# Obtaining polyhydroxyalkanoates (PHA) from lignocellulosic biomass: a review study

## Obtención polihidroxialcanoatos (PHA) a partir de biomasa lignocelulósica: un estudio de revisión

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## Abstract

Polyhydroxyalkanoates (PHA) are considered biodegradable polymers that are obtained by microbial fermentation from different substrates, mainly sugars, which are synthesized from diverse natural sources. An alternative is obtaining it from lignocellulose biomass. Therefore, this paper presents a compilation of research related to the methods used for pretreatment, saccharification, its subsequent conversion to PHA, and the final extraction process and characterization applied to identify it to identify it. Regarding pretreatment methods, it was found that the hydrothermal process and the application of ultrasound (US) combined with chemical agents that are acidic or alkaline are the most used to eliminate inhibitors, mainly lignin and its derivatives. Hydrothermal pretreatment is presented as a promising method under the concept of biorefinery, due to its short treatment times and ease of by-product recovery. In the saccharification process, it evidenced that the enzymatic method with cellulose, combined in some cases with (3-glucosidase), is the most reported. Concerning the fermentation process from hydrolyzed lignocellulosic residues such as rice straw, wheat, kenaf, and inter alia, the *Ralstonia eutropha* strain is the most used for the synthesis of poly (3-hydroxybutyrate) (PHB). It was found that the variables that most influence the performance of the fermentation process are the C/N ratio, concentration of the substrate source (concentration of hydrolysate), oxygenation, and the presence of inhibitors, which are byproducts formed during the biomass pretreatment process.

***Keywords:*** fermentation; PHA*;* polyhydroxyalkanoates; pretreatment; saccharification; *Ralstonia eutropha;* sugars.

## Resumen

Los polihidroxialcanoatos (PHA) son considerados polímeros biodegradables que se obtienen por fermentación microbiana, a partir de diferentes sustratos principalmente azúcares, los cuales se sintetizan de diversas fuentes naturales. Una alternativa es la obtención a partir de biomasa lignocelulósica. Por lo anterior, este documento presenta una recopilación de investigaciones relacionadas con métodos utilizados para el pretratamiento, sacarificación, su posterior conversión a PHA y el proceso final de extracción y caracterización, aplicados para su identificación. En cuanto a los métodos de pretratamiento, se encontró que el proceso hidrotermal y la aplicación de ultrasonido (US) combinado con agentes químicos ácidos o alcalinos son los más utilizados para eliminar inhibidores, principalmente lignina y sus derivados. El pretratamiento hidrotermal se presenta como un método promisorio bajo el concepto de biorrefinería, debido a que se requieren tiempos cortos de tratamiento, y a la facilidad de recuperación de subproductos. En el proceso de sacarificación se evidenció que el método enzimático con celulasa combinada, en algunos casos con β-glucosidasa, es el más reportado. En relación con el proceso fermentativo, a partir de hidrolizados de residuos lignocelulósicos como paja de arroz, trigo, kenaf entre otros, la cepa *Ralstonia eutropha* es la más utilizada para la síntesis de poli(3-hidroxibutirato) (PHB). Se encontró que las variables que más influyen en el rendimiento del proceso fermentativo son la relación C/N, concentración de la fuente del sustrato (concentración de hidrolizados), la oxigenación y la presencia de inhibidores, que son subproductos formados durante el proceso de pretratamiento de la biomasa.

***Palabras clave:*** polihidroxialcanoatos; PHA; *Ralstonia eutropha;* sacarificación; fermentación; pretratamiento; azúcares.

# 1. Introduction

Plastics are derived from oil and are composed of chains of carbon atoms with additions of nitrogen (N), oxygen (O), or sulfur (S); they are characterized by their wide applicability, because they are easy to manufacture and also due to their properties of flexibility, strength, low weight, impermeability, and low cost (Report European Bioplastic, 2017). Pursuant to the Plastic Market Size (2020) report, the global plastic market for 2019 was USD 450.88 billion and it's expected to reach USD 579.19 billion by 2027. The main use is oriented towards the packaging and wrapping, automotive, construction, final goods, medical and pharmaceutical, electricity and electronics, and textiles sectors. The packaging industry showed the highest demand on the market for the year 2019, mainly on the food and drinks sector, followed by the construction sector.

Due to the environmental pollution, caused by the use of non-biodegradable polymers, and the decrease in sources of natural gas and oil, alternatives are being sought to replace the use of synthetic polymers with biodegradable ones, such as polylactic acid (PLA) and polyhydroxyalkanoate (PHA) (Anjum *et al.,* 2016). These polymers, according to García, Novoa, Franco, and Higuita (2015) are considered of microbial origin and can be synthesized from renewable natural sources (Saratale *et al.,* 2020).

PHA is a biopolymer that stands out for its biodegradability and compostability, low activity with water, resistance to hydrolytic degradation and exposure to UV rays, high modulus of elasticity, and water barrier properties. These features enable versatility for their possible applications. Consequently, PHA production capacity is expected to increase from 2.1 million tons in 2019 to 2.4 million tons in 2022, which results in an opportunity to develop research processes focused on the synthesis of PHA using high availability and low-cost raw materials, as well as for production optimization (Saratale *et al.,* 2020).

Although PHA has various properties and characteristics that make it an ideal material for developing high value-added polymeric products, mainly packaging and medical devices, there are limitations to its large-scale production and marketing due to the high cost of production compared to conventional polymeric

materials. According to Raza, Abid, and Banat (2018), such a cost is 5 to 19 times higher. This difference is mainly caused by the type of carbon source, the fermentation, extraction, and purification stages, and the process productivity (Govil *et al.*, 2020; Raza *et al.*, 2018). Therefore, in order to reduce production cost gaps, it is necessary to establish cost-effective processes with high PHA yields. The alternatives include the use of lignocellulosic biomass which, according to Govil *et al.* (2020) and Raza *et al.* (2018), would reduce the total cost of production by up to 50 %.

Currently, residues of lignocellulosic biomass, such as sugarcane and corn bagasse, kenaf (*Hibiscus cannabinus*), rice straw, wood, wheat, inter alia, have become the main alternative for the PHA synthesis (Liu; Zhang; An; Chen; Yang, 2015), and are considered an economically viable and environmentally sustainable resource for large-scale and low-cost production, which makes it competitive with oil derivatives (Yang; Tao; Wayman, 2018).

According to Kant, Gurav, Choi, Jung, and Yang (2019), the disadvantages of lignocellulosic materials for the synthesis of PHA, include the fact that the pretreatments (either physical, chemical, or biological) are required to remove lignin and make cellulose and hemicellulose accessible for the subsequent saccharification process. Additionally, during pretreatment, by-products such as furfural, vanillin, hydroxymethylfurfural (HMF), and acetate are generated, which have an inhibitory effect during microbial fermentation, mainly for cell growth and PHA production. Jonsson, Alriksson, and Nilvebrant (2013) reported various methods to reduce the effect of inhibitors after pretreatment, which include the detoxification of hydrolysates by physical, chemical, physicochemical, and/or biological methods, along with the use of genetically modified microorganisms with resistance to inhibitors and improvement of cellular metabolism to increase performance.

The use of lignocellulosic biomass has generated research opportunities in three stages of the process: pretreatment, fermentation, and PHA purification. Among the most promising pretreatment techniques are those that use clean technology, preferably those that do not require the addition and recovery of chemicals other than water or, in lieu thereof, those that use low concentrations, to avoid equipment corrosion, and those that process is easy to operate, that are economical, with production of fewer inhibitors and respectful of the environment, such processes include the hydrothermal pretreatment (Capolupo; Faraco, 2016; Ruiz; Rodriguez; Fernandes; Vincent; Teixeira, 2013).

At the same time, it is important to explore the synthesis processes with continuous fermentation, using thermotolerant microorganisms of pure or mixed strains (Sabapathy *et al.*, 2020), where the former have yields exceeding 90 %, but require special conditions in terms of nutrients purity and aseptic environments for fermentation, which is reflected in the costs and difficulty of scaling the process. On the contrary, the use of mixed strains allows working fermentations with different substrates, with variability in sugars composition (Pakalapati; Chang; Show; Arumugasamy; Lan, 2018), which would be an alternative to scale the process (Li; Wilkins, 2020). Finally, the current trend is that the process of PHA synthesis from biomass is focused on the concept of circular economy and biorefinery, whose main objective is to reduce $CO_2$ emissions and the conversion of waste into products that re-enter the production cycle after their elimination (Adeleye *et al.*, 2020).

In this paper, considering the current importance of PHA as an alternative for the production of biopolymers, it is discussed the research work of recent years pertaining to obtain PHA from lignocellulosic materials, whose process includes several stages, such as pretreatment for the isolation of cellulose, the saccharification process, followed by the fermentation process and finally, the methods of extraction and characterization of the PHA.

Among the current challenges to improve the efficiency of its production, studies show that the use of lignocellulosic waste is a viable alternative due to its availability and low cost. However, pursuant to the analysis carried out for the PHA production, several works indicate that pretreatment is decisive for the PHA synthesis process, due to the recalcitrant nature of this material (Ganesh *et al.*, 2019; Saratale *et al.*, 2020). Therefore, this

document is intended to establish which are the pretreatments used that allow to cellulose be isolated in less time, with the reduction of chemical agents and the minimum formation of inhibitors. Additionally, this work is intended to acknowledge the different factors that influence the process of obtaining PHA, ranging from the hydrolysis conditions of the cellulose isolation to the formation of sugars, the cultivation methods and the kind of microorganism used in the fermentation stage on the production yield of PHA.

# 2. Methodology

For the literature review pertaining to the production of PHA from lignocellulosic materials, it is resorted to the Scopus database. The search terms were "*Production PHA* and *lignocellulosic*", "*Production PHA* and *Ralstonia eutropha*", "*Lignocellulosic pretreatment*" and "*Lignocellulosic saccharification*". With the results thereof, an information matrix was elaborated with the name of the article, publication year, and the journal or database where it was published. The main databases where the publications regarding the appropriate topics are Science Direct, Springer Link, Wiley Online Library, SciELO, and Web of Science.

To consult the research results at the national level, it was searched the repository of the National University of Colombia and the University of Antioquia. The search margin was defined for publications of the last 10 years. For the analysis of the information collected, common factors of study by the researchers were considered, such as generalities, production and characterization of PHA, and pretreatment methods of lignocellulosic materials and saccharification. Abstracts of around 100 papers were reviewed between articles and graduation works, of which 58 were selected.

## *2.1. Overview of PHA*

PHAs belong to the family of polyesters, which are made up of hydroxyalcanoic acid (Rojas; Holes; Mosquera, 2016), and are synthesized by intracellular accumulation in several microorganisms (bacteria, archaea, and microalgae), as a result of environmental stress and nutrient imbalance, especially nitrogen (N) and phosphorus (P). Depending on the substrate and type of microorganism, intracellular accumulation can reach up to 90 % yield of PHA in dry weight (Acosta; Alcaraz; Cardona, 2018; Gonzalez; Meza; Gonzalez; Córdoba, 2013).

PHA are classified depending on the number of carbon atoms: short-chain ones are composed of 3 to 5 carbon atoms (C); those of medium chain are composed of 6 to 14; and those of long chain are composed of 15 or more Atoms of C (González *et al.,* 2013). Short-chain PHA are classified into poly(3-hydroxybutyrate) (PHB), poly(4-hydroxybutyrate) (P4HB), poly(3-hydroxyvalerate) (P3HV), and the heteropolymer composed of poly(3-hydroxybutyrate-copolymer-3-hydroxyvalerate) (PHB-co-3HV). Medium-chain ones are composed of poly(3-hydroxyhexanoate) (P3HHx), poly(3-hydroxyoctanoate) (P3HO) and heteropolymers, such as poly(3-hydroxyhexanoate-copolymer-3-hydroxyoctanoate) (P3HHx-co-3HO) (Kumar *et al.*, 2020). The chain size is directly pertaining to hydrophobicity, melting point, glass transition temperature, and degree of crystallization; where PHB is the most studied and characterized, because it has properties similar to those of polyethylene and polypropylene.

For the synthesis of PHB the most used microorganism is the bacterium *Ralstonia eutropha,* due to its ability to store up to 96 % of the polymer in dry weight, using carbon sources from agro-industrial waste. This bacterium, according to the latest taxonomic classification, is also known as Cupriavidus necátor (Dey; Rangarajan, 2017; Madeiros-Garcia *et al.,* 2020). Research reports that R. eutropha is able to metabolize several hydrolysates obtained from lignocellulosic sources, whose sugar composition are glucose and xylose, mainly (Ranganathan; Dutta; Moses; Anandharamakrishnan, 2020). Pursuant to Chanprateep *et al.* (2008) and Saratale *et al.* (2020), the *R. eutropha* strain has been used for the production of PHB from wheat residual biomass, rice straw, cane bagasse, paper residues and kenaf.

This research focuses on evaluating the production of the polymer in the stationary phase under limited concentrations of Nitrogen (N), Phosphorus (P), Magnesium (Mg), and Oxygen (O) (Kerketta; Vasanth, 2019). Before the fermentation stage of hydrolysates, lignocellulosic biomass must go through the pre-treatment and saccharification stages, which determine the quality of the sugars that are used as Alexander-Guancha, Realpe-Delgado, García-Celis. Obtaining polyhydroxyalkanoates (PHA) from biomass lignocellulosic: a review study substrate in the synthesis of PHA. Therefore, it is necessary to establish the most efficient method of biomass pretreatment. The techniques used for this process are discussed below.

## 2.2. Pretreatment of lignocellulosic materials and saccharification

The goal of the pretreatment in lignocellulosic material is to alter or eliminate the chemical structures that inhibit the saccharification process, which come mainly from lignin. This process allows to increase the hydrolysis speed of the cellulose and hemicellulose chains and to increase the yields in fermentable sugars. According to Adeleye, *et al.* (2020) and Yu and Stahl (2008), an effective pretreatment method must ensure high conversion to fermentable sugars, minimal degradation of the cellulose and hemicellulose chains during the pretreatment process, reduction of the formation of by-products that inhibit the hydrolysis process, and finally, it must ensure that the process is inexpensive.

Several methodologies have been used to remove lignin from lignocellulosic materials in order to improve the efficiency of saccharification, either by acid or enzymatic hydrolysis, the most common ones are physical (grinding, ultrasound), chemical (acidic and alkaline), physicochemical (steam explosion), and biological (Wu *et al.,* 2017). Physicochemical pretreatment is the most efficient combination to remove lignin since the saccharification performance is increased by 4 to 10 times; however, the main disadvantages include the use of expensive reactors and aggressive chemicals such as acid, increased water and energy consumption, the formation of inhibitors and environmental impact limitation.

On the other hand, microbial pretreatment is considered a simpler and more environmentally friendly process; however, it takes a long time (from 15 to 40 days), which reduces profitability. Consequently, it is necessary to develop universal, profitable, and sustainable pretreatment techniques (simple or combined) under the biorefinery model, whose objective is to take advantage of all the components of lignocellulosic biomass to obtain value-added products using transformation processes with a sustainable approach (from Bhowmick; Sarmah; Sen, 2018). The model consists of the recovery of chemicals, enzymes, and by-products of biomass for reuse, conversion into valuable products and reduction of waste production. Galbe and Wallberg (2019) and Vu *et al.* (2020) mention that an alternative that is coincident with the concept of biorefinery is the use of hydrothermal pretreatment, which allows the recovery of most of the by-products of lignocellulosic material after treatment, besides gradually reducing the impact on the environment.

## 2.3. Chemical pretreatment and treatment combined with US

Ganesh *et al.* (2019) performed the chemical pretreatment of kenaf biomass using sodium carbonate ($Na_2CO_3$) and sodium sulfite ($Na_2SO_3$) to obtain fermentable sugars by enzymatic hydrolysis, using commercial Cellulase from *Trichoderma reesei*. The results reported that treatment with $Na_2CO_3$ and $Na_2SO_3$ at 4 % (w/v) for 3 h at 100 °C has a higher saccharification yield (598.4 mg/g kenaf), 65 % of delignification, hydrolysis performance of 74.8 %, glucose yield of 74.8 % and a biomass recovery between 50 % to 54 %. The authors conclude that treatments with $Na_2CO_3$ and $Na_2SO_3$ have higher saccharification performance. Severe chemical treatments, with strong acids and alkalis, allow greater saccharification of biomass and a high presence of inhibitors that can affect microbial fermentation processes in the PHA production. Therefore, the authors recommended using combined methods.

Dattatraya *et al.* (2020) evaluated the process of delignification of wheat residual biomass using the alkaline method combined with US for the subsequent obtaining of fermentable sugars by enzymatic hydrolysis, and the production of PHB using the *R. eutropha* bacterium (ATCC 17699). For the lignin removal process, alkaline pretreatments were carried out, with sodium hydroxide (NaOH) and calcium hydroxide ($Ca(OH)_2$); with US and combined (US+NaOH). The authors report that the conditions for increased performance during enzymatic hydrolysis were defined as a combined treatment (US+NaOH) at a concentration of 2 % at 20 kHz, for 60 min and 2.0 W/mL, at a temperature of 100 °C for 30 min, with a solid-solvent ratio of 10 % (w/V).

These conditions are reported to be 70 % delignified, hydrolysis yield of 84.5 %, and glucose and xylose yield of 90 % and 65 %, respectively. The authors recommend working with moderate concentrations of NaOH, since when they are severe there is a hydrolysis of the hemicellulose and an increase in the presence of inhibitors from lignin, which affects the efficiency of the enzymatic reaction. Additionally, the concentrations of NaOH for pretreatment were reduced when combined with US, due to the cavitation process that enables the generation of turbulence that affects the surface structure of the biomass and improves the mass transfer; therefore, pretreatment times and accessibility of enzymes to the substrate during the enzymatic hydrolysis stage are reduced.

Velmurugan and Muthukumar (2012) performed sugarcane bagasse (BCA) pretreatment using US-assisted NaOH. They evaluated particle size, solid-liquid ratio, NaOH concentration, temperature, and sonication time. They found that the maximum yield in reducing sugars obtained was 96.27 %, using a particle size of 0.27 mm, solid-liquid ratio 1:25 and NaOH concentration of 2.89 %, keeping the temperature at 70 °C and a sonication time of 47.42 min. It is clear that, when using the US technique, there is a lignin elimination and a yield of reducing sugars of 82.32 % and 92.11 %, respectively, compared to pretreatment using autoclave, where a percentage of 46.50 % and 72.00 % was obtained, respectively. Similar results were reported by Wu *et al.* (2017), who evaluated the process of alkaline pretreatment with NaOH in rice straw using US, and observed that the yield of saccharification by enzymatic hydrolysis was increased, due to porosity and modification of the wheat straw surface area, caused by the effect of the US.

## 2.4. Hydrothermal pretreatment

Hydrothermal pretreatment is a physicochemical method that uses water at high temperatures, usually between 150 °C and 300 °C, and pressures between 0 to 60 bar, under these conditions, it is possible to break the hydrogen bonds of the interactions of the lignin, hemicellulose and cellulose complex. Therefore, it is important to determine the pressure and temperature conditions of the process, depending on the composition of the lignocellulosic biomass, the solubilization temperature, and degradation of its components.

In the case of hemicellulose and lignin, solubilization is reached at 150 °C and 180 °C, respectively, and cellulose degradation is above 230 °C (Vu *et al*., 2020). Additionally, according to Yang *et al.* (2018), this pretreatment reduces operating costs, because low concentrations of chemical agents are required, larger particle sizes with higher concentrations of solids can be used, compared to conventional pretreatments, and shorter residence times of 10 to 50 min are required (Ruiz *et al.*, 2020). Generally, depending on the type of biomass, it is necessary to establish the conditions of temperature, pressure, residence time, pH, and solid-liquid ratio, which allow the greatest amount of lignin to be removed (Yang *et al*., 2018).

According to Jonsson *et al.* (2013), during the pretreatment of lignocellulosic materials, regardless of the method applied, inhibitors are formed in greater or lesser concentration, which are maintained during the hydrolysis process. Therefore, it is necessary to look for alternatives to detoxify the hydrolysate before fermentation, in order to reduce its effect on metabolism and the growth of microorganisms, as well as increase the production yield of the polymer. This process requires the application of chemical, biological or physical

methods, consisting of liquid-liquid, liquid-solid extraction, heating and evaporation, and treatments with microbial and enzymatic biocatalysts. The authors report that alkaline treatment with $Ca(OH)_2$ is the most effective, due to its low cost and increased removal of inhibitors.

## 2.5. Enzymatic hydrolysis

López, Acosta, and Zambrano (2013) performed pretreatment with sulfuric acid ($H_2SO_4$), NaOH, and $H_2O_2$ in cassava leaves and stems separately, and saccharification by enzymatic hydrolysis. For the pretreatment process, two particle sizes, and different concentrations of chemical and substrate were studied. The enzymatic hydrolysis process was performed with the enzyme accellerase 1,500 (cellulase complex). It was found that with the pretreatment with NaOH the highest concentration of total sugars was obtained (3.7 g/L), followed by the pretreatment with $H_2SO_4$ (2.11 g/L) and with $H_2O_2$ (1.54 g/L). Cellulose digestibility increased with alkaline pretreatment, because lignin removal is more effective and shows less solubilization of hemicellulose and cellulose, in contrast to acid or hydrothermal pretreatment. Likewise, the size of the substrate particle influences the release of sugars during hydrolysis. On the other hand, the concentration of the chemical agent showed a significant difference, since the higher the concentration, the greater the removal of lignin.

Annamalai and Sivakumar, (2016) obtained fermentable sugars from wheat bran for the production of PHB by enzymatic hydrolysis. Pretreatment was performed in two stages: initially, the wheat bran was treated with 1 % NaOH, in a 1:10 ratio, and sterilized by autoclave at 121 °C for 30 min, for lignin removal. For enzymatic hydrolysis, the enzymes cellulase and β-glucosidase were used, with an activity of 185 FPU/mL and 518 CBU/mL, respectively. Incubation was performed at 50 °C, 150 rpm for 96 h for NaOH-treated and untreated wheat bran. A hydrolysis percentage of 83.75 %, a maximum total sugar concentration of 63 g/L at 72 h, and a sugar yield of 629 mg/g of wheat bran were obtained. The aforementioned authors report that sugar production was four times higher for wheat bran treated with NaOH compared to the untreated sample. Similar results were obtained according to Saratale and Oh (2015), who performed alkaline pretreatment using NaOH, potassium hydroxide (KOH) or sodium hypochlorite (NaClO) in rice straw, for the subsequent obtaining of sugars by enzymatic hydrolysis using commercial Trichoderma reesei cellulase. Pretreatment with 2 % NaOH (w/V) at 121 °C per 30 min showed a higher yield of total reducing sugars (703 mg/g of rice straw), with a hydrolysis percentage of 84.19 %.

Table 1 describes the results of certain research related to obtaining sugars from lignocellulosic biomass. According to the result reported in the studies, the saccharification performance depends on the conditions of the pretreatment process of the lignocellulosic biomass, mainly the particle size, the solid-liquid ratio, chemical agent, applied physical method, and saccharification method. Treatment with US with NaOH and hydrothermal requires less than 60 min of pretreatment, compared to treatment with green liquor, which requires approximately 3 h.

Additionally, hydrothermal requires much shorter times (less than 30 min), with the advantage that the concentration of sugars in the final hydrolysate is triple compared to the untreated sample according to the study reported by Amaro; Barreto; Lopes; Edward; Vinicius (2019). As for the saccharification process, it is evident that the method of enzymatic hydrolysis with cellulase is common in all reported studies. Finally, according to Vu *et al.* (2020), pre-treatment technologies are selected considering the cost, efficiency of the method, its level of technological development, and its environmental impact.

Chemical methods are the most widely used, which include acids and alkalines, and are characterized by high efficiency and high level of technological development; however, they are considered expensive and unfriendly for the environment processes. This can be contrasted with hydrothermal pretreatment, which is an alternative and can be explored, because, although its cost ranges between medium and high, it is efficient and has a high level of technological development on a pilot scale, with a reduced environmental impact.

**Table 1.**
*Methods of plant biomass delignification and saccharification*

| Material type and particle size (tp) | Relation | Agent and method of treatment | Saccharification method | Saccharification performance | Reference |
|---|---|---|---|---|---|
| Sugarcane bagasse (*Saccharum officinarum*) 0.27 mm | 1:25 | *Ultrasound:* 2<br>4 kHz<br>Power 400 W Amplitude 100 %<br>*Alkaline treatment:* NaOH 2.89 %<br>T 70 °C (maintained)<br>sonication time of 47.42 min | *Enzymatic hydrolysis*<br>Cellulase: 25 FPU/g dry matter<br>β-glucosidase: 0.46-CBU/g cellulase | Max. concentration of total reducing sugars: 92.11 % | Velmurugan and Muthukumar (2012) |
| Rice straw 0.841 a 0.177 mm | 1:20 | *Ultrasound*:<br>22 kHz<br>Time on: 2 s<br>Time off: 4 s<br>Power: 300 W<br>T 23 °C<br>for 60 min<br>*Alkaline treatment with ultrasound*: NaOH concentration: 0.4 %, 0.6 %, 0.8 % and 1.0 % | Enzymatic hydrolysis<br>Cellulase: 10000 UI/g | Max. concentration of reducing sugars:<br>2.91 g/L | Wu *et al.* (2017) |
| Sugarcane bagasse (*Saccharum officinarum*) 0.27 mm | 1:25 | *Ultrasound:* 2<br>4 kHz<br>Power 400 W Amplitude 100 %<br>*Alkaline treatment:* NaOH 2.89 %<br>T 70 °C (maintained)<br>sonication time of 47.42 min | *Enzymatic hydrolysis*<br>Cellulase: 25 FPU/g dry matter<br>β-glucosidase: 0.46-CBU/g cellulase | Max. concentration of total reducing sugars: 92.11 % | Velmurugan and Muthukumar (2012) |
| Rice straw 0.841 a 0.177 mm | 1:20 | *Ultrasound*:<br>22 kHz<br>Time on: 2 s<br>Time off: 4 s<br>Power: 300 W<br>T 23 °C<br>for 60 min<br>*Alkaline treatment with ultrasound*: NaOH concentration: 0.4 %, 0.6 %, 0.8 % and 1.0 % | Enzymatic hydrolysis<br>Cellulase: 10000 UI/g | Max. concentration of reducing sugars:<br>2.91 g/L | Wu *et al.* (2017) |
| Kenaf (*Hibiscus cannabinus L.*) 0.2 mm | 1:10 | *Pre-treatment with green liquor:*<br>$Na_2CO_3$+$Na_2SO_3$ at 4 % (w/v)<br>T 100° C<br>for 3 h | *Enzymatic hydrolysis*<br>*Trichoderma reesei* cellulase | Saccharification yield: 598.4 mg/g kenaf<br>Hydrolysis performance of 74.8 %<br>Glucose of 74.8 %<br>Biomass recovery: 50 % to 54 % | Ganesh *et al.* (2019) |

| Material type and particle size (tp) | Relation | Agent and method of treatment | Saccharification method | Saccharification performance | Reference |
|---|---|---|---|---|---|
| Unreported tp sugarcane bagasse | 1:3.94 and 1:10 | Batch Reagent: 1.9 L Hydrothermal Solid-water ratio T 183 °C Pressure: 11.7 atm time: 17 min *Alkaline treatment* NaOH 0.2 M Agitation: 2800 rpm for 30 s | Cellic HTec2® Enzymes 15 % Cellic CTec2®) 85 % Enzymatic activity 264.29 FPU/mL. Cellulase: 10 FPU/g β-glucosidase: 52.2 UI/g | Removal of 79 % lignin with combined pretreatment. Enzymatic conversion No pre-treatment: 14.86 % Hydrothermal treatment: 34.55 ± 2.52 % Hydrothermal treatment combined with NaOH: 46.82 ± 2.05 % | Amaro *et al.* (2019) |
| Wheat biomass 0.3-0.4 mm | 10 % w/V | *Ultrasound:* 20 kHz 2.0 W/mL for 60 min *Alkaline treatment:* NaOH 2 % T 100 °C for 30 min | Enzymatic Hydrolysis Trichoderma reesei cellulase, with activity 30 FPU/g of WS | Hydrolysis Performance: 84.5 % Glucose: 90 % Xylose: 65 % | Dattatraya *et al.* (2020) |
| Unreported tp sugarcane bagasse | 1:10 and 1:1 | Batch reactor Hydrothermal T 200 °C Pressure: 15.8 atm time: 10 min Auto hydrolysis T 200 °C Pressure: 15.8 atm time: 10 min | Cellic CTec2 Enzymes + purified thermoset xylanase Enzymatic activity 1000 IU/g | With the hydrothermal method, a higher concentration of sugars was obtained in comparison to the autohydrolysis method | Kaur *et al.* (2020) |
| Rice straw: 2-3 cm | 1:10 | High temperature and thermal pressure reactor with glycerol T 130 °C a 250 °C time: 10, 20, 30 min Hydrothermal T 100, 130 to 210 °C Time: 10, 20, 30 min Alkaline pretreatment NaOH 0.5 % T 100 °C time: 30 min | Cellic CTec2 enzymatic hydrolysis 10 FPU/g (Novozyme) | Pretreatment with glycerol higher yield in reducing sugars. | Gabhane, Kumar and, Sarma (2020) |

Source: own elaboration.

## 2.6. Fermentation conditions and inhibition effects during the PHA synthesis

According to Adeleye *et al.* (2020), three factors must be considered for the production of PHA: the energy used for the sterilization of the fermentation equipment, the production performance of PHA in the substrate, and the efficiency of the extraction of the polymer after fermentation. PHA is a secondary metabolite, so in the fermentation process it is initially required to induce cell growth and in a second phase, the formation of the polymer, in which the limitation of nutrients is required, mainly N, P, and in some, O (Madeiros-Garcia *et al.*, 2020; Koller, 2018; Schmid; Raschbauer; Song; Bauer; Neureiter, 2021). Therefore, the use of interconnected stirred tank reactor blocks is the most explored alternative for the synthesis of PHA (Ganesh *et al.*, 2019; Koller, 2018).

Annamalai and Sivakumar (2016) studied the process of obtaining PHB by varying the C/N ratio (20:1, 20:2, 20:3, 20:4 and 20:5) and the effect of the N source on production yield, using the *R. eutropha* strain (NCIMB 11599). They used ammonium chloride ($NH_4C_l$), ammonium nitrate ($NH_4NO_3$), ammonium sulfate $(NH_4)_2SO_4$, yeast extract, meat extract, peptone, and tryptone, respectively, as sources of inorganic and organic nitrogen. Regarding the nitrogen source, it was found that $(NH_4)_2SO_4$, in a ratio of C/N 20:1, it presented the highest cell growth and the highest accumulation of PHB, using hydrolyzed wheat bran substrates. The study was found, additionally, that cell growth was constant up to 60 h, and the maximum accumulation of PHB was reached at 48 h. Also, more than 90 % of glucose was metabolized during the first 36 h, in contrast to xylose, wherein it was not metabolized. According to Favaro, Basaglia, and Casella (2019), this type of bacteria cannot metabolize pentose, the probable cause thereof is the absence of enzymes necessary for the degradation of these sugars.

Amini, Yousefi-Massumabad, Younesi, Abyar, and Bahramifar (2020) obtained PHB by fermentation, from industrial-grade maltose and brewery wastewater, using the bacterium Cupriavidus necátor (DSMZ 454) as a microorganism. For the synthesis process, an optimization was initially carried out by modifying the C/N/P ratio and the concentration of industrial-grade maltose, in order to establish the maximum biomass yield and volumetric productivity of PHB. A maximum PHB yield of 92 % was obtained, with a ratio of C/N/P 100:2:10 and a maltose concentration of 40 g/L. Subsequently, these results were extrapolated to obtain the polymer with brewery wastewater. Biomass and PHB production were 7.9 and 3.0 g/L. It was found that the C/N/P ratio is a factor that must be determined in order to increase PHB production.

According to Li and Wilkins (2020), nutrient limitation based on N and P induces an increase in the accumulation of PHA in the bacterial strain. Moreover, the limitation of dissolved oxygen during fermentation leads to the production and accumulation of PHB. On the other hand, according to Hermann *et al.* (2013); Nath, Dixit, Bandiya, Chavda and Desai (2008) and Raposo *et al.* (2014), an oxygen concentration of 20 % in the fermentative means increased PHB production. A low concentration of dissolved oxygen promotes the synthesis of PHB; insofar the metabolism of consumption of 5-carbon sugars is induced. Conversely, high values promote only cell growth (Dey; Rangarajan, 2017).

Saratale and Oh (2015) synthesized PHB with the *R. eutropha* strain (ATCC 17699), using rice straw hydrolysates. Initially, they determined the operating conditions of the fermentation process and found that the maximum PHB production was reached at a temperature of 30 °C, in a pH range of 6 to 8 and at a stirring speed of 200 rpm. These parameters were used for the production of PHB under two fermentation conditions with and without supplements. Additionally, they compared PHB production, using commercial sugars (glucose, xylose and arabinose) with wheat straw hydrolysates.

*R. eutropha* produced a higher accumulation of biomass and PHB yield with glucose, 15.10 g/L and 0.53 g PHB/g sugar, respectively, in contrast to xylose, with 8.25 g/L and 0.19 g PHB/g of sugar, and arabinose, with 9.15 g/L and 0.16 g PHB/g of sugar, respectively. On the other hand, it is evident that R. eutropha presents a higher yield, when using pure glucose compared to mixed sugars. Apparently, xylose and arabinose are not essential carbon sources for the metabolism of the strain, which was confirmed in the fermentation carried out with rice straw, whereas after 36 hours of fermentation full consumption of glucose was identified without the use of xylose and arabinose. Regarding the use of supplements, the studies show that corn maceration liquor produced the maximum accumulation of PHA (75.45 %) and PHB production (11.42 g/L), followed by yeast extract, with 71.5 % and 10.26 g/L, respectively. This could be explained by the contribution of amino acids and peptides present in the corn maceration liquor.

Kant *et al.* (2019) studied the production of PHA using hydrolysates of barley, *miscanthus* and pine, with the strain *R. eutropha* (5119), where they evaluated the effect of different concentrations of inhibitors (furfural, hydroxymethylfurfural, vanillin and acetate) on cell growth and PHA production. They noted that vanillin has inhibitory effects on bacterial growth and PHA accumulation in all ranges of concentrations used (0.02 % to 0.1 % w/w). On the other hand, the concentration of acetate, between 0.2 % and 0.1 % w/w favors cell growth and

the accumulation of PHA. Finally, furfural and hydroxymethylfurfural (HMF) have no significant effects on the production and accumulation of PHA up to concentrations of 0.02 % and 0.04 % w/w, respectively.

Additionally, the authors reported that the maximum biomass production and accumulation of PHA was obtained at a glucose ratio: $NH_4Cl$:furfural:HMF:acetate of 1.5:0.05:0.005:0.005:0.25 % w/w, respectively. It was established that the accumulation of PHA is directly related to the source of N, since, at high concentrations, the accumulation of PHA is decreased.

Another effect reported in different studies is related to the concentration of the carbon source, since, at certain concentrations, it can act as an inhibitor. Studies such as Dey and Rangarajan (2017), who evaluated the inhibitory effect of the substrate for the production of PHB, varying the concentration of the source of C (sucrose) and N ($NH_4SO_4$), have found that at sucrose concentrations above 20 g/L, the specific microbial growth rate decreased. The same behavior occurred with $NH_4SO_4$ at concentrations above 2 g/L. High sugar concentrations can probably cause cell lysis (Dey; Rangarajan, 2017) and decreased cell growth or cell starvation (Islam; de Wever; Volcke; Garcia-Gonzalez, 2014).

Likewise, Dattatraya *et al.* (2020) reported that *R. eutropha* (ATCC 17699) has a sugar assimilation of 85 %, bacterial growth of 10.5 g/L, a PHB concentration of 7.1 g/L and PHB yield of 0.417 g/g of reducing sugars for wheat hydrolysates pretreated with US and NaOH. It was reported that *R. eutropha* can metabolize sugars up to a concentration of 30 g/L, with significant bacterial growth and PHB production. At higher concentrations of sugars, the growth of the strain is inhibited, possibly by the presence of five-carbon sugars or inhibitors. The aforementioned authors suggest that, before fermentation, it is important to establish methods of detoxification of hydrolysates to ensure greater assimilation of sugars and achieve greater conversion into PHB.

For the production of PHA it is necessary to reduce production costs. Some alternatives has been proposed such as the search for additives and substitute supplements as a source of nitrogen or precursors, including acetate. Dattatraya *et al.* (2020) studied different sources of nitrogen (organic and inorganic) on the production of PHB, and showed that corn maceration liquor increased sugar consumption (89 %), cell growth (11.45 g/L), PHA accumulation (74 %) and PHB concentration (7.85 g/L). They also reported a possible inhibiting effect on cell growth and PHB production in the presence of $(NH_4)_2SO_4$. Regarding the use of acetate, the results showed that concentrations up to 1 g/L increase cell growth and the accumulation of PHB, because this precursor acts as an intermediate metabolite of the metabolic pathway of PHB production.

Ganesh *et al.* (2019) obtained PHB from kenaf lignocellulosic hydrolysates using the *R. eutropha* strain (ATCC 17699). To evaluate PHB production, they made a comparison of pretreated hydrolysates with $Na_2CO_3$+$Na_2SO_3$ and NaOH+$H_2O_2$, which were treated with $Na_2CO_3$+$Na_2SO_3$. As a result, higher sugar consumption (88 %), higher biomass production and higher PHB accumulation were obtained, compared to those treated with NaOH+$H_2O_2$. Additionally, the effect of PHB production by increasing the concentration of sugars between 20, 39 and 40 g/L compared to the increase in hydrolysate concentration, increased cell growth, accumulation and PHB yield was also studied. At concentrations above 40 g/L, a decrease in PHB production was observed due to the inhibitory effect on the metabolization process exerted by a higher concentration of 5-carbon (5C) sugars (xylose and arabinose) on the bacterium and the increased presence of fermentation inhibitors.

Kerketta and Vasanth (2019) studied fermentation variables such as incubation time, temperature, pH and inoculum size on the production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) using *R. eutropha*. At 48 h, the highest growth and concentration of PHBV were obtained. Similarly, studies indicated that at pH 6, maximum cell growth and PHBV production is obtained. With pH above 6, the accumulation of the polymer was reduced, probably caused by the cellular degrading enzyme, which breaks down the PHA chain. As for the inoculum, they found that at a concentration of 5 % v/v, the maximum amount of polymer and cell

growth is produced. Above this concentration, the production of the polymer is reduced. This can be attributed to the fact that a high population of bacteria leads to competition between them for the use of nutrients and space.

Dey and Rangarajan (2017) studied eleven factors to determine which were the most significant in the synthesis of PHB during the fermentation process using the *C. necator* strain. The parameters studied were sucrose concentration, $MgSO_{4\cdot7}H2O$, $Na_2HPO_4$, $KH_2PO_4$, trace element solution, $(NH_4)_2\ SO_4$, yeast extract, peptone, $CaCl_2$, temperature and stirring speed. It was found that the most significant factors in PHB production were the concentration of peptone, yeast extract, trace metals and $(NH_4)_2SO_4$. These nutrients provide biomolecules (peptides, amino acids, nucleotides, vitamins and trace elements) that directly influence cell growth, the speed of product formation and performance.

Table 2 presents a summary of research work related to obtaining PHA from sugars present in hydrolysates obtained from lignocellulosic biomass. The trend observed in the reported research is the use of the bacterium *Ralstonia eutropha* for the fermentation process.

**Table 2.**
*Fermentation conditions of lignocellulosic hydrolysates for the production of PHA*

| Substrate | Microorganisms Used | Fermentation variables | Yield | Reference |
|---|---|---|---|---|
| Rice straw hydrolysates | Ralstonia eutropha ATCC 17699 (Cupriavidus necator) Inoculum 1 % | Erlenmeyer Reactor 250 mL<br>Volume: 100 mL<br>Carbon source<br>Rice straw hydrolysate: 20 g/L ATR.<br>Means: salt mixture (g/L)<br>$NaH_2PO_4$, 3.6<br>$Na_2HPO_4$, 2.84<br>$K_2SO_4$, 3.486<br>NaOH, 0.4<br>Yeast extract, 0.2<br>MgSO 4-7$H_2O$, 0.39<br>$CaCl_2$, 0.062<br>$(NH_4)_2SO_4$, 0.1<br>CuSO 4-5$H_2O$, 0.005<br>ZnSO 4-7$H_2O$, 0.024<br>MnS0 4-$H_2O$, 0.024<br>Fes 4-7$H_2O$, 0.15<br>Sugar concentration: 5, 10, 20 and 30 g/L<br>pH: 4.0 to 11.0<br>Stirring speed from 50 to 250 rpm.<br>Incubation time: 12 to 96 h<br>T incubation 25 to 40 °C | Increased performance<br>PHB yield (g/L): 11.42<br>Cumulative percentage: 75.45 %<br>Conditions:<br>incubation time 48<br>T 30 °C<br>pH: 7,0<br>Stirring speed 200 rpm | Saratale and Oh (2015) |

| Substrate | Microorganisms Used | Fermentation variables | Yield | Reference |
|---|---|---|---|---|
| Wheat bran hydrolysate | *Ralstonia eutropha* (NCIMB 11599) Inoculum 1 % | C/N ratio: 20<br>Carbon source: Wheat bran hydrolysates (48 g/L)<br>Source of N: $(NH_4)_2SO_4$: 4.8 g/L<br>Means:<br>$KH_2PO_4$ (g/L): 2.4<br>$Na_2HPO_4$ (g/L): 2.5<br>$MgSO_4$ (g/L): 0.5<br>$(NH_4)5[Fe(C_6H_4O_7)^2]$ (g/L): 0.1<br>pH: 6.8<br>Stirring speed: 150 rpm<br>Time: 72 h<br>T 30 °C | PHB (g/L) yield: 24.5<br>Cell growth (g/L): 14.82<br>Cumulative percentage: 62.5 %<br>Sugar concentration residual: 0.319 g/g sugar | Annamalai and Sivakumar (2016) |
| Hydrolysates of kenaf residues (*Hibiscus cannabinus L.*) | *Ralstonia eutropha* (ATCC 17699) Inoculum: 5 % | Reagent: Erlenmeyer 500 mL<br>Volume: 100 mL<br>Carbon source: Hydrolysates of wheat residues (g/L): 20, 30, 40<br>Salt mixture according to Saratale and Oh (2015)<br>pH: 7.0<br>Stirring speed: 200 rpm<br>Incubation time: 36 h<br>T 30 °C | Maximum performance at a hydrolysate concentration of 30 g/L<br>PHB content: 81 %<br>PHB (g/L) yield: 10.09<br>Cumulative percentage: 69.8 %<br>PHB yield (ATR): 0.488 g/g sugar. | Ganesh *et al.* (2019) |
| Miscanthus Hydrolysate (MBH)<br><br>Barley Biomass Hydrolysate (BBH)<br><br>Pine Biomass Hydrolysate (PBH) | *Ralstonia eutropha* (5119) Inoculum: 0.1 % | Reagent: Erlenmeyer 500 mL<br>Volume: 200 mL<br>Carbon source: Hydrolysate (MBH, BBH or LBH): 1.5 %<br>Commercial means of culture M9:<br>$NH_4Cl$ (%): 0.043<br>Furfural (%): 0.004<br>HMF (%): 0.005<br>Acetate (%): 0.234<br>Stirring speed: 200 rpm<br>Incubation time 72 h<br>T 30 °C | MBH<br>Cell growth (g/L): 4.6<br>Cumulative percentage: 44 %<br>BBH<br>Cell growth (g/L): 3.2<br>Cumulative percentage: 54 %<br>PBH<br>Cell growth (g/L): 2.7<br>Accumulation percentage: 63 % | Kant *et al.* (2019) |

| Substrate | Microorganisms Used | Fermentation variables | Yield | Reference |
|---|---|---|---|---|
| Hydrolysates of wheat residues | *Ralstonia eutropha* (ATCC 17699) | Reagent: Erlenmeyer 500 mL<br>Carbon source: Wheat residue hydrolysates (g/L): 30<br>Nitrogen source: corn liquor hydrolysates<br>Salt mixture according to Saratale and Oh (2015)<br>pH: 7.0<br>Stirring speed: 200 rpm<br>Incubation time 36 h<br>T 30 °C | PHB yield (g/L): 7.85<br>Cumulative percentage: 74 %<br>PHB performance (ATR): 0.441 g/g sugar | Dattatraya *et al.* (2020) |
| Corn husk hydrolysates | *Bacillus megaterium* Ti3<br>Inoculum at 2 % | Reagent: Erlenmeyer 250 mL<br>Volume: 100 mL<br>Carbon source: Lignocellulosic hydrolysates:<br>10 g/L ATR.<br>Nitrogen-deficient means (g/L):<br>$MgSO_4$: 0.2 NaCl: 0 4.5<br>Peptone: 2.5<br>Yeast extract: 2.5<br>pH: 7<br>Stirring speed: 120 rpm.<br>Time: 48 h T 30 °C. | PHA yield (g/L): 1<br>Cell growth (g/L): 1.73<br>Cumulative percentage: 57.8 % | De Souza, Manasa, and Shivakumar (2020) |

Source: own elaboration.

## 2.7. Methods of extraction and characterization of PHA

Among the important aspects of the PHA extraction stage is to achieve maximum polymer recovery and reasonable purity with the use of inexpensive chemicals and with largely reducing the generation of hazardous waste. Separation and purification operations must be minimal and inexpensive (Heinrich; Madkour; Al-Ghamdi; Shabbaj; Steinbüchel, 2012). Different methods have been proposed for the PHA extraction, including the extraction with organic solvents, flotation, chemical and enzymatic digestion, use of supercritical fluids and US (Shahid; Razzaq; Farooq; Nazli, 2021).

As for solvent extraction, chloroform, ethanol, and hexane are generally used, achieving a high removal of impurities and residues from the fermentation product. It is a highly polluting method due to the nature and quantity of the chemicals used. Chloroform is used for the flotation method, which has minimal degradation of the polymer and is of lower cost due to the low use of chemical agents. The chemical digestion method uses NaClO or sodium dodecyl sulfate (SDS) combined with chloroform and ethanol. It is useful for the extraction of large volumes and a pure PHA is obtained without degradation.

In SDS enzymatic digestion, EDTA with enzymes (alkalase and lysozyme) is used, and this method features high recovery of the polymer, but the process is expensive and complex. The supercritical fluid method using $CO_2$ is a low-cost process with high yields, although pressure conditions around 350 bar must be obtained for extraction. Finally, with the ultrasound method, a highly pure polymer is obtained. However, the extraction yield is low due to the concentration- solvent cell ratio (Shahid *et al.*, 2021).

Kant *et al.* (2019) started by centrifuging the solid sample, suspending it in water and drying it by lyophilization. Finally, the extraction was performed with chloroform and sodium hypochlorite at 30 % 1:1 ratio, allowed it to act for 2.5 h, and then centrifuged again. They recovered the chloroform phase and the polymer was precipitated using 80 % methanol. The precipitate was dried at room temperature and characterized using Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), and thermogravimetric analysis (TGA). Isak *et al.* (2016) studied a model of quantification of PHA mixtures (pure and mixed) by FTIR. They used the results of the spectra to determine the PHA concentration in the sample, based on the heights and band areas of the main groups: carbonyl (C = O), amide (N-H), protein (C-O-C and C-O); the results were compared with the data obtained by the gas chromatography method.

Dey and Rangarajan (2017) performed PHB extraction using the surfactant method of dodecyl sulfate sodium (SDS) and sodium hypochlorite, considered the most efficient and lowest cost method. They obtained an extraction yield of 71 % and a PHB purity of 98 %. Alternative methods have been proposed such as the one reported by Arikawa, Sato, Fujiki, and Matsumoto (2017), who evaluated the PHA extraction using US combined with different concentrations of SDS. The purity of the PHA reached 96 %, using an SDS concentration of 3.3 %. According to the results obtained, the authors assert that this method can be applied on an industrial scale, because no initial centrifugation or use of organic solvents is required, and it could be applied with frozen or dried cells suspended in water, adapted to a sonicator. Table 3 presents the PHA extraction and characterization methods used in the different studies.

Finally, it is important to establish how to increase the efficiency of the PHA extraction process focused on reducing costs, energy, and environmental impact. Adeleye *et al.* (2020) carried out an analysis of the environmental impact and cost of the extraction of PHA after fermentation, where the alkaline treatment is the most favorable in terms of costs, global warming potential, and use of non-renewable energy with 1.40 €/ kg PHB, 2.4 kg $CO_2$-eq/kg PHB and 106 MJ/kg PHB, respectively; compared to the extraction process using solvents whose reported values for the same variables were €1.95/kg PHB, 4.30 kg $CO_2$-eq/kg PHB and 156 MJ/kg PHB. However, the overall costs of the process remain high compared to petroleum-derived polymers. Bioplastics are 2.5 to 7.5 times more expensive than major traditional petroleum-based plastics. It is noteworthy that a few years ago bioplastics were between 35 and 100 times more expensive than existing equivalents based on non-renewable fossil fuels.

**Table 3.**
*PHA Extraction and characterization*

| Substrate | Microorganisms Used | Fermentation variables | Yield | Reference |
|---|---|---|---|---|
| PHB | Extraction:<br>Digestion:<br>30 % mixture sodium chloroform and hypochlorite in 1:1 v/v ratio<br>Precipitation: 80 % methanol. | Crystallinity<br><br>Thermogravimetric analysis (TGA)<br><br>Differential Scanning Calorimetry (DSC)<br><br>FTIR Analysis<br><br>Raman Analysis<br><br>SEM Microscopy<br><br>X-ray spectroscopy | **TGA Analysis**<br>Td: 283.5 °C<br><br>**DSC Analysis**<br>Vitreous transition temperature (Tg): 10 °C<br>Tm: 172 °C<br><br>**FTIR Analysis**<br>Wavelength (cm-1):<br>2910-3300 vibration C H,<br>1721 stretch C = O,<br>1379 groups $CH_3$,<br>1455 asymmetric deformation $CH_3$.<br>1120-1280 stretching C-O-C,<br>1043 stretching of the -CO of the ester group and 3444 by the OH water adsorption groups.<br><br>**X-ray spectroscopy**<br>C: 60.94 %<br>Or: 39.5 % | Saratale and Oh (2015) |

| Substrate | Microorganisms Used | Fermentation variables | Yield | Reference |
|---|---|---|---|---|
| P (3HB-co-3HV) | Extraction: Initial centrifugation followed by 0.1 % sodium dodecylsulfate (SDS) treatment | Differential Scanning Calorimetry (DSC) | **DSC Analysis**<br>Transition temperature (Tg): 2.6 °C<br>T crystallization (Tc): 65.4 °C<br>Tm1 140.1 °C<br>Tm2 154.4 °C | Alsafadi and Al-Mashaqbeh (2017) |
| | Cell lysis using vortex. | FTIR Analysis | **FTIR Analysis**<br>Wavelength:<br>1730 stretching of the carbonyl group (C = O) of the ester, 2924 and 2858 bonds of groups $CH_3$ and $CH_2$ respectively and 1450-1000 corresponds to the stretching of C-O, C-C and C-O-C. | |
| | Incubation for 3 h at 25 °C. Centrifugation and incubation with 3 % sodium hypochlorite for 30 min at 25 °C.<br><br>Final centrifugation and washing with water and ethanol. 10 ml of chloroform were added, the result was evaporated which produced a film. | PHA content and purity by gas chromatography. Standard: 8 % poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) in Sigma-Aldrich moles Internal standard: Benzoic acid Column: Agilent DB 23, 60mx0.25mm, 0.15µm. | PHA content and purity by gas chromatography polymer composed of 6.5 % in moles of 3-hydroxyvalarate (3HV). | |
| PHB | Extraction: Digestion mixture of 30 % sodium chloroform and hypochlorite in 1:1 v/v ratio | Crystallinity<br><br>Thermogravimetric analysis (TGA) | **Crystallinity**<br>peaks at 2 θ = 13.4; 16.8; 22.4; 25.3 and 27.1<br><br>**TGA Analysis**<br>Decomposition temperature (Td): 292.89 °C | Ganesh *et al.* (2019) |
| | Precipitation: 80 % methanol" | Differential Scanning Calorimetry (DSC)" | **DSC Analysis**<br>Melting temperature (Tm): 171.65 °C | |

| Substrate | Microorganisms Used | Fermentation variables | Yield | Reference |
|---|---|---|---|---|
| P(3HB-co-3HV) | Extraction: digestion mixture chloroform and 30 % sodium hypochlorite in 1:1 v/v ratio<br><br>Precipitation: 80 % methanol. | Thermogravimetric analysis (TGA)<br><br>Differential Scanning Calorimetry (DSC)<br><br>FTIR Analysis | TGA Analysis<br>Td: 299.24 °C<br><br>DSC Analysis<br>Tm1 116.9 °C and Tm2 175 °C<br><br>FTIR Analysis<br>Wavelength (cm-1): 3000 vibration C-H,<br>1720 group C = O,<br>1000 and 1300 correspond to C-O, of the ester bonds and<br>3386 presence of the -OH group. | Kant *et al.* (2019) |
| PHB | Extraction: digestion mixture chloroform and 30 % sodium hypochlorite in 1:1 v/v ratio<br><br>Precipitation: 80 % methanol. | Crystallinity<br><br>Thermogravimetric analysis (TGA)<br><br>Differential Scanning Calorimetry (DSC)<br><br>FTIR Analysis<br><br>Chemical structure nuclear magnetic resonance (NMR) | **Crystallinity**<br>Peaks at 2 θ = 13.3; 16.4; 22.4 and 25.4 and small peaks at 26.9 and 30.0°.<br><br>**TGA Analysis**<br>Td: 291.5 °C<br><br>**DSC Analysis**<br>Tm: 175.4 °C<br><br>**FTIR Analysis**<br>Wavelength (cm-1):<br>3450 corresponds to the bending of the OH,<br>2974 and 2933 CH stretch,<br>acute peak 1720 stretch C = O (carbonyl) and -COOR (ester),<br>1454 and 1379 stretch $CH_3$ and $CH_2$,<br>1456 and 1378 flexion of the asymmetric and symmetrical CH bond in $CH_2$<br>1378 for $CH_3$,<br>those of 1277 at 1049 bonds of carbonyl (COC) and CO.<br><br>**NMR analysis**<br>Functional groups present -$CH_3$, $CH_2$ -CH-, hydroxybutyrate (HB), -$CH_2$ - of the HB unit, -$CH_3$ of the HB unit and the ester group (O-CH-) carbonyl (-C-) of HB. | Saratale *et al.* (2020) |

Source: own elaboration.

### 2.8. Determination of biodegradability

PHAs are considered biodegradable materials due to the hydrolysis of the polymer by the PHA depolymerase enzymes and lipases, which act on the chiral center of the monomer in the R configuration. The aforementioned enzymes act intra and extracellularly, allowing the degradation of PHA in environments with high microbial activity. The way of degradation begins with the growth of microorganisms on the surface of the polymer and enzymes secretion resulting at the end in water-soluble oligomers metabolized by microorganisms (Utsunomia; Ren; Zinn, 2020). The factors that affect the PHA degradation include molecular weight, molecular structure and content of the comonomer unit, crystallinity, porosity, pH and temperature. Furthermore, the presence of catalytic molecules, enzymes, and alkaline substances in the means produces the degradation of the polymer through a mechanism of surface erosion (Sultana; Khan, 2012).

Sultana and Khan (2012) studied the degradation of polyhydroxybutyrate-co-hydroxyvalerate (PHBV), using ASTM F 1635-04a. Among the results found, the authors reported that the molecular weight decreased by approximately 47 % in 44 weeks. Likewise, crystallinity increased by approximately 8 %, due to the hydrolysis of the amorphous structure, a weight loss of approximately 10 % was also evidenced, which is greater in the polymeric structure with greater porosity. Finally, there was a decrease of about 40 % in mechanical properties after 20 weeks at 20 °C.

## 3. Conclusions

Hydrothermal and ultrasound methods are promising alternatives for the pretreatment of lignocellulosic biomass due to the reduction of process time, ease of operation, and reduction of the formation of inhibitory by-products.

It is important to highlight that the different investigations carried out, indicate that lignocellulosic biomass is an alternative for the synthesis of PHA, considering the amount and diversity available in the different sectors of the economy.

In the results obtained in laboratory-scale investigations, it evidences that in the saccharification process hydrolysis yields above 70 % are achieved up to a maximum of 92 %, and during fermentation, a percentage of PHA accumulation is obtained ranging from 44 % to 74 %. Therefore, it is necessary to continue these studies on a larger scale, to evaluate nutritional variables, alternative sources and cost-efficient obtaining methods.

# References

Acosta, Alejandro; Alcaraz, Wilman; Cardona, Mariana (2018). Sugarcane molasses and vinasse as a substrate for polyhydroxyalkanoates (PHA) production. *Revista Dyna, 85*(206), 220-225. https://doi.org/10.15446/dyna.v85n206.68279

Adeleye, Aderemi; Kenneth, Chuks; Christian, Obieze; Oluwabunmi, Oluwakemi; Oludare, Osigbeminiyi; Toluwalope, Emmanuel; Louis, Hitler (2020). Sustainable synthesis and applications of polyhydroxyalkanoates (PHAs) from biomass. *Process Biochemistry, 96*(marzo), 174-193. https://doi.org/10.1016/j.procbio.2020.05.032

Alsafadi, Diya; Al-Mashaqbeh, Othman (2017). A one-stage cultivation process for the production of poly-3-(hydroxybutyrate- co - hydroxyvalerate) from olive mill wastewater by Haloferax mediterranei. *New Biotechnology, 34*, 47-53. https://doi.org/10.1016/j.nbt.2016.05.003

Amaro, Gustavo; Barreto, Elisa; Lopes, Rogelio; Eduardo, Bruno; Vinícius, Leandro (2019). Fractionation of sugarcane bagasse using hydrothermal and advanced oxidative pretreatments for bioethanol and biogas production in lignocellulose biorefineries. *Bioresource Technology, 292*(agosto), 121963. https://doi.org/10.1016/j.biortech.2019.121963

Amini, Malihe; Yousefi-Massumabad, Hassan; Younesi, Habibollah; Abyar, Hajar; Bahramifar, Nader (2020). Production of the polyhydroxyalkanoate biopolymer by Cupriavidus necator using beer brewery wastewater containing maltose as a primary carbon source. *Journal of Environmental Chemical Engineering, 8*(1), 1-9. https://doi.org/10.1016/j.jece.2019.103588

Anjum, Anbreen; Zuber, Mohammad; Zia, Khalid; Noreen, Aqdas; Anjum, Muhammad; Tabasum, Shazia (2016). Microbial production of polyhydroxyalkanoates (PHAs) and its copolymers: A review of recent advancements. *International Journal of Biological Macromolecules, 89*, 161-174. https://doi.org/10.1016/j.ijbiomac.2016.04.069

Annamalai, Neelamegam; Sivakumar, Nallusamy (2016). Production of polyhydroxybutyrate from wheat bran hydrolysate using *Ralstonia eutropha* through microbial fermentation. *Journal of Biotechnology, 237*, 13-17. https://doi.org/10.1016/j.jbiotec.2016.09.001

Arikawa, Hisashi; Sato, Shunsuke; Fujiki, Tetsuya; Matsumoto, Keiji (2017). Simple and rapid method for isolation and quantitation of polyhydroxyalkanoate by SDS-sonication treatment. *Journal of Bioscience and Bioengineering, 124*(2), 250-254. https://doi.org/10.1016/j.jbiosc.2017.03.003

Capolupo, Laura; Faraco, Vicenza (2016). Green methods of lignocellulose pretreatment for biorefinery development. *Applied Microbiology and Biotechnology, 100*, 9451-9467. https://doi.org/10.1007/s00253-016-7884-y

Chanprateep, Suchada; Katakura, Yoshio; Visetkoop, Sirirat; Shimizu, Hiroshi; Kulpreecha, Songsri; Shioya, Suteaki (2008). Characterization of new isolated *Ralstonia eutropha* strain A-04 and kinetic study of biodegradable copolyester poly(3-hydroxybutyrate-co-4-hydroxybutyrate) production. *Journal of Industrial Microbiology and Biotechnology, 35*(11), 1205-1215. https://doi.org/10.1007/s10295-008-0427-5

Dattatraya, Ganesh; Ganesh, Rijuta; Varjani, Sunita; Cho, Si-Kyung; Ghodake, Gajanan; Kadam, Avinash; Mulla, Sikandar; Naresh, Ram; Kim, Dong-Su; Seung, Han (2020). Development of ultrasound aided chemical pretreatment methods to enrich saccharifi cation of wheat waste biomass for polyhydroxybutyrate production and its characterization. *Industrial Crops & Products, 150*, 1-13. https://doi.org/10.1016/j.indcrop.2020.112425

De Bhowmick, Goldy; Sarmah, Ajit; Sen, Ramkrishna (2018). Lignocellulosic biorefinery as a model for sustainable development of biofuels and value added products. *Bioresource Technology, 247*, 1144-1154. https://doi.org/10.1016/j.biortech.2017.09.163

De Souza, Larissa; Manasa, Y.; Shivakumar, Srividya (2020). Biocatalysis and Agricultural Biotechnology Bioconversion of lignocellulosic substrates for the production of polyhydroxyalkanoates. *Biocatalysis and Agricultural Biotechnology, 28*(julio), 101754. https://doi.org/10.1016/j.bcab.2020.101754

Dey, Pinaki; Rangarajan, Vivek (2017). Improved fed-batch production of high-purity PHB ( poly-3 hydroxy butyrate ) by *Cupriavidus necator* ( MTCC 1472 ) from sucrose-based cheap substrates under response surface- optimized conditions. *3 Biotech, 310*(7), 1-14. https://doi.org/10.1007/s13205-017-0948-6

Favaro, Lorenzo; Basaglia, Marina; Casella, Sergio (2019). Improving polyhydroxyalkanoate production from inexpensive carbon sources by genetic approaches: a review. *Biofuels, Bioproducts and Biorefining, 13*(1), 208-227. https://doi.org/10.1002/bbb.1944

Gabhane, Jagdish; Kumar, Sachin; Sarma, A. K. (2020). Effect of glycerol thermal and hydrothermal pretreatments on lignin degradation and enzymatic hydrolysis in paddy straw. *Renewable Energy, 154*, 1304-1313. https://doi.org/10.1016/j.renene.2020.03.035

Galbe, Mats; Wallberg, Ola (2019). Pretreatment for biorefineries: a review of common methods for efficient utilisation of lignocellulosic materials. *Biotechnology for Biofuels, 12*(294), 1-26. https://doi.org/10.1186/s13068-019-1634-1

Ganesh, Rijuta; Dattatraya, Ganesh; Kyung, SI; Su, Dong; Ghodake, Gajanan; Kadam, Avinash; Kumar, Gopalakrishanan; Naresh, Ram (2019). Pretreatment of kenaf (*Hibiscus cannabinus L* .) biomass feedstock for polyhydroxybutyrate (PHB) production and characterization. *Bioresource Technology, 282*, 75-80. https://doi.org/10.1016/j.biortech.2019.02.083

García, L.; Novoa, J.; Franco, A.; Higuita, L. (2015). Estudio de la síntesis de biopolímeros de origen microbiano. *Revista QUID,* 25, 69-78.

González, Y.; Meza, J.; González, O.; Córdoba, J. (2013). Síntesis y biodegradación de polihidroxialcanoatos: plásticos de origen microbiano. *Revista Internacional de Contaminación Ambiental, 29*(1), 77-115.

Govil, Tanvi; Wang, Jia; Samanta, Dipayan; David, Aditi;Tripathi, Abhilash; Rauniyar, Shailabh; Salem, David; Sani, Rajesh (2020). Lignocellulosic feedstock: A review of a sustainable platform for cleaner production of nature's plastics. *Journal of Cleaner Production, 270,* 1-19. https://doi.org/10.1016/j.jclepro.2020.122521

Heinrich, Daniel; Madkour, Mohamed; Al-Ghamdi, Mansour; Shabbaj, Ibraheem; Steinbüchel, Alexander (2012). Large scale extraction of poly ( 3-hydroxybutyrate ) from *Ralstonia eutropha* H16 using sodium hypochlorite. *AMB Express, 2*(59), 1-6. https://doi.org/10.1186/2191-0855-2-59

Hermann, Carmen; Koller, Martin; Muhr, Alexander; Fasl, Hubert; Stelzer, Franz; Braunegg, Gerhart (2013). Archaeal Production of Polyhydroxyalkanoate (PHA) Co- and Terpolyesters from Biodiesel Industry-Derived By-Products. *Hindawi, 2013,* 1-10. https://doi.org/10.1155/2013/129268

Isak, I.; Patel, M.; Riddell, M.; West, M.; Bowers, T.; Wijeyekoon, S.; Lloyd, J. (2016). Quantification of polyhydroxyalkanoates in mixed and pure cultures biomass by Fourier transform infrared spectroscopy : comparison of different approaches. *Letters in Applied Microbiology, 63*(2), 139-146. https://doi.org/10.1111/lam.12605

Islam, Salatul; de Wever, Heileen; Volcke, Eveline; Garcia-Gonzalez, Linsey (2014). A robust fed-batch feeding strategy independent of the carbon source for optimal polyhydroxybutyrate production. *Process Biochemistry, 49*(3), 365-373. https://doi.org/10.1016/j.procbio.2013.12.004

Jonsson, Leif; Alriksson, Björn; Nilvebrant, Nils-Olof (2013). Bioconversion of lignocellulose: inhibitors and detoxification. *Biotechnology for Biofuels, 6*(16), 1-10. https://doi.org/10.1186/1754-6834-6-16

Kant, Shashi; Gurav, Ranjit; Choi, Tae-Rim; Jung, Hye-Rim; Yang, Yun-Hun (2019). Bioconversion of plant biomass hydrolysate into bioplastic (polyhydroxyalkanoates) using Ralstonia eutropha 5119. *Bioresource Technology, 271*, 306-315. https://doi.org/10.1016/j.biortech.2018.09.122

Kaur, Kamalpreet; Espirito, Melissa; Pellegrini, Vanessa; de Azevedo, Eduardo; Guimaraes, Francisco; Polikarpov, Igor (2020). Biomass and Bioenergy Enhanced hydrolysis of hydrothermally and autohydrolytically treated sugarcane bagasse and understanding the structural changes leading to improved saccharification. *Biomass and Bioenergy, 139,* 105639. https://doi.org/10.1016/j.biombioe.2020.105639

Kerketta, Ankush; Vasanth, D. (2019). Madhuca indica flower extract as cheaper carbon source for production of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) using *Ralstonia eutropha*. *Process Biochemistry, 87*, 1-9. https://doi.org/10.1016/j.procbio.2019.09.013

Koller, Martin (2018). A review on established and emerging fermentation schemes for microbial production of polyhydroxyalkanoate (PHA) biopolyesters. *Fermentation, 4*(20), 1-30. https://doi.org/10.3390/fermentation4020030

Kumar, Manish; Rathour, Rashmi; Singh, Rashmi; Sun, Yaquing; Pandey, Ashok; Gnansounou, Edgard; Lin, Kun-Yi; Tsang, Daniel; Shekhar, Indu (2020). Bacterial polyhydroxyalkanoates: Opportunities, challenges, and prospects. *Journal of Cleaner Production, 263*, 1-20. https://doi.org/10.1016/j.jclepro.2020.121500

Li, Mengxing; Wilkins, Mark (2020). Recent advances in polyhydroxyalkanoate production : Feedstocks , strains and process developments. *International Journal of Biological Macromolecules, 156*, 691-703. https://doi.org/10.1016/j.ijbiomac.2020.04.082

Liu, C.; Zhang, L.; An, J.; Chen, B.; Yang, H. (2015). Recent strategies for efficient production of polyhydroxyalkanoates by micro-organisms. *Letters in Applied Microbiology, 1558*, 9-15. https://doi.org/10.1111/lam.12511

López, L.; Acosta, A.; Zambrano, R. (2013). Evaluación de pretratamientos químicos para la hidrólisis enzimática de residuos lignocelulósicos de yuca (Manihot esculenta Crantz). *Revista Facultad Ingenieria Universidad de Antioquia, 69*, 317-326.

Madeiros-Garcia, João; Distante, Franceso; Storti, Giuseppe; Moscatelli, Davide; Morbidelli, Massimo; Sponchioni, Mattia (2020). Current trends in the production of biodegradable bioplastics: The case of polyhydroxyalkanoates. *Biotechnology Advances, 42*, 1-9. https://doi.org/10.1016/j.biotechadv.2020.107582

Nath, A.; Dixit, M.; Bandiya, A.; Chavda, S.; Desai, A. J. (2008). Enhanced PHB production and scale up studies using cheese whey in fed batch culture of *Methylobacterium* sp. ZP24. 99. *Bioresource Technology, 99*(13), 5749-5755. https://doi.org/10.1016/j.biortech.2007.10.017

Pakalapati, Harshini; Chang, Chi-Kai; Show, Pau; Arumugasamy, Senthil; Lan, John (2018). Development of polyhydroxyalkanoates production from waste feedstocks and applications. *Journal of Bioscience and Bioengineering, 126*(3), 282-292. https://doi.org/10.1016/j.jbiosc.2018.03.016

Plastic Market Size (2020). *Plastics Polymers & Resins.* https://www.fortunebusinessinsights.com/plastics-market-102176

Ranganathan, Saranya; Dutta, Sayantani; Moses, J. A.; Anandharamakrishnan, C. (2020). Utilization of food waste streams for the production of biopolymers. *Heliyon, 6*(9), 1-13. https://doi.org/10.1016/j.heliyon.2020.e04891

Raposo, Rodrigo; Cesário,Teresa; Almeida, Catarina; van Keulen, Frederik; Ferreira, Bruno; de Fonseca, Manuela R. (2014). Enhanced bioproduction of poly-3-hydroxybutyrate from wheat straw lignocellulosic hydrolysates. *New Biotechnology, 31*(1), 104-113. https://doi.org/10.1016/j.nbt.2013.10.004

Raza, Zulfiqar; Abid, Sharjeel; Banat, Ibrahim (2018). Polyhydroxyalkanoates: Characteristics, production, recent developments and applications. *International Biodeterioration and Biodegradation, 126*, 45-56. https://doi.org/10.1016/j.ibiod.2017.10.001

Report European Bioplastic (2017). *Bioplastic market data 2017.* http://docs.european-bioplastics.org/publications/market_data/2017/Report_Bioplastics_Market_Data_2017.pdf

Rojas, E.; Hoyos, J.; Mosquera, S. (2016). Producción de polihidroxialcanoatos (PHAs) a partir de *Ralstonia eutropha* en un medio con harina de yuca como fuente de carbono. *Biotecnología en el Sector Agripecuario y Agroindustrial, 14*(1), 19-26.

Ruiz, Héctor; Rodríguez, Rosa; Fernandes, Bruno; Vicente, António; Teixeira, José (2013). Hydrothermal processing , as an alternative for upgrading agriculture residues and marine biomass according to the biorefinery concept : A review. *Renewable and Sustainable Energy Reviews, 21,* 35-51. https://doi.org/10.1016/j.rser.2012.11.069

Ruiz, Héctor; Conrad, Marc; Sun, Shao-Ni; Sánchez, Arturo; Rocha, George; Romaní, Aloia; Castro, Eulogio; Torres, Ana; ... Meyer, Anne (2020). Engineering aspects of hydrothermal pretreatment : From batch to continuous operation , scale-up and pilot reactor under biorefinery concept. *Bioresource Technology, 299*, 1-16. https://doi.org/10.1016/j.biortech.2019.122685

Sabapathy, Poorna; Devaraj, Sabarinathan; Meixner, Katharina; Anburajan, Parthiban; Kathirvel, Preethi; Ravikumar, Yuvaraj; Zabed, Hossain; Qi, Xianghui (2020). Recent developments in Polyhydroxyalkanoates (PHAs) production - A review. *Bioresource Technology, 306,* 1-14. https://doi.org/10.1016/j.biortech.2020.123132

Saratale, Ganesh; Oh, Min-Kyu (2015). Characterization of poly-3-hydroxybutyrate (PHB) produced from Ralstonia eutropha using an alkali-pretreated biomass feedstock. *International Journal of Biological Macromolecules, 80,* 627-635. https://doi.org/10.1016/j.ijbiomac.2015.07.034

Saratale, Ganesh; Saratale, Rijuta; Varjani, Sunita; Cho, Si-Kyung; Ghodake, Gajanan; Kadam, Avinash; Mulla, Sikandar; ...Shin, Han (2020). Development of ultrasound aided chemical pretreatment methods to enrich saccharification of wheat waste biomass for polyhydroxybutyrate production and its characterization. *Industrial Crops and Products, 150,* 1-13. https://doi.org/10.1016/j.indcrop.2020.112425

Schmid, Maximilian; Raschbauer, Michaela; Song, Hyunjeong ; Bauer, Cornelia; Neureiter, Markus (2021). Effects of nutrient and oxygen limitation, salinity and type of salt on the accumulation of poly(3-hydroxybutyrate) in *Bacillus megaterium* uyuni S29 with sucrose as a carbon source. *New Biotechnology, 61,* 137-144. https://doi.org/10.1016/j.nbt.2020.11.012

Shahid, Salma; Razzaq, Sadia; Farooq, Robina; Nazli, Zill-i-Huma (2021). Polyhydroxyalkanoates: Next generation natural biomolecules and a solution for the world's future economy. *International Journal of Biological Macromolecules, 166,* 297-321. https://doi.org/10.1016/j.ijbiomac.2020.10.187

Sultana, Naznin; Khan, Tareef (2012). In vitro degradation of PHBV scaffolds and nHA/PHBV composite scaffolds containing hydroxyapatite nanoparticles for bone tissue engineering. *Journal of Nanomaterials, 2012,* 1-12. https://doi.org/10.1155/2012/190950

Utsunomia, Camila; Ren, Qun; Zinn, Manfred (2020). Poly(4-Hydroxybutyrate): Current State and Perspectives. *Frontiers in Bioengineering and Biotechnology, 8*(257), 1-20. https://doi.org/10.3389/fbioe.2020.00257

Velmurugan, Rajendran; Muthukumar, Karuppan (2012). Ultrasound-assisted alkaline pretreatment of sugarcane bagasse for fermentable sugar production : Optimization through response surface methodology. *Bioresource Technology, 112,* 293-299. https://doi.org/10.1016/j.biortech.2012.01.168

Vu, Hang; Nguyen, Luong; Vu, Mihn; Johir, Abu; McLaughlan, Robert; Nghiem, Long (2020). A comprehensive review on the framework to valorise lignocellulosic biomass as biorefinery feedstocks. *Science of the Total Environment, 743*, 1-16. https://doi.org/10.1016/j.scitotenv.2020.140630

Wu, Han; Dai, Xiao; Zhou, Si-Li; Gan, Yu-Yan; Xiong, Zi-Yao; Qin, Yuan-Hang; Ma, Jiayu; …Wang, Cun-Wen (2017). Ultrasound-assisted alkaline pretreatment for enhancing the enzymatic hydrolysis of rice straw by using the heat energy dissipated from ultrasonication. *Bioresource Technology, 241*, 70-74. https://doi.org/10.1016/j.biortech.2017.05.090

Yang, Bin; Tao, Ling; Wayman, Charles (2018). Strengths, challenges, and opportunities for hydrothermal pretreatment in lignocellulosic biorefineries. *Biofuels, Bioproducts and Biorefining, 12,* 125-138. https://doi.org/10.1002/bbb.1825

Yu, Jian; Stahl, Heiko (2008). Microbial utilization and biopolyester synthesis of bagasse hydrolysates. *Bioresource Technology, 99*(17), 8042-8048. https://doi.org/10.1016/j.biortech.2008.03.071