acid (PLA) which is a thermoplastic polyester produced from annually renewable biomass such as corn, sugar beet or sugar cane. It is a 100 % biological material, biodegradable and industrially compostable. It has good rigidity, although it is fragile and transparent. (Mediapilote 2020.) As for its applications, it is suitable for the preparation of containers and packaging that will be in contact with food. The cellulose-based bioplastics, which are rigid and of considerable resistance, are mainly used for making labels and caps. Starch-based bioplastics will degrade more quickly but are water-soluble. (RAJAPACK 2019.)

Finally, there is the material on which this work is focused, polyhydroxyalkanoates (PHA). It is a material derived from the bacterial fermentation of vegetable raw materials and is a totally biodegradable and biocompatible plastic. This polymer is a completely different alternative to petrochemical plastics, but with very similar characteristics. PHAs are biopolyesters that accumulate as intracellular carbon, energy, and have certain physical properties very similar to petroleum-based plastics. PHA can be biosynthesized from renewable resources, which allows the process to be sustainable. (Álvarez da Silva 2016,3-8.)

Polyhydroxyalkanoates are obtained on an industrial scale, focusing on microbial strains and substrates through renewable waste materials and activated sludge. Polyhydroxybutyrate (or polyhydroxybutyric acid) (PHB) and polyhydroxyvalerate (or polyhydroxyvaleric acid), or the mixture of these two subunits are the most common polymers of the polyhydroksyalkanoates. As a subgroup of the PHAs, PHB polymer can be obtained, which is an intracellular microbial thermoplastic produced by different types of bacteria. In relation to thermoplastics derived from petrochemistry, it has quite a few characteristics in common with those mentioned above such as molecular weight, stiffness and brittleness, melting point and glass transition temperature. Therefore, in certain applications, PHB can replace traditional, non-biodegradable polymers. (Mariano 2012.)

PHB, in combination with other biocompatible and non-toxic polymers, can have improved properties in biomedical applications since it is resistant to water and ultraviolet radiation and is also impermeable to oxygen. PHB degrades very easily in soil. In some cases, a wastewater treatment plant is needed to obtain these plastics, but they are not the only possibility. Other organic waste material can also be used as a carbon source for microbial production of PHA. Nowadays most of commercial PHAs are produced from sugarbeet/sugarcane waste residues using a pure microbial culture. In those fermentation processes sugar is the carbon source. It is easier to degrade by microorganisms than cellulose, hemicellulose or lignin. (Mariano 2012.) The option of using a type of bioplastic produced by fermentation is now available. The only drawback so far is that the price of PHA is still quite high compared to that of crude oil-based plastics. This is why measures are being taken to make them more accessible to the population. (Mexpolímeros 2020.)

3 WASTEWATER TREATMENT

3.1 Process in Lahti, Finland

There are currently two wastewater treatment plants (WWTP's) in Lahti, the Kariniemi plant and the Ali-Juhakkala plant (Palmer 2016). The Kariniemi plant has supplied the activated sludge samples required for the practical part of this thesis through Lahti Aqua, a water supply company located in the Lahti region of Finland with over 100 years of experience (Lahti Aqua 2020). These samples were taken on 3 March 2020 at the Kariniemi plant, in one of the activated sludge pools, with the help of authorised Lahti Aqua personnel.

The wastewater treatment plant was built in 1975, and today it treats the wastewater from the Lahti area north of Salpausselkä, which accumulates approximately 20,000 m^3 of wastewater per day. The wastewater treatment plant is located underground in a cave with a volume of 120,000 m^3. The biological treatment based on the activated sludge process is carried out there. (Palmer 2016.)

Image 1. Kariniemi wastewater treatment plant (Palmer 2016)

The entire process of the WWTP is carried out from the control room, from where we have both the control of pumps and equipment and the control of flows, temperature, pH, oxygen, phosphorus and nitrogen content in the water. In addition, in failure situations, alarms are immediately sent to the control room. As far as the wastewater treatment process is concerned, screening is carried out first, which removes large particles (e.g. paper) from

the wastewater. The screens are washed, dried and transported to a landfill. Secondly, sand is removed by sedimentation, which means the separation of solid, insoluble particles into a concentrated mixture. The sand is then washed, dried and transported to a landfill. (Palmer 2016.)

Next, primary clarification is carried out, where the sludge is deposited at the bottom of some pools. The sedimented sludge is pumped to a sludge treatment, while the effluent goes to aeration (biological treatment). In the aeration phase, the necessary air is pumped into the aeration tanks by compressors (blowers). In this phase the bacteria use the wastewater as food. Thus, at this point where the activated sludge is accumulated, this is where the samples for the present experiment were taken. Then, the secondary clarification is carried out, where the biomass is deposited at the bottom of some pools and is pumped back to the aeration, being called return sludge. In turn, the wastewater treated on the surface is conveyed through gutters to the discharge tunnel. Treated wastewater is hygienized by UV-light (48 UV-lamps) after Nikula basin. There is also nitrogen removal by nitrification-denitrification aside activated sludge pools. Ferrosulfate is added to precipitate phosphorus and also organic matter. The addition of polymer helps in separating solid material. (Palmer 2016.)

The effluent goes through a tunnel to the Nikula basin via the Porvoo River. Treated wastewater from both the Kariniemi and Ali-Juhakkala plants is pumped into the Nikula basin, which has a volume of 50 000 - 60 000 m^3. In addition, biogas is collected and used to heat the wastewater treatment plant. The surplus gas is sold to the electricity company. The air is changed once an hour in the cave, which is ventilated by two blowers working at 20 m^3/second. (Palmer 2016.) Figure 3 shows the complete wastewater treatment process carried out in the plant.

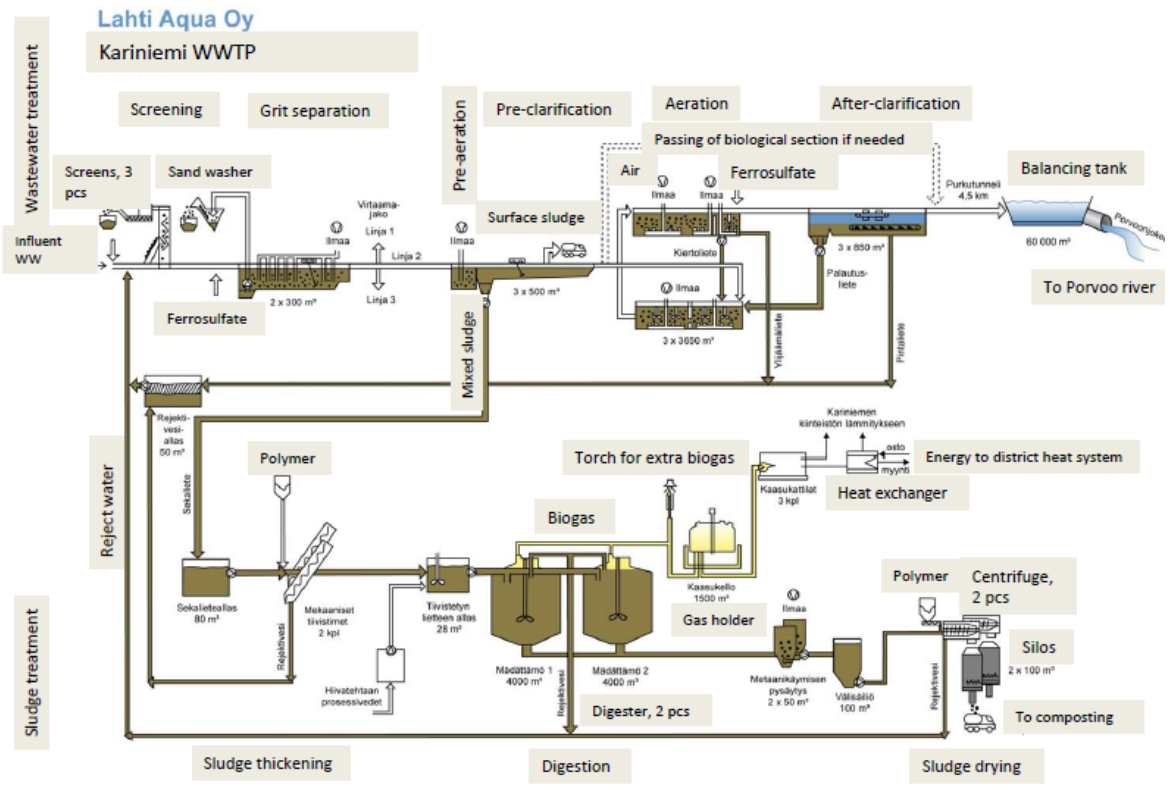

Figure 3. Wastewater treatment process (Palmer 2016)

3.2 Activated sludge

The activated sludge, or also called mixed liquor, is the content of the aeration tank or biological reactor, composed of living organisms along with the material introduced into the tank through the primary effluent (Estévez Pastor 2006, 11). The activated sludge process is an aerobic process of suspended biomass, which requires intimate contact between the wastewater, the active biomass and the oxygen. The activated sludge treatment system used for the treatment of wastewater consists of bringing into contact in an aerobic medium, normally in an aerated pool or aeration tank, the wastewater with previously formed biological flocs in which organic matter is adsorbed and where it is degraded by the bacteria present. The bacterial culture in charge of purification is suspended inside the biological reactor. In order to accelerate the natural processes, they are supplied with dissolved oxygen, thus increasing the treatment capacity and obtaining a better quality of the effluent and less sludge. (Cyclus 2020.)

The main component of the different types of sludge is the content of microorganisms present in them; these organisms use the nutrients of the resource for their development

and cell growth, thus contributing to the cleaning of the wastewater. The main components that make up activated sludge are bacteria, which are unicellular microorganisms that use the nutrients for their own reproduction without the need for a thermal source. These organisms, are bio-reducers and fulfill the function of degrading organic matter, allowing the stabilization of organic waste present in treatment plants. Bacteria are responsible for the growth of sludge in wastewater treatment plants. On the other hand, there is the water content present in the active sludge, which depends on factors such as the type of sludge and the type of stabilisation used in it. The dry matter, present in the sludge, is made up of organic and inorganic matter. Finally, the sludge has a content of trace elements that are extracted from the wastewater and remain concentrated in the material. These elements are mostly carbon with a concentration between 50 and 70 % of the total, oxygen with a percentage between 21 and 24 %, nitrogen (15 – 18 %) and hydrogen (6.5 - 7.3 %), and to a lesser extent phosphorus and sulfides, with a concentration between 0 and 2.5 %. As general characteristics, the active sludge presents a dark brown color. The humidity percentage is high, around 99 %. The concentration of total solids is between 0.5 and 2 %, and the concentration of volatile solids varies from 70 to 80 %. The partially stabilized organic matter generates less odors. (Fibras y Normas de Colombia S.A.S. 2020.)

There are a series of factors, both design and environmental, that have a considerable influence on the generation of these activated sludges. The most important factors are the existence of nitrification processes, which generate not only the oxidation of organic matter from carbon, but also from nitrogen, so there is an increase in nitrogen eliminated in the sludge. The composition of the water to be treated, wastewater with higher concentrations of organic matter produce more sludge. Finally, the considerable increase in the temperature of the sludge generating medium produces a greater reaction speed and, therefore, a greater production of sludge. (Fibras y Normas de Colombia S.A.S. 2020.)

One piece of information to take into account every time we talk about activated sludge or sludge in general is the age of the sludge, SRT (Sludge retention time), which represents the relationship, expressed in days, between the mass of sludge in the reactor and the mass of sludge removed from the reactor daily. It also coincides with the sludge retention time in the reactor. The age of the sludge makes it possible to limit the influence of inert materials; if they increase, the extraction of the sludge will increase and in order to keep the same age, the mass of sludge will have to be increased. Knowing the age of the sludge, one can compare the treatment of two waters with very different inert fractions. The age of the sludge is used in the other biological treatment processes, with the exception of fixed culture systems, such as nitrification, denitrification, aerobic stabilisation of the sludge and anaerobic digestion when there is a joint thickening. (Ronzano & Dapena 2020, 1-2.)

3.3 Process in Spain

Wastewater treatment includes a number of physical, chemical and biological processes that aim to remove pollutants from water used for domestic and economic activities. WWTPs are the facilities where wastewater is received and treated to return it to the environment in an optimal state or, where appropriate, to reuse it for other uses. (iAgua 2020.) During the process of purification of the wastewater generated, there are two factors to take into account when carrying out the treatment, the components of this water and the order in which it is eliminated during the process. The order of elimination ranges from the largest to the smallest components, large objects, sand, fat, sedimentable organic matter, dissolved or colloidal organic matter, nutrients and pathogens. In conventional urban wastewater treatment plants, there are two treatment lines, the water line, which includes the processes or treatments that reduce the contaminants present in the wastewater, and the sludge line, where most of the by-products originating from the water line are treated. (Martín García, Betancort Rodríguez, Salas Rodríguez, Peñate Suárez, Pidre Bocardo & Sardón Martín 2006, 42.)

Firstly, the water treatment line follows a process chain that is divided into 4 phases. In the first phase, water pre-treatment is carried out, which consists of eliminating the constituents of the fats, sands and objects with a certain thickness that make up the wastewater, whose presence may cause maintenance and operating problems. For this purpose, various procedures are carried out, such as roughing by grids, homogenisation and regulation of the flow, desanding by sedimentation and degreasing by flotation. The second phase is that of primary water treatment, which is understood as the process that has the mission of separating by physical and/or chemical means the substances in suspension not retained by the pre-treatment, especially sedimentary and floating matter. These treatments require the addition of chemicals or coagulants that break up the colloidal state of the particles and form large flocs so that they decant more quickly. The processes used in this phase are coagulation by flocculation, sedimentation and flotation. Phase 3 is the secondary water treatment phase, which consists of both aerobic and anaerobic biological processes. This is a key stage for the removal of non-sedimentable colloidal substances and dissolved organic matter, where secondary sedimentation is included. Within the aerobic treatment the most common methods are the use of activated sludge, biodiscs and aerated lagoons. In the anaerobic treatment methods such as fluidized beds and anaerobes are used among others. Finally, phase 4 is the tertiary water treatment, which consists of a more rigorous type of treatment, used in cases where the wastewater discharge has certain concentrations of certain substances that need to be eliminated or reduced by the subsequent use that will be given to the water. This type of treatment basically removes suspended solids, organic

waste matter, nutrients and pathogens, and therefore it is necessary to use methods such as reverse osmosis, electrodialysis, ion exchange, absorption of active carbon or chemical oxidation processes. (Gerdisa 2017.)

Secondly, there is the treatment of sludge, or sludge line, which is the set of operations that aim to reduce the volume of sludge produced from primary and secondary sedimentation prior to the evacuation of water (Gerdisa 2017). A distinction must be made between primary sludge, which is the solids decanted in the primary water treatment, and secondary or biological sludge, which corresponds to the solids retained in the decanter after the water passes through the biological reactor, carried out in the third phase of the water line (Martín García et al. 2006, 50).

There are a series of stages in the treatment of sludge generated in the treatment of urban wastewater. The first is thickening, where the concentration of the sludge is increased by eliminating the water it contains. The most common methods are by gravity and by flotation. The second stage is stabilisation, where the biodegradable fraction present in the sludge is reduced to prevent rotting and the consequent generation of unpleasant odours. Stabilization can be done by aerobic or anaerobic digestion, chemical stabilization or thermal treatment. The third stage consists of conditioning, where the addition of chemicals improves the dehydration of the sludge, facilitating the elimination of water. This is the same as polymer addition in Finland. As the last stage, dewatering is the phase where some of the water contained in the sludge is removed, in order to transform it into easily manageable and transportable solids. The most common methods are centrifugation, filtration, thermal drying, or drying beds. In a wastewater treatment plant, the incoming flow, as a result of the treatment processes to which it is subjected, is transformed into two outgoing flows, treated effluents and sludge. With the evacuation of both currents, the treatment of wastewater is completed. (Martín García et al. 2006, 50-51.)

4 METHODS AND MATERIALS

4.1 Volatile solids and fixed solids

One of the most important physical characteristics of wastewater is total solids, which are determined by gravimetric methods and depending on the treatment before weighing, are classified into different types of solids (Torres 2019, 31). Total solids in wastewater are, by definition, the waste that remains once the liquid part has evaporated, and the surplus has dried to constant weight at a temperature between 103 and 105 ºC. In this evaporation process, the solids that have a low vapour pressure are lost. Thus, a first distinction is made between dissolved solids and undissolved solids, also called suspended solids. (Glynn Henry & Heinke 1999, 423.) This first phase allows us to know the total amount of solids that enter the WWTP or a specific process, regardless of the nature of the process. In the case that we have in mind, this step allows us to calculate the dry weight of the samples since the humidity they contained has been eliminated. (Torres 2020, 33.)

In the second phase, the samples are incinerated in a muffle furnace at a temperature of 550 ºC until they reach a constant weight. Once the samples are removed from the furnace, it is possible to differentiate between two types of suspended solids, fixed and variable. The former, fixed suspended solids (SSF), are the number of suspended solids that remain after the incineration process. These are the residual ashes and represent the inorganic or non-volatile solids. On the other hand, there are the volatile suspended solids (VSS), which are the number of suspended solids that are volatilized after the incineration process. These represent the organic matter in the sludge and are used to evaluate the stability of the sludge. This second determination is useful for process control, as it provides an approximate calculation of the amount of organic matter present in the solid fraction of the wastewater which the activated sludge is being worked with in this case. (Torres 2020, 39.)

4.1.1 Materials

The materials used for the calculation of organic matter or volatile solids (VS) and ash content (FS) are those presented in Table 1.

Table 1. Materials for the VS-FS method

Personal protective equipment	**Products and reagents**
• Lab coat • Latex or nitrile gloves • Laboratory mask • Laboratory glasses • Oven gloves	• Activated sludge to work with
Instrumental	**Technological equipment**
• 5 porcelain crucibles • Tool to remove the sludge (homogenize it) • 5 mL micropipette • Tools for recording and calculating results	• Precision Scale • Oven at 105 ºC • Oven at 550 ºC

4.1.2 Method

To calculate the organic matter or volatile solids (VS) and ash content (FS) of the activated sludge sample collected from the Lahti Aqua plant, the dry weight of the sample (dw) must first be calculated. To do this, 5 empty crucibles were weighed one by one (5 parallel samples were made) on a precision scale, noting the result obtained to four decimal places. This weight of each empty crucible will be the so-called "M_crucible" weight. Before adding the activated sludge, it is very important to mix it well so that it is homogeneous in all the samples taken. Next, 5 ml of activated sludge was pipetted and deposited in each crucible and weighed again noting its weight as "M_crucible + sample ww", i.e. the weight of the crucible plus the weight of the wet sample. Then the 5 samples were kept in the oven at 105 °C for approximately 16 hours. Afterwards, the samples were taken, allowed to cool to the room temperature of 21±2 °C and weighed, noting this weight as "M_crucible + sample

dw", i.e. the weight of the crucible plus the weight of the dry sample. With the weight data you have so far, you can calculate the dry weight of each sample according to Formula 1.

$$dw\ (\frac{g}{g}) = \frac{(M\_crucible+sample\ dw)-M\_crucible}{(M\_crucible+sample\ ww)-M\_crucible} \quad (1)$$

Following Formula 2, the result can also be calculated as a percentage.

$$dw\ (\%) = dw\ \left(\frac{g}{g}\right) * 100 \quad (2)$$

Once the dry weight (dw) of each sample has been calculated, the procedure continued by placing the samples taken from the oven at 105 ºC in another oven that is at 550 ºC, this time for 4 hours. After this time, the samples were taken out and cooled down to the temperature of the laboratory, about 21 °C. Afterwards, the samples were weighed again and recorded. This was the weight called "M_crucible + sample after 550 ºC", i.e. the weight of the crucible plus the weight of the sample taken from the oven at 550 ºC.

Finally, the two desired parameters were calculated using Formula 3 to calculate the organic matter content (VS) and Formula 4 to calculate the ash content (FS) in the 5 samples taken.

$$VS(g) = (M_{crucible} + sample\ dw) - (M\_crucible + sample\ after\ 550ºC) \quad (3)$$

$$FS(g) = (M\_crucible + sample\ after\ 550ºC) - (M\_crucible) \quad (4)$$

The percentage of VS and FS is calculated according to Formula 5 and 6, respectively.

$$VS(\%) = \frac{VS(g)}{VS(g)+FS(g)} \quad (5)$$

$$FS(\%) = \frac{FS(g)}{VS(g)+FS(g)} \quad (6)$$

4.2 Bioreactors

A bioreactor is a system or vessel in which a biological conversion of carbon to polyhydroxyalkanoates is carried out in an active environment. In general, a chemical process involving biochemically active organisms or substances derived from such organisms as enzymes, microorganisms, animal, or plant cells is carried out in the bioreactor. This process can be aerobic if the presence of oxygen is required for the process to take place, or anaerobic if the process can do without the presence of oxygen to run. (Mihelcic & Beth Zimmerman 2012.) The most outstanding characteristic of biological processes is their synthetic capacity to carry out complicated chemical reactions in a single process (Definición XYZ 2020). Based on another definition, the term "bioreactor" can be differentiated from the term "fermenter", since fermenters provide the systems that are used to improve the growth and maintenance of a population of bacterial or fungal cells in a controlled mode, whereas it is the bioreactor system that is responsible for the growth and maintenance of mammalian and insect cells only (Hernandez Chavez 2020).

A bioreactor seeks to maintain certain favourable environmental conditions such as pH, temperature, or oxygen concentration, so that the organism grows, and the products are formed correctly. In general terms, a bioreactor must provide internally a controlled environment that guarantees and maximizes the production and growth of a live culture.

Externally, the bioreactor is the frontier that protects the culture from the polluted and uncontrolled external environment. The environmental conditions of a biological reactor such as gas flow, temperature, pH, dissolved oxygen and agitation or circulation speed, must be carefully controlled so that the operation or process is carried out with economy, high performance and in the shortest time possible. (Mihelcic & Zimmerman 2012.) Bioreactors range from simple open vessels with or without agitation to complex integrated aseptic systems involving varying levels of advanced computer control (Smith 2004). These bioreactors are commonly cylindrical, and their diameter size can vary from millimetres to cubic meters. Those used industrially are usually manufactured in stainless steel. (Definición XYZ 2020.)

Depending on the mode of operation, three types of bioreactors can be found. The first type is, the batch bioreactor, which consists of closed systems that are characterized by changing physiological and environmental conditions, and in which there is no entry or exit of culture medium. The second type is the semi-continuous bioreactor, which consists of removing, at the end of the operation, between 80 and 90 % of the culture and replacing it with fresh medium. These are very useful when a high cell density is required at the initiation stage of a process which involves a high consumption of nutrients. Finally, there is the continuous biological reactor, in which the input of fresh medium is equal to the output of the medium used. This type of reactor is used in cultures where the cell growth rate is constant. (Biorreactores 2020, 23-26.)

In the present experiment, we were working with six small bioreactors with a capacity of 500 mL, of the discontinuous or batch type, since, once started, there was no input or output of culture medium. Only daily samples were taken for analysis, but no more feed was added.

4.2.1 Materials

The materials used for the start-up of the different bioreactors are shown in Table 2.

Table 2. Materials for the bioreactors

Personal protective equipment	**Products and reagents**
• Lab coat • Latex or nitrile gloves • Laboratory mask • Laboratory glasses	• Activated sludge • Cellulose • Hemicellulose • Lignin
Instrumental	**Technological equipment**
• 18 beakers of 250 ml • 1 l beaker • 500 ml glass bottles • Aluminium foil • 500 ml graduated cylinder • 6 small cylindrical magnets • Tool to remove the sludge (to homogenize)	• Precision Scale • Laboratory Autoclave • Magnetic stirrer

4.2.2 Method

Next, it will be explained how the preparation and commissioning of the different bioreactors used to accumulate PHA was carried out. In this case, a total of six bioreactors were started up.

The activated sludge, with which the aim was to work, was well mixed homogeneously, and added. A mixture of cellulose, hemicellulose and lignin was added as nutrients for this sludge. These three polymers will provide the necessary carbon to nitrogen ratio to the sludge of each bioreactor, and simultaneously the biopolymers that degrade the microbial community can be enriched.

First, the quantities of cellulose, hemicellulose, and lignin to be weighed and added to each bioreactor were calculated in order to guarantee an optimal amount of nutrients and growth

of sludge bacteria. The result obtained was 6.715 g of cellulose, 7.043 g of hemicellulose and 4.467 g of lignin, which were weighed separately on a well-calibrated precision balance. Once the three carbon sources were weighed separately a total of 6 times, as there are 6 bioreactors, there will be 6 samples of each carbon source, that is, a total of 18 samples. Then, all 18 weighed samples were mixed in six decanter glasses until the powder was completely homogeneous.

Six 0.5 L glass bottles were prepared, which will act as bioreactors in this experiment. The bottles were washed well outside and inside without the cap, and once clean and dry, they were covered with a sheet folded in four layers of aluminium foil that will be used as a cap. Once the bottles were covered with the aluminium foil, they were placed in a bag and sterilised in the laboratory autoclave. The autoclave is a thick-walled metal container with a hermetic seal that allows the work to be done with high-pressure, and high-temperature steam, which is used to sterilize the laboratory material. The sterilization conditions were 15 min at 121 ºC.

Once all the separate carbon sources needed to set up the bioreactors were prepared, they were assembled. To do this, 300 mL of homogeneously mixed activated sludge was added to each previously cleaned and sterilised 0.5 L bottle covered with aluminium foil. From the decanter glass containing three carbon sources (18,224 g), the mixture was added to each bioreactor together with the previously deposited sludge. Then, a cylindrical laboratory magnet was introduced into each of the prepared reactors. The six bottles were placed in a magnetic stirrer and this stirrer was started up. It was checked that the mixing started in the bottles with their respective magnets correctly to guarantee a correct and continuous mixing of the products introduced into the bottles. Then the six bioreactors were in operation, three of which were adjusted to a basic pH between values 9 and 10, and the remaining three were adjusted to pH 7, i.e. neutral. This pH adjustment will be explained in detail in the next section.

The pH of the bioreactors was adjusted twice a day, once at 8:30 in the morning and once at 15:30 in the afternoon. Each time this pH adjustment was made, a sample must be taken from each bioreactor, so a total of 12 samples were taken daily, 6 in the morning and 6 in the afternoon. These twelve daily samples were subjected to the PHA analysis procedure, which will be explained later, in order to follow microbial growth and to determine whether PHA are accumulated during the initial hydrolysis stage.

4.3 pH adjustment

The meaning of pH in its acronym is hydrogen potential, and it is a unit of measurement that serves to establish the level of acidity or alkalinity of a substance or solution. More specifically, the pH allows to determine the concentration of hydrogen ions in a given solution. In chemistry, the determination of acidity or alkalinity of a substance is one of the most important procedures, since through the results of this procedure a lot of data can be obtained with respect to the structure and activity of the molecules and at the same time to know more with respect to the cells of the body. (Yirda 2019.) The mathematical formula for calculating the pH is expressed as the negative logarithm based on 10 of the hydrogen ion activity, as presenting in the following Formula 7 and 8 (Estela Raffino 2020).

$$\mathrm{pH} = -\log[\mathrm{H}^+] \tag{7}$$

$$[\mathrm{H}^+] = 10^{-pH} \tag{8}$$

The pH will be more acidic the more positive hydrogen ion activity there is in the solution. When there is less activity, the sample will be alkaline. (Olarte Romero 2017.) The pH scale is composed of 14 units numbered from 0 to 14, with zero being the indicator of maximum acidity, and 14 being the indicator of maximum basicity or alkalinity. The pH 7 represents the midpoint of the table and is the neutral pH, which means that the solutions with a value below 7 are acidic and those above are basic. Examples of acidic pHs would be battery acids between 0 and 1, lemon juice between 2 and 3 or coffee at 5. As for substances with a neutral pH, these are found in blood or milk. Examples of bases are the milk of magnesia, found between 10 and 11, or the bleach or chlorine with an alkalinity level of 13. (Estela Raffino 2020.)

There are several different methods of measuring pH. On the one hand, there are drops and pH indicator paper. In the case of the droplets, they are poured into the sample and depending on the colour of the liquid, it can be determined whether it is acid, alkaline or

neutral. A similar procedure is followed with the strips, which will change colour when introduced into a solution. Each different colour indicates a different pH value. It is very easy to use these two methods, but as they are not very accurate, they are not suitable for determining exact pH values. (Lenntech 2020.)

The most effective and accurate way to measure pH is by using a tool known as a pH meter, which is a sensor that measures the activity of the hydrogen ion in aqueous solutions, indicating its degree of acidity or alkalinity expressed as pH. The pH meter measures the difference in electrical potential between a pH electrode and a reference electrode. This electrical potential difference is related to the acidity or pH of the solution. Potentiometric pH meters measure the voltage between two electrodes and display the result converted to the corresponding pH value. It consists of a simple electronic amplifier and a pair of electrodes, or alternatively a combination electrode, and display calibrated in pH units. The electrodes, or probes, are inserted into the solution to be tested. One of the electrodes is usually made of silver or silver chloride and the other is usually made of glass which is sensitive to hydrogenation. (Laboratorio Químico 2020.)

The electrodes are rod structures, usually made of glass, with a bulb containing the sensor at the bottom and attached to the pH meter by a cable. The glass pH electrode has a glass bulb specifically designed to be selective for hydrogen ion concentration. When immersed in the solution to be tested, the hydrogen ions in the test solution are exchanged for other positively charged ions in the glass bulb, creating an electrochemical potential across the bulb. The electronic amplifier detects the electrical potential difference between the two electrodes generated in the measurement and converts the potential difference into pH units. (Laboratorio Químico 2020.) The potential of the charges determines the number of H^+ and OH^- ions and when this has been determined the pH will appear digitally on the pH meter. The potential depends on the temperature of the solution. That is why the pH-meter also displays the temperature. (Lenntech 2020.)

4.3.1 Materials

The materials used for the correct adjustment of the pH of the samples in each bioreactor are those mentioned in Table 3.

Table 3. Materials for pH adjustment

Personal protective equipment	**Products and reagents**
• Lab coat • Latex or nitrile gloves • Laboratory mask • Laboratory glasses	• Bioreactors in operation • Distilled water • Buffer solution at pH 7 • Buffer solution at pH 4 • Acidic solution (H+) • Basic Solution (OH-)
Instrumental	**Technological equipment**
• Paper to dry the electrode • 5 ml micropipette • 1000 µl micropipette • Several plastic tips for the micropipette • Tools for recording values	• pH-meter

4.3.2 Method

In order to make a correct pH adjustment of our samples, first, the calibration of the pH meter to be used must be carried out. To begin with, we will explain how to calibrate the pH meter properly.

Like any measuring instrument (ION85 Ion Analyzer; Radiometer, Copenhagen, Danmark)) the electrode of a pH-meter must be calibrated at regular intervals to maintain its reliability. Standard solutions with a known pH value called calibration solutions, buffers or pH buffers were used for calibration. The calibration was carried out at the usual place of work and within the appropriate temperature ranges. First, the pH meter was switched on, the electrode sleeve was removed, and it was washed with plenty of distilled water. The electrode

was dried with a cloth and immersed in the temperature-controlled buffer solution. The solution used should be the one closest to the internal pH of the glass electrode, which is usually pH 7. The thermal equilibrium for about 1 minute. Once the reading has stabilised, the neutral point control calibration-standardisation-asymmetry was activated until the pH of the buffer solution is indicated. The electrode was removed from the solution, washed with plenty of distilled water and dried it with paper. Then the electrode was immersed in another beaker containing a different pH buffer solution, usually pH 4. Once the reading has stabilised, the slope-scale control was used to adjust the display to the pH value of the buffer solution used.

In this way the instrument was calibrated to its full scale and it was ready to carry out pH measurements on substances of unknown pH. The scale was set at two points on the line that relates the potentials (mV) generated by the electrode to the pH of the solutions in which it was immersed. In the case of the present experiment, a calibration in two points was made (pH 7 and pH 4), but there are simpler equipment that are enough with a point of calibration, and of other more complex ones that require three points.

Once the calibration of the pH-meter was finished, we proceeded to the adjustment of the pH of the six bioreactors that we had in operation. The first three bottles were adjusted to a pH between 9 and 10, and the remaining three bottles were adjusted to a pH as close to 7 as possible.

To make the pH adjustment, the electrode was washed in abundant distilled water and dried with paper before introducing it in the first bottle. When the measurement value was stabilized, a note was made to know the pH values of each bioreactor before adjusting them. Once these initial values were obtained, certain amounts of acid solution was added, to lower the value if the pH was higher than was aimed, or basic solution was added to raise the pH value if it was below the required one. These solutions were added with a micropipette, changing the plastic tip each time we changed the sample or solution to avoid contamination. The amount of acid or basic solution added in each case depends on the difference between the pH you have and the pH you want to have. For example, if you have a pH of 8.90, and you require a pH between 9 and 10, you will add a smaller amount of basic solution than if you had a pH of 8.00, which would require more basic solution. The same thing happens with the acid solution. Once the six bioreactors were adjusted to the pH they were intended for, the final pH values were written down so that the specific pH of the bottles was known when the experiment bottles were left in agitation until the next adjustment.

In addition, the time of the adjustment was also noted in order to have a daily control and to always repeat the same time ranges. Thus, this pH adjustment will be made twice a day,

once at about 8:30 a.m. and the other at about 3:30 p.m., thus achieving daily control of the pH of each bioreactor.

4.4 Polyhydroxyalkanoate analysis

Polyhydroxyalkanoates or PHA comprise a group of biodegradable linear polyesters produced in nature and synthesized by microorganisms or bacteria by fermentation of sugar or lipids in order to store carbon and energy (Mariano 2012). They are a type of polymer that is classified into so-called biopolymers. A biopolymer is a polymer obtained from renewable materials of biological origin, being polymerized by chemical or biological methods. Thus, biopolymers can be divided into three main groups: bio-chemosynthetic polymers, biosynthetic polymers, and natural modified polymers. PHA are linear (R)-3-hydroxy acid polymers in which polymerization by the action of intracellular enzymes takes place by condensation of the carboxyl group of one monomer (hydroxyalkanoic acid), with the hydroxyl group of the next monomer, forming an ester bond, hence they are also known as biopolyesters. (Álvarez da Silva 2016, 12.) Figure 4 shows the chemical structure of the PHA.

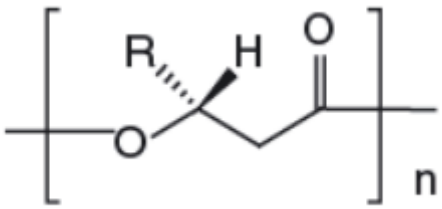

Figure 4. Chemical structure of PHA (Andler & Díaz-Barrera 2013, 31)

Currently, around 150 different monomers can be found in the structures of polyhydroxyalkanoates that can be combined within this family to give materials with extremely different properties (Mariano 2012). The reason why the formation of different types of PHA is possible is due to the wide variety of specific substrates for the synthesis of PHA, and the metabolic pathways that they activate (Mexpolímeros 2020). They can have very diverse structures such as linear, branched, saturated, unsaturated and aromatic. (Álvarez da Silva 2016, 15). As mentioned above, these polymers are biodegradable and

are used in the production of bioplastics. They can be thermoplastic or elastomer materials, with melting points between 40 and 180 °C. The mechanics and biocompatibility of PHA can also be changed by mixing, surface modification or combination of PHA with other polymers, enzymes, and inorganic materials, making a wider range of products and applications possible. (Mariano 2012.)

PHAs accumulate as liquid, mobile and amorphous polymers that are lodged in the microbial cytoplasm as water-insoluble, liquid and mobile granules of different sizes. PHA granules are surrounded by a phospholipid monolayer containing polymerase and depolymerase enzymes. Research on the accumulation process of PHAs indicates that the number of granules per cell is defined in the early stages of accumulation and that the production of the polymer ceases when its content reaches about 70 % of the cell weight on a dry weight basis. This phenomenon has led to the conclusion that there are physical restrictions that prevent the cell from accumulating more polymer, despite the availability of substrate and activity of the PHA polymerase enzyme. These inclusions are observed under the microscope as spherical granules of different sizes. (González García, Meza Contreras, Gonzalez Reynoso & Córdova López 2013, 79.)

The most common polyhydroxyalkanoates are poly(3-hydroxybutytate) (PHB), poly(3-hydroxivalerate) (PHV) and poly(3-hydroxyhexanoate) (PHHx). Depending on the length of the side-chain, three types of PHAs may differ: short side-chain PHAs (the chain contains 1 to 2 monomers), medium side-chain PHAs (the chain contains 3 to 13 monomers), or long side-chain PHAs (the chain contains more than 14 monomers). (Lemos Delgado & Mina Cordoba 2015.)

Polyhydroxyalkanoates possess physicochemical properties very similar to petrochemical polymers (Mexpolímeros 2020). PHAs are thermoplastics such as polypropylene and polyethylene, can be processed on conventional processing equipment, and are, depending on their composition, ductile and more or less elastic. They are highly crystalline with 70 % and have mechanical properties similar to polypropylene, although they are a little more fragile and rigid than polypropylene. They have good barrier properties similar to PET, against humidity, aroma and flavours, and can be moulded by injection or extruded. They are stable to UV light, in contrast to other bioplastics, and have low water permeability. It is a material with a very low viscosity and quite brittle, but whose mechanical properties do not differ from those of polystyrene, although it is more solid and resistant to temperatures. (Mariano 2012.) It also has antioxidant, optical and piezoelectric properties. The most important properties are that they are biocompatible and biodegradable, since PHA and related copolymers degrade easily in soil, sludge and sea water. (Mexpolímeros 2020.) PHA

is degraded by two main routes, one intracellular and one extracellular, by means of PHA hydrolases and PHA depolymerases (Mariano 2012).

However, several disadvantages limit its competition with traditional synthetic plastics or its application as an ideal biomaterial. These disadvantages include their high production cost, limited functionality, incompatibility with conventional thermal processing techniques, and susceptibility to thermal degradation. To avoid these drawbacks, PHA's must be modified to ensure improved performance in specific applications. (Mexpolímeros 2020.)

As stated, PHAs are linear hydroxy acid polymers, and are obtained from microorganisms that accumulate them as reservoir substances. Because these microorganisms are sometimes difficult to grow, it is very interesting to use better characterized laboratory bacteria that have been genetically engineered to carry the genes needed for PHA synthesis. (Mariano 2012.) Several bacterial species such as *Azospirillum brasilense, Alcaligenes eutrophus* or *Bacillus subtilis* among others, have been tested for commercial production of PHA. There is also a great variety of genetic manipulations that can be carried out on the colonies to promote the production of PHA. (Textos Científicos 2009a.) In the same way, these genes could be introduced into plants and thus lower production costs (Mariano 2012).

Some studies on the production of PHAs in fermentation show the great influence of the type and concentration of the carbon source and of the levels of aeration on the productive success. Carbohydrates such as glucose, sucrose and fructose, as well as vegetable oils and glycerin derived from biodiesel production have been used as base materials for fermentation. There are parallel investigations to develop transgenic plants (corn and soybean) that synthesize PHA in their tissues. Another aspect to consider is the level of phosphorus in the culture medium, since reducing the level of this in the medium almost doubles the PHA production/biomass, indicating a better physiological response. This also applies to nitrogen. (Textos Científicos 2009a.)

The polyesters are deposited in the form of highly refractive granules in the cells. The PHA granules are then recovered by disrupting the cells, extracted by appropriate solvent means according to patented methods. The weight percentage of the polymer can exceed 70 % of the body's dry weight. (Textos Científicos 2009a.) In the industrial production of PHA, polyester is extracted and purified from the bacteria by optimizing the conditions of microbial fermentation of sugar or glucose. PHA is mainly processed through injection moulding, extrusion (including blown film) (Mariano 2012).

PHA is processed according to its properties, but mostly according to the chemical composition and molecular weight it can be processed into a variety of finished products

including films, sheets, printed products, fibres, elastics, painted articles, non-woven fabrics (Mexpolímeros 2020). Some PHA producers are in Italy, USA or China (Mariano 2012). Although the advantages of PHA's are evident, the cost of producing these biopolymers is high. The two most common and commercially available classes of PHA are the homopolymer polyhydroxybutyrate (PHB) and the copolymer of polyhydroxybutyrate and polyhydroxivalerate known as polyhydroxybutyrate-valerate (PHBV). (Mexpolímeros 2020.)

Polyhydroxyalkanoates are versatile biopolymers with diverse applications in industries such as pharmaceutical, biomedical, food, packaging, among others, mainly due to their biodegradability. Although they are not yet widely commercialized or in great demand worldwide, some industrial applications for PHAs have already been described, including the manufacture of thin coating films; binding agents in water-based ink formulations; as a source of chiral monomers for the synthesis of active compounds and as a support for tissue engineering and temporary medical implants. PHAs can be used in flexible films of various thicknesses, including semi-permeable membranes, filaments, fibres, packaging materials, gels, and adhesives. In addition, they can be used in the covering of fibrous materials such as paper or cardboard from the watery latex form. In this way, due to its high-water resistance, this cover protects the paper or cardboard against deterioration caused by moisture. (Mexpolímeros 2020.)

4.4.1 Materials

The materials used for the analysis of the accumulated PHA in the activated sludge samples of each bioreactor are those mentioned in Table 4.

Table 4. Materials for PHA analysis

Personal protective equipment	**Products and reagents**
• Lab coat • Latex or nitrile gloves • Laboratory mask • Laboratory glasses • Oven gloves	• Samples of the bioreactors in operation • Methanol • H_2SO_4 • Chloroform • Na-benzoate • β-hydroxybutyrate • Tridecanoid acid • Nonadecanoid acid • N_2 gas • Distilled water • Na_2SO_4 • anhydrous
Instrumental	**Technological equipment**
• Kimax tubes • Permanent marker • 1 L volumetric flask • 100 ml volumetric flask • 2 x 30 ml volumetric flasks • 1 ml micropipette • 5 ml micropipette • 1000 µl micropipette • Pasteur pipette • Plastic vials • GC-MS sample bottles • Several plastic tips for the different micropipettes • Tools for recording values • Metal cooking rack • Plastic Rack	• Precision Scale • Freezer at -20 ºC • Lab Sonicator • Oven at 105 ºC • Test tube shaker • Pot with water at 100 ºC • Agitator (at 240 rpm) • Centrifuge (2000 x g) • GC-MS equipment • Stopwatch

4.4.2 Method

Before proceeding with the analysis of polyhydroxyalkanoate, the reagents and standards were prepared for the determination of PHAs. Reagent 1 consisted of a mixture of 970 mL of methanol and 30 mL of H_2SO_4 (3 %; vol/vol), and reagent 2 was chloroform.

The Na-benzoate standard was prepared by weighing approximately 10 mg of Na-benzoate in a 100 mL volumetric flask and adding 100 mL of reagent 1. The mixture was divided in 25 mL portions into 35 mL Kimax tubes, about 3 or 4 tubes. The height of the solution was marked with a black marker. If there was evaporation from the solution reagent 1 was added up to the mark and the solution was mixed well. To perform the PHA standard curve, 10 mg/10 mL (1 µg/µL) of β-hydroxybutyrate and reagent 1 (concentration 1.0 mg/ml = 1.0 µg/µL) was pipetted (about 1-2 mL) to a GC-MS sample bottle, the height of the surface was marked with a black marker and the bottle was stored at -20 ºC. A new bottle must always be taken when preparing new standards. For the preparation of tridecanoid and nonadecanoid acid standards, about 30 mg of tridecanoid acid was weight into a 30 mL volumetric flask (1.0 mg/mL = 1.0 µg/µL) and the flask was filled with chloroform, and then about 1-2 mL was pipetted to a GC-MS sample bottle. The height of the surface was marked with a black marker and, like the previous standard, the bottle was kept at -20 ºC. To prepare the nonadecanoid acid standard, about 30 mg of nonadecanoid acid was weight into a 30 mL volumetric flask (1.0 mg/mL = 1.0 µg/µL), the flask was filled with chloroform, and about 1.0 mL of the standard was pipetted to a GC-MS sample bottle. The height of the surface was marked with a black marker, and the standard was stored at -20 ºC. It should be noted that whenever a standard bottle is used, it must be warmed up to the room temperature of 21±2 °C and sonicated for 5 min before it can be used.

With the prepared reagents and standards, the analysis of the polyhydroxyalkanoates present in the bioreactors was followed. Firstly, a sample of about 400 µl was taken from each of the bioreactors in operation (a total of 6) and deposited in a Kimax tube, which was weighed before adding the sample. Then the dry weight was determined as presented in Chapter 4.2. With the aid of a micropipette, 0.8 mL of sodium benzoate was added to the samples (0.1 g/L * 0.0008 L = 80 µg/ sample; in solution of methanol-3 % H_2SO_4) as the internal standard (IS). Then 1.2 mL of the previously prepared reagent 1 was added to each tube. The fatty acid standards, 30 µl of tridecanoic acid and 30 µl of nonadecanoic acid were added to the samples and standards. The standards were always sonicated for 5 min before addition (40 kHz, 320 W; Branson 8510, W.A. Brown Industrial Sales Inc., Richmond, VA, USA). The sonicator is an apparatus used to subject a biological sample to ultrasonic vibration in order to fragment the cells, macromolecules, and membranes.

Chloroform (1.94 mL) was added to the samples and standards and the tubes were mixed carefully on the test tube shaker. The Kimax tubes were flushed with N_2 and placed on a metal rack for boiling at 100 °C water bath for 3.5 hours. It was important to check the samples after 5 min, to verify the tightness of the Kimax tube caps (no bubbles should appear inside the tube). If bubbles appeared, the caps were tightened. After 30 min boiling, the metal rack was taken out of the bath, the tubes were mixed carefully one by one, and put back into the boiling water path. After 3.5 hours, the tubes were removed and cooled down to the room temperature (21±2 °C) in a cold-water bath. Water (1 mL) was added to each sample, and the samples in a plastic rack were shaken for 5 min at 240 rpm, followed by centrifugation at 2000 × g for 20 min. Once the samples were centrifuged, two phases were formed inside the Kimax tubes. The lower phase of the tubes was removed with a Pasteur pipette, and transferred to an Eppendorf tube with anhydrous Na_2SO_4. It was important to use a different Pasteur pipette for each sample, and to ensure that no solid particles or particles of the other phase to be separated were taken, as this may make the subsequent GC-MS analysis difficult. These tubes were centrifuged at 2000 × g for 20 min and then the chloroform phase, i.e. the liquid phase, was separated to a GC-MS sample vial.

Finally, the samples were analysed by the Gas Chromatography and Mass Spectrometry (GC-MS), with the aid of a computer program, the graphs representing the final results of the determination of the PHA present in the samples taken from the six bioreactors in operation are obtained. The Shimadzu GCMS-QP-2010Ultra GC-MS (Shimadzu, Kyoto, Japan) was equipped with an AOC-20i+s autosampler and a ZB-5MS capillary column (29.8 m, 0.25 mm, 0.25 µm), carrier gas helium (1 mL/min), injector temperature 290 °C, and 2 µL split in-jection. The ions followed in selective ion monitoring were m/z 74, 77 and 136 for Na-benzoate, and m/z 74, 87 and 103 for hydroxybuturate. The chromatographic separation was carried out using the following program: The oven temperature was held at 70 °C for 1 min, increased 10 °C/min to 100 °C, 30 °C/min to 160 °C, and 5 °C/min to 270 °C, held for 5 min.

5 RESULTS AND DISCUSSION

PHA biosynthesis is a four-step reaction, which starts from biopolymer substrates, such as proteins, lignin or carbohydrate polymers cellulose or hemicellulose, which provides the carbon source necessary for the biosynthesis process to take place. First, this carbon is converted into soluble organic compounds such as sugars, amino acids and fatty acids. The soluble organic compounds, on the other hand, go on to the third step and are transformed into alcohols or acids such as alcohols, carbonic acids and volatile fatty acids. These volatile fatty acids are the ones needed in this case to produce PHA. Therefore, two phases are differentiated: the first phase where the carbon source is converted into acid through the process of hydrolysis, and the second phase where the production of PHA takes place. Figure 5 shows the steps preceding PHA biosynthesis.

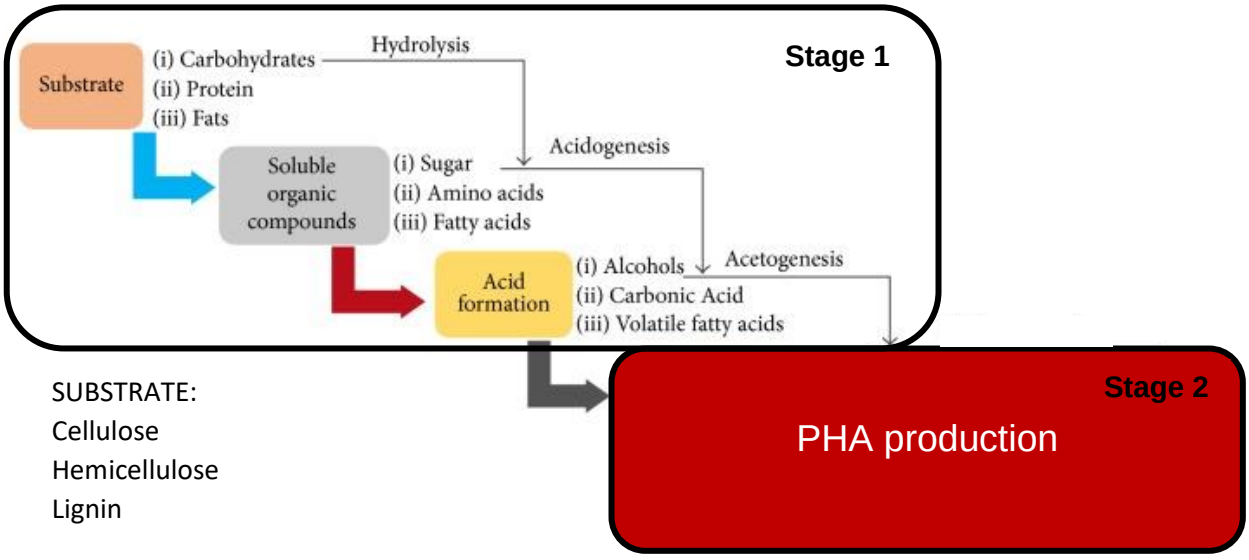

Figure 5. PHA biosynthesis

As shown in Figure 6, the production of PHA starts from agricultural residues, forest and marine biomass, and waste from industry and municipalities. From these residues, cellulose, hemicellulose and lignin are extracted, the main sources of carbon that feed this process, and from which a series of processes such as hydrolysis are carried out. With hydrolysis, the first phase ends, and a second phase is differentiated where, through detoxification and fermentation, the production of PHA is finally reached.

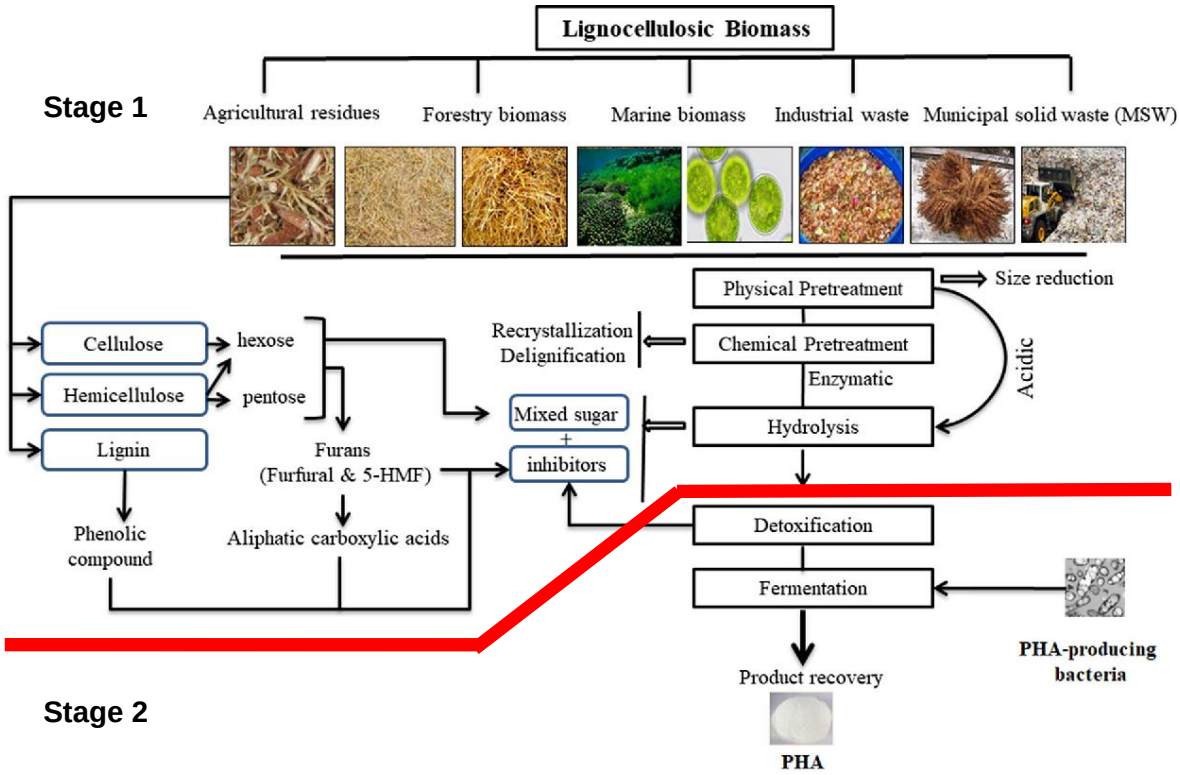

Figure 6. PHA production from waste residues (Rao, Haque, El-Enshasy, Singh & Mishra 2019)

The production of PHA is a process that is divided into two stages, which coincide with the stages shown and explained previously. In this case, as shown in Figure 7, each stage consists of a different tank or reactor. In the first stage, there is a fermentation tank where the hydrolysis of the biomass takes place, while in the second stage accumulation reactor PHA accumulation inside the cell is carried out.

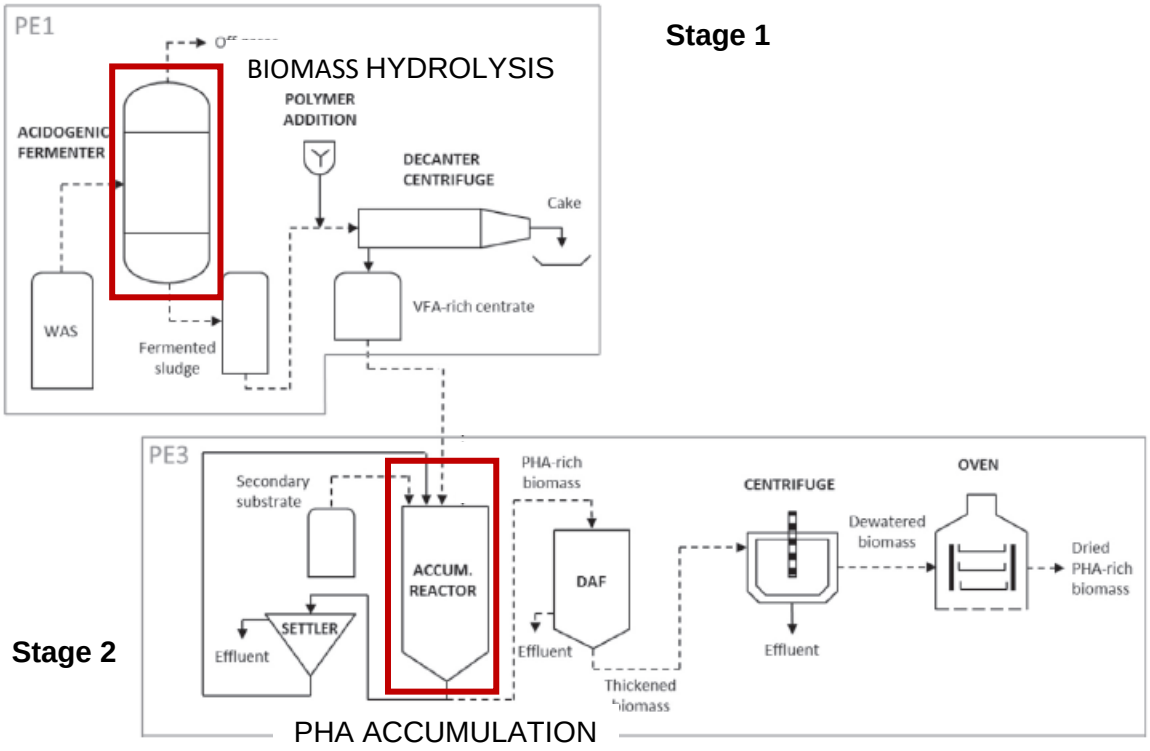

Figure 7. Two-stage PHA production (Albuquerque, Concas, Bengtsson, & Reis 2010)

5.1 Volatile solids and fixed solids

All the resulting values in the method of calculating the organic matter and ash content of the five samples analysed are summarised in Appendix 1. The values of organic matter content ranged from 73.30% to 74.50% of the total of each sample. As for the ash content, the results fall within a range between 25.50% and 26.70% of the total composition of each analysed sample.

5.2 Results from bioreactors

During the days that occupied the practical part of the present experiment, the pH of the six bioreactors was analysed twice a day, adjusting three of them to a pH 9-10 and the remaining three to a pH 7. The results noted in each pH adjustment are found in Appendices 4 and 5, dealing with the adjustment to pH 9-10 and pH 7, respectively. In these tables of results, the date and time at which each adjustment was made, the bioreactors at which it was

made, and the pH values taken before and after making the adjustment can be found. Table 5 shows both the reduction in pH during the day and during the night and the total average of the pH values taken at all the settings made.

Table 5. pH adjustment

	Adjustment to pH 9-10	**Adjustment to pH 7**
pH reduction during day	7.23	5.92
pH reduction during night	6.48	5.63
TOTAL	9.55	7.15

The conclusion that can be drawn from the results of the bioreactors is that the substrate composed of cellulose, hemicellulose and lignin was degraded to volatile fatty acids, which are the cause of the reduction in pH.

5.3 Polyhydroxyalkanoates accumulation during hydrolysis

Each time a pH adjustment was made, a sample was taken from each of the bioreactors for further analysis. The results and calculations carried out for both bioreactors adjusted to pH 9-10 and those adjusted to pH 7, respectively, are shown in Appendices 2 and 3.

Within each appendix, there are two tables. The first one contains the weight values taken for the calculation of the volatile solids, the results of which are reflected in the same table and are around 23 mg. The second table contains the values resulting from the analysis of each sample with the GC-MS and the average PHA concentration. Table 6 shows the average of the PHA concentration of all samples with its standard deviation.

Table 6. PHA concentration in mg/g dry weight.

	Average PHA concentration	**S.D.**
pH 9-10	0.187	0.028
pH 7	0.545	0.253

From the results of PHA concentration in the samples it can be said that when cellulose, hemicellulose and lignin are degraded, volatile fatty acids are formed and PHA accumulation is low. Therefore, the incorporation of another bioreactor would be necessary for a greater accumulation of PHA.

6 CONCLUSIONS

From the results obtained, some general conclusions can be drawn about the current experiment that was carried out. Firstly, the degradation of cellulose, hemicellulose and lignin, through hydrolysis, to volatile fatty acids was in progress in the six bioreactors that were set up. As a result, the conditions in which they were found, allowed the design of the first hydrolysis bioreactor in high carbon with VS conditions.

Secondly, during the hydrolysis process, less PHA was accumulated at a basic pH of 9-10, with an average concentration of 0.187 mg/g dry weight, which may be appropriate for efficient hydrolysis of biomass since there is less energy consumption for PHA synthesis during hydrolysis than at pH 7. On the other hand, more PHA was accumulated at a neutral pH, with an average PHA concentration of 0.545 mg/g dry weight. Although the results are quite different with different pH settings, both show a low concentration of PHA in the activated sludge samples. These samples were treated in different bioreactors, adjusted with the desired pHs and finally analyzed to obtain the present results.

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APPENDICES

Appendix 1. Table of the results of the calculation of organic matter and ash content.

Sample	M_crucible (g)	M_crucible + sample ww (g)	M_crucible + sample dw (g)	dw (g/g)	dw (%)	M_crucible + sample after 550ºC (g)	Organic matter, VS (g/g)	Ash content, FS (g/g)	Organic matter (%)	Ash content (%)
1	22.0033	27.1256	22.0242	0.0041	0.41	22.0088	0.0154	0.0055	73.68	26.32
2	27.9740	32.8743	27.9931	0.0039	0.39	27.9791	0.014	0.0051	73.30	26.70
3	26.4846	31.4362	26.5046	0.0040	0.40	26.4897	0.0149	0.0051	74.50	25.50
4	20.3128	25.2695	20.3340	0.0043	0.43	20.3183	0.0157	0.0055	74.06	25.94
5	23.148	28.0550	23.1686	0.0042	0.42	23.1534	0.0152	0.0054	73.79	26.21

Appendix 2. Table of the results of the volatile solids calculation and the average of PHA concentration in pH 9-10 bioreactors.

Sampling time	Bioreactor	Number Sample	Kimax tube weight (g)	Tube + dry sludge weight (g)	Sludge weight (g)	Sludge weight (mg)	Sample volume (µL)	Volatile Solids (mg)
04/03/2020 15:30 pH 9-10	13	91	11.2075	11.2210	0.014	13.5	200	13.2
	14	92	11.2494	11.2624	0.013	13.0	200	12.8
	15	93	11.1803	11.1962	0.016	15.9	200	15.6
05/03/2020 9:30 pH 9-10	13	97	11.2285	11.2406	0.012	12.1	200	11.9
	14	98	11.1645	11.1773	0.013	12.8	200	12.6
	15	99	11.1967	11.2087	0.012	12.0	200	11.8
05/03/2020 15:30 pH 9-10	13	103	11.2217	11.2343	0.013	12.6	200	12.4
	14	104	11.2023	11.2140	0.012	11.7	200	11.5
	15	105	11.2232	11.2353	0.012	12.1	200	11.9
07/03/2020 15:30 pH 9-10	13	121	11.2068	11.2284	0.022	21.6	400	21.2
	14	122	11.2187	11.2406	0.022	21.9	400	21.5
	15	123	11.2353	11.2584	0.023	23.1	400	22.7
08/03/2020 17:00 pH 9-10	13	127	11.2424	11.2645	0.022	22.1	400	21.7
	14	128	11.3362	11.3576	0.021	21.4	400	21.0
	15	129	11.2204	11.2435	0.023	23.1	400	22.7

09/03/2020 8:30 pH 9-10	13	139	11.2424	11.2650	0.023	22.6	400	22.2
	14	140	11.1950	11.2174	0.022	22.4	400	22.0
	15	141	11.2606	11.2820	0.021	21.4	400	21.0
09/03/2020 15:30 pH 9-10	13	145	11.1996	11.2214	0.022	21.8	400	21.4
	14	146	11.2416	11.2646	0.023	23.0	400	22.6
	15	147	11.2920	11.3143	0.022	22.3	400	21.9
10/03/2020 9:30 pH 9-10	13	157	10.7441	10.7676	0.024	23.5	400	23.1
	14	158	10.9024	10.9241	0.022	21.7	400	21.3
	15	159	10.8464	10.8686	0.022	22.2	400	21.8
10/03/2020 15:30 pH 9-10	13	163	11.1822	11.2059	0.024	23.7	400	23.2
	14	164	11.2079	11.2302	0.022	22.3	400	21.9
	15	165	11.1572	11.1802	0.023	23.0	400	22.6
11/03/2020 9:30 pH 9-10	13	175	11.2379	11.2610	0.023	23.1	400	22.7
	14	176	11.2252	11.2482	0.023	23.0	400	22.6
	15	177	11.2133	11.2360	0.023	22.7	400	22.3
11/03/2020 15:30 pH 9-10	13	181	10.8813	10.9046	0.023	23.3	400	22.9
	14	182	11.1756	11.1991	0.024	23.5	400	23.1
	15	183	11.1416	11.1644	0.023	22.8	400	22.4
12/03/2020 10:45 pH 9-10	13	193	11.1386	11.1614	0.023	22.8	400	22.4
	14	194	11.1982	11.2205	0.022	22.3	400	21.9
	15	195	11.0052	11.0279	0.023	22.7	400	22.3
12/03/2020 15:40 pH 9-10	13	199	11.1876	11.2109	0.023	23.3	400	22.9
	14	200	11.1779	11.2010	0.023	23.1	400	22.7
	15	201	11.2194	11.2427	0.023	23.3	400	22.9

Sampling time	Sample identification	HB Area	NaBe n/z 105	(Ahb/Ast)*Cst (µg)	HB (µg)	mg HB g dry wt	mg HB g VS	Average PHA concentration	S.D.
05/03/2020 15:30 pH 9-10	20200306_34	2750	225970	1.064	5.96	0.473	0.482	0.497	0.099
	20200306_35	2514	177712	1.237	6.92	0.592	0.603		
	20200306_36	1978	200313	0.863	4.83	0.399	0.407		
08/03/2020 17:00 pH 9-10								0.198	0.002
	20200311_10	4037	269886	1.222	4.19	0.196	0.199		
	20200311_11	2576	162350	1.296	4.44	0.192	0.196		
10/03/2020 9:30 pH 9-10	20200312_16	2108	145371	1.184	4.68	0.199	0.203	0.217	0.029
	20200312_17	2532	153379	1.348	5.33	0.246	0.250		
	20200312_18	2541	191664	1.083	4.28	0.193	0.197		
10/03/2020 15:30 pH 9-10	20200316_10	3282	199356	1.345	4.77	0.201	0.205	0.212	0.021
	20200316_11	2806	157198	1.458	5.17	0.232	0.236		
	20200316_12	3553	233496	1.243	4.41	0.192	0.195		
11/03/2020 9:30 pH 9-10	20200313_16	2167	171643	1.031	3.79	0.164	0.167	0.177	0.022
	20200313_17	3146	207629	1.238	4.55	0.198	0.202		
	20200313_18	1836	153506	0.977	3.59	0.158	0.161		
11/03/2020 15:30 pH 9-10	20200313_22	1720	129162	1.088	4.00	0.172	0.175	0.181	0.014
	20200313_23	3234	212685	1.242	4.57	0.194	0.198		
	20200313_24	3115	243939	1.043	3.83	0.168	0.171		
12/03/2020 10:45 pH 9-10	20200316_22	2128	168162	1.034	3.67	0.161	0.164	0.181	0.020
	20200316_23	1786	116269	1.255	4.45	0.200	0.204		
	20200316_24	2022	149455	1.105	3.92	0.173	0.176		
12/03/2020 15:40 pH 9-10	20200316_28	697	168986	0.337	1.20	0.051	0.052	0.143	0.085
	20200316_29	2156	125377	1.405	4.98	0.216	0.220		
	20200316_30	2876	234063	1.004	3.56	0.153	0.156		
							TOTAL	0.187	0.028

Appendix 3. Table of the results of the volatile solids calculation and the average of PHA concentration in pH 7 bioreactors.

Sampling time	Bioreactor	Number Sample	Kimax tube weight (g)	Tube + dry sludge weight (g)	Sludge weight (g)	Sludge weight (mg)	Sample volume (µL)	Volatile Solids (mg)
04/03/2020 15:30 pH 7	16	94	11.1981	11.2121	0.014	14.0	200	13.7
	17	95	11.1654	11.1789	0.014	13.5	200	13.2
	18	96	11.1982	11.2109	0.013	12.7	200	12.5
05/03/2020 9:30 pH 7	16	100	11.2111	11.2229	0.012	11.8	200	11.6
	17	101	11.1714	11.1842	0.013	12.8	200	12.6
	18	102	11.1983	11.2108	0.013	12.5	200	12.3
05/03/2020 15:30 pH 7	16	106	11.0071	11.0186	0.011	11.5	200	11.3
	17	107	11.1991	11.2106	0.011	11.5	200	11.3
	18	108	11.2205	11.2325	0.012	12.0	200	11.8
07/03/2020 15:30 pH 7	16	124	11.1417	11.1634	0.022	21.7	400	21.3
	17	125	11.2282	11.2496	0.021	21.4	400	21.0
	18	126	11.1508	11.1719	0.021	21.1	400	20.7
08/03/2020 17:00 pH 7	16	130	11.2327	11.2532	0.021	20.5	400	20.1
	17	131	11.2146	11.2360	0.021	21.4	400	21.0
	18	132	11.1931	11.2130	0.020	19.9	400	19.5

09/03/2020 8:30 pH 7	16	142	11.1390	11.1606	0.022	21.6	400	21.2
	17	143	11.2008	11.2246	0.024	23.8	400	23.3
	18	144	11.1184	11.1398	0.021	21.4	400	21.0
09/03/2020 15:30 pH 7	16	148	11.2265	11.2478	0.021	21.3	400	20.9
	17	149	11.1924	11.2136	0.021	21.2	400	20.8
	18	150	11.2721	11.2932	0.021	21.1	400	20.7
10/03/2020 9:30 pH 7	16	160	10.8770	10.8983	0.021	21.3	400	20.9
	17	161	10.7671	10.7877	0.021	20.6	400	20.2
	18	162	10.7916	10.8122	0.021	20.6	400	20.2
10/03/2020 15:30 pH 7	16	166	11.2396	11.2611	0.022	21.5	400	21.1
	17	167	11.1977	11.2194	0.022	21.7	400	21.3
	18	168	11.1638	11.1852	0.021	21.4	400	21.0
11/03/2020 9:30 pH 7	16	178	11.1718	11.1937	0.022	21.9	400	21.5
	17	179	11.2277	11.2491	0.021	21.4	400	21.0
	18	180	11.2035	11.2251	0.022	21.6	400	21.2
11/03/2020 15:30 pH 7	16	184	11.2454	11.2673	0.022	21.9	400	21.5
	17	185	11.1338	11.1556	0.022	21.8	400	21.4
	18	186	11.2154	11.2372	0.022	21.8	400	21.4
12/03/2020 10:45 pH 7	16	196	11.1838	11.2048	0.021	21	400	20.6
	17	197	11.1911	11.2119	0.021	20.8	400	20.4
	18	198	11.2140	11.2343	0.020	20.3	400	19.9
12/03/2020 15:40 pH 7	16	202	11.1934	11.2149	0.021	21.5	400	21.1
	17	203	11.2314	11.2527	0.021	21.3	400	20.9
	18	204	10.7735	10.7939	0.020	20.4	400	20.0

Sampling time	Sample identification	HB Area	NaBe n/z 105	(Ahb/Ast)*Cst (µg)	HB (µg)	mg HB	mg HB	Average PHA concentration	S.D.
						g dry wt	g VS		
05/03/2020 15:30 pH 7	20200306_37	2433	174375	1.220	6.83	0.594	0.605	1.551	0.825
	20200306_38	12439	280856	3.873	21.67	1.885	1.921		
	20200306_39	16257	317811	4.473	25.03	2.086	2.126		
08/03/2020 17:00 pH 7	20200311_12	3723	173639	1.751	6.00	0.293	0.298	0.638	0.316
	20200311_13	11192	216213	4.228	14.49	0.677	0.690		
	20200311_14	11934	185063	5.267	18.05	0.907	0.925		
10/03/2020 9:30 pH 7	20200312_19	2913	122928	1.936	7.65	0.359	0.366	0.711	0.299
	20200312_20	10668	193539	4.502	17.80	0.864	0.881		
	20200312_21	10086	181931	4.528	17.90	0.869	0.886		
10/03/2020 15:30 pH 7	20200316_13	3967	178487	1.815	6.44	0.300	0.305	0.459	0.240
	20200316_14	6487	120097	4.412	15.66	0.721	0.735		
	20200316_15	2417	99243	1.989	7.06	0.330	0.336		
11/03/2020 9:30 pH 7	20200313_19	3358	167465	1.638	6.02	0.275	0.280	0.537	0.232
	20200313_20	5905	140603	3.430	12.61	0.589	0.601		
	20200313_21	6680	129430	4.216	15.50	0.718	0.731		
11/03/2020 15:30 pH 7	20200313_25	2833	154030	1.502	5.52	0.252	0.257	0.585	0.300
	20200313_26	9208	197346	3.811	14.01	0.643	0.655		
	20200313_27	6440	107168	4.908	18.05	0.828	0.844		
12/03/2020 10:45 pH 7	20200316_25	3636	193224	1.537	5.45	0.260	0.265	0.514	0.218
	20200316_26	5444	115882	3.837	13.62	0.655	0.667		
	20200316_27	11713	279680	3.421	12.14	0.598	0.610		
12/03/2020 15:40 pH 7	20200316_31	3315	181973	1.488	5.28	0.246	0.250	0.368	0.166
	20200316_32	13420	400961	2.734	9.70	0.476	0.485		
							TOTAL	0.545	0.253

Appendix 4. pH adjustment to pH 9-10.

Sampling time	Bioreactor	pH before adjustment	pH after adjustment
06/03/2020 9:30 pH 9-10	13, 14, 15	6.01	10.32
06/03/2020 15:30 pH 9-10	13, 14, 15	8.97	9.68
07/03/2020 15:30 pH 9-10	13, 14, 15	6.04	9.06
08/03/2020 17:00 pH 9-10	13, 14, 15	6.09	9.30
09/03/2020 8:30 pH 9-10	13, 14, 15	6.18	9.22
09/03/2020 15:30 pH 9-10	13, 14, 15	6.87	9.88
10/03/2020 9:30 pH 9-10	13, 14, 15	6.35	9.31
10/03/2020 15:30 pH 9-10	13, 14, 15	6.54	9.95
11/03/2020 9:30 pH 9-10	13, 14, 15	6.86	9.29
11/03/2020 15:30 pH 9-10	13, 14, 15	7.88	9.70
12/03/2020 10:45 pH 9-10	13, 14, 15	7.02	9.53
12/03/2020 15:40 pH 9-10	13, 14, 15	8.25	9.40

Appendix 5. pH adjustment to pH 7.

Sampling time	Bioreactor	pH before adjustment	pH after adjustment
06/03/2020 9:30 pH 7	16, 17, 18	4.65	7.22
06/03/2020 15:30 pH 7	16, 17, 18	5.94	7.12
07/03/2020 15:30 pH 7	16, 17, 18	4.80	7.41
08/03/2020 17:00 pH 7	16, 17, 18	5.02	7.28
09/03/2020 8:30 pH 7	16, 17, 18	5.72	7.18
09/03/2020 15:30 pH 7	16, 17, 18	6.20	7.11
10/03/2020 9:30 pH 7	16, 17, 18	5.92	7.11
10/03/2020 15:30 pH 7	16, 17, 18	6.45	7.20
11/03/2020 9:30 pH 7	16, 17, 18	5.83	7.00
11/03/2020 15:30 pH 7	16, 17, 18	6.50	7.16
12/03/2020 10:45 pH 7	16, 17, 18	6.02	7.00
12/03/2020 15:40 pH 7	16, 17, 18	6.52	7.01