CHAPTER 1: STATE OF THE ART

1.1. Overview: circular economy and climate change

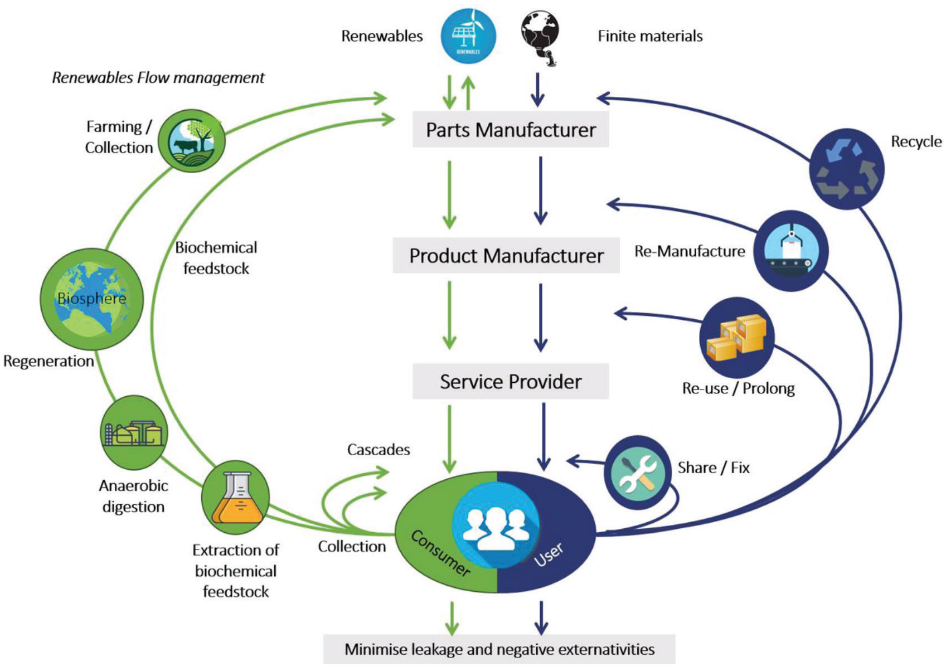
Climate change is the greatest challenge facing our society in this century. Anthropogenic emissions of Greenhouse Gases (GHG) have led to an increase in atmospheric CO2 by 47% above pre-industrial levels, which is higher than the natural increase over the past 20,000 years (NASA, 2021a). The increase of the CO2 concentration in the atmosphere contributes to the greenhouse effect, altering the planet's climate and ecosystems and increasing the temperature during the last decades by more than 1°C (NASA, 2021b). Therefore, the international environmental policies are seeking a universal agreement to keep global warming below a critical threshold, that is, to limit temperature rise in the next decade to 1.5 °C (IPCC, 2018), thus avoiding a biosphere breakdown. This objective entails a radical reduction of emissions through the decarbonization of the production model, migrating to a zero-emission economy. Emission reduction policies generally focus on decreasing GHG generation from energy production, but they should also limit the number of raw materials used in manufacturing since they account for 45% of total GHG emissions (Ellen MacArthur Foundation, 2019; IRP, 2019). In a context of a growing population, increasing demand for scarce resources, and climate burden, changing our current "make - use - discard" production model to one based on the precepts of the circular economy is imperative.

A transition to a circular economy requires a drastic shift in our approach to the production and consumption of products. The circular economy concept is based on optimizing manufacturing through product reuse, life extension, and recycling loops of its components (Kalmykova *et al.*, 2018). Figure 1.1 shows a diagram of the resource flows in a circular economy, illustrating the potential for extending the life of both renewable flows and finite resources while minimizing losses or negative externalities to a minimum. Improving resource efficiency and material reduction should be an essential element of climate policy (Rizos *et al.*, 2019). According to a Club of Rome's report, estimating the effects of a shift to a circular economy in 5 EU countries (Finland, France, the Netherlands, Spain, and Sweden) could lead to a reduction of CO2 emissions of up to 75% (Wijkman and Skånberg, 2016). For example, applying this production model to the food industry could reduce emissions by 49% (Ellen MacArthur Foundation, 2019). Another main driver of the circular economy is reducing and recycling waste, whereby the European Commission's Waste Package estimated that it could reduce 443 million tons of GHG between 2014 and 2030 (European Commission, 2014). Conservation and closed loops in resource value chains are implicit in the circular economy. Resources from one sector are converted into raw materials in another sector, attempting to maintain the quality and status of the resources or even upgrading them (upcycling) (Korley *et al.*, 2021). The increasing urban expansion of industrialized countries has led to a steady increase in Municipal Solid Waste (MSW) generation, which is a complex problem for our society but can be turned into valuable resources due to the scarcity and high prices of certain raw materials.

1.2. Municipal Solid Waste in a Circular Economy

The management of MSW challenges the EU as a critical element to realize a circular economy. The EU 27 has produced 505 kg of municipal waste per capita in 2020 (Eurostat, 2020). Therefore, it is essential to reduce the percentage of MSW deposited in landfills and increase waste recycling. However, the reduction of landfilling was only 58% in 2020 compared to 1995 but this value must be lower than 10% (Figure 1.2). On the other hand, 27% of MSW is managed by incineration (no distinction is made between incineration and incineration with energy recovery) and 18% by composting. Only 30% of MSW is being recycled, far from the EU target of 55% by weight settled by 2025. In the Waste Framework Directive and the Landfill Directive, high emphasis

Figure 1.1. Resource flows through a value chain in a circular economy Adapted from Ellen MacArthur Foundation, (2019).



is placed on the **organic fraction of municipal solid waste (OFMSW)**, which is a key element in the planning of the sustainable MSW management system (Council Directive 1999/31/EC on the Landfill of Waste, 1999). OFMSW must be collected separately from 2023 onwards, a major step in the establishment of a circular bioeconomy in the EU (Sherwood, 2020).

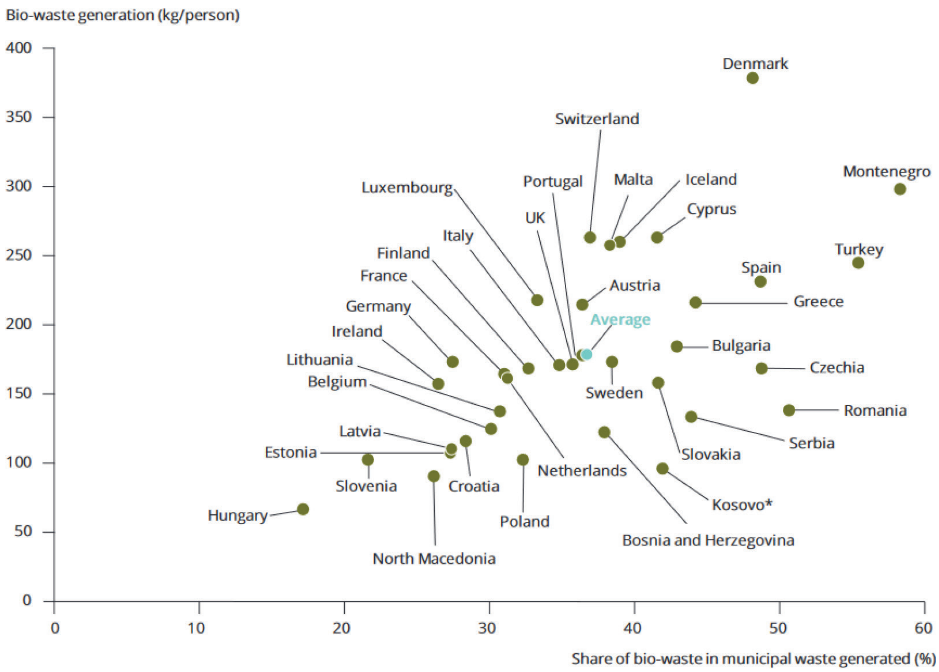
The OFMSW includes food and kitchen waste from households, restaurants, supermarkets, food processing plants, and garden and park waste. Depending on their origin, they may contain different proportions of readily biodegradable simple organic matter like sugars, starch, proteins, and lipids, more recalcitrant compounds like lignocellulosic matter, or unwanted fractions like plastic packaging or inorganic materials (Moretti *et al.*, 2020). Biowaste percentage

Figure 1.2. EU legislation targets MSW management. Source: (Council Directive 1999/31/EC on the Landfill of Waste, 1999; EC. Com. 397, 2014; European Commission, 2020; Eurostat, 2020)

in the overall MSW varies significantly among European countries (Figure 1.3), averaging 37% (EEA, 2020). These variations rely on the urbanization level and the country-specific data collection and reporting system. For example, in Spain the percentage is higher, being in the range of 40 to 60% of the total MSW (MAGRAMA, 2015). In a circular economy, organic waste is directed to treatment options that use the waste as a source of valuable resources such as nutrients, organic substances, and energy.

The separated collection of the OFMSW is beneficial when valorizing the organic matter adequately. The two most common treatments for OFMSW are composting and anaerobic digestion (AD). Composting generates a humic product through aerobic oxidation that can be used as a fertilizer or soil improver, while in AD anaerobic consortia conduct a series of syntrophic chemical reactions that produce a digestate and biogas that can be used to generate electricity or heat. In any case, both alternatives are considered low value and do not take advantage of the high potential of this resource. However, the EU has defined a treatment hierarchy that prioritizes the valorization of organic wastes into biomaterials rather than compost or energy (Figure 1.4), thus defining the scope of the biorefineries for OFMSW

Figure 1.3. OFMSW generation per person and share of bio-waste in MSW generated by country, 2017. Source: (EEA, 2020).



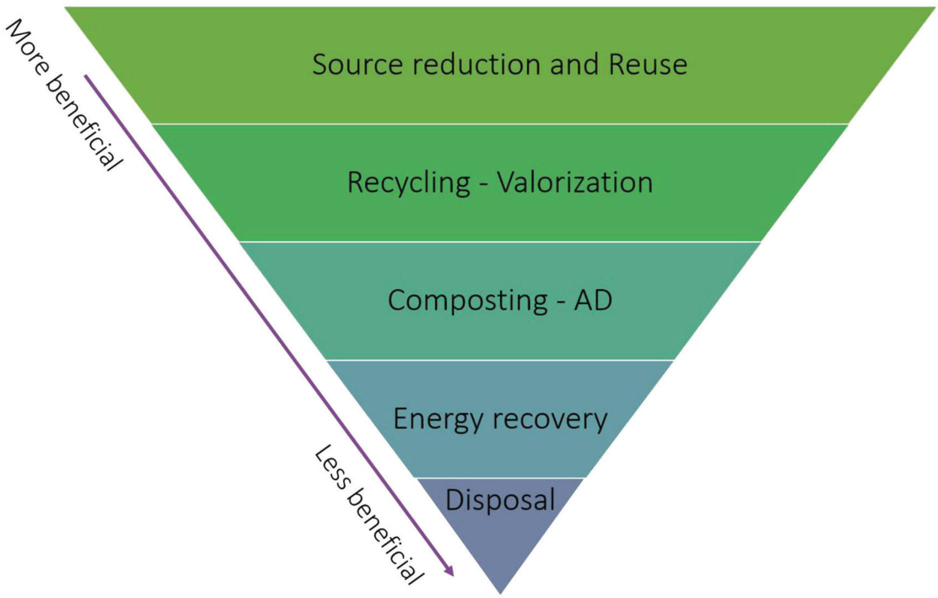
(European Commission, 2018). Indeed, the fundamental contribution of biorefineries to the concept of circular economy is the 'pyramidal approach,' where the extraction and production of an extensive portfolio of high added-value products have greater priority than bioenergy production.

1.3. Biorefineries: the workhorse of the bioeconomy

In the transition to a circular economy, we must reassess our approach to environmental management and the exploitation of the planet's resources, embracing waste as a valuable resource and closing the loops around industrial ecosystems. Biorefineries combine innovative technologies to produce biofuels, high value-added products, and bioenergy to achieve this goal from different organic wastes. This feedstock is of significant interest since it does not compete with the food industry and the need for arable land is not required. The most challenging aspect of this kind of feedstock is its seasonal variability, heterogeneity, moisture content, and the recalcitrance of the organic material compared to the fossil-based feedstock that has traditionally been used to manufacture fuels and chemicals in conventional refineries.

The most widely used platforms for the transformation of organic waste into value-added products are (Holtzapple *et al.*, 2022):

Figure 1.4. Waste hierarchy adapted from European Commission, (2018, 2020).



1. Thermochemical platform: It consists of thermal degradation of recalcitrant biomass such as: pyrolysis, a thermal process in absence of oxygen that generates mainly bio-oil but also biochar; gasification, a thermal process in presence of low levels of oxygen that yields syngas (CO_2 + H_2) as main product; hydrothermal carbonization (HTC) where wet biomass is converted into a rich-carbon solid product called hydrochar; and hydrothermal liquefaction (HTL) to obtain as primary product a biocrude. These thermochemical processes have as main drawback the requirement of high energy input (even higher for pyrolysis and gasification where the biomass needs to be previously dried).

2. Sugar platform: In this biological approach exogenous enzymes hydrolyze polysaccharides (cellulose and hemicellulose) to simple sugars that are subsequently fermented to the corresponding alcohols. Nowadays, this technology has low yields which makes it not competitive against the fermentation process using starch and edible sugars (Tonini and Astrup, 2012).

3. Carboxylate platform: A set of processes where a mixed culture of microorganisms uses endogenic enzymes in a fermentation process to obtain carboxylic acids with a carbon range from C1 (formate) to C8 (octanoate) or even higher. These compounds can later be transformed into fuels, heat, electricity and high value-added products such as bioplastics or biochemicals (Agler *et al.*, 2011).

The carboxylate platform has the advantages of being resilient and environmentally sound, having higher product yields (Holtzapple *et al.*, 2022), and being industrially applicable and scalable (Jones *et al.*, 2021).

1.3.1. The carboxylate platform

AD is still the core process technology of modern biorefineries, with the potential of waste stabilization, biogas production, recovery of chemical building blocks for the carboxylate platform and nutrients. The methane produced in the biogas can be used directly to produce heat and electricity in a combined heat and power (CHP) plant or upgraded to natural gas quality by removing water vapor and CO2 and fed into the natural gas grid (Kumar and Samadder, 2020). In recent years, the upcycling of biogas obtained through the valorization of CO2 by using microalgae or the cultivation of methanotrophs for their use as feed ingredients has also been gaining ground (Tsapekos *et al.*, 2021). However, separating the processes occurring within the AD to maximize the production of carboxylates represents an advantageous configuration, as it does not require the dissolution of feedstock and maximizes the concentration of products and nutrients for recovery in downstream processes, as well as avoids leakage and the potential emission of methane into the atmosphere if biogas is not handled adequately (Kleerebezem *et al.*, 2015). Therefore, a thorough understanding of the different phases within the anaerobic degradation process of organic waste is key for developing the carboxylate platform.

The carboxylate platform describes an anaerobic fermentation process (either named as dark fermentation or acidogenic fermentation) that produces carboxylates as building blocks to generate higher-value products (Figure 1.5). Carboxylates are organic acids having at least one carboxyl group and up to 20 carbon atoms. Depending on the pH, they can be in the form of carboxylate salts or carboxylic acids. Short-chain carboxylic acids (SCCA) have one to eight carbon atoms, commonly referred to as volatile fatty acids (VFA), when reduced to 6 carbon atoms.

Figure 1.5 shows the main different biochemical pathways between AD and acidogenic fermentation for converting complex organic matter into metabolic

Figure 1.5. Schematic representation of the main differences between AD and acidogenic fermentation biochemical pathways.

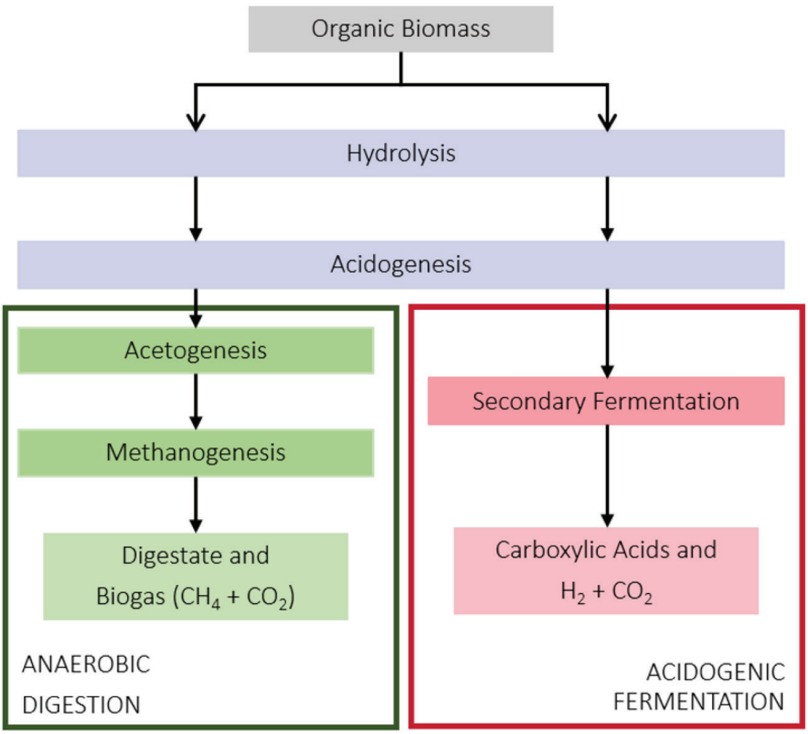
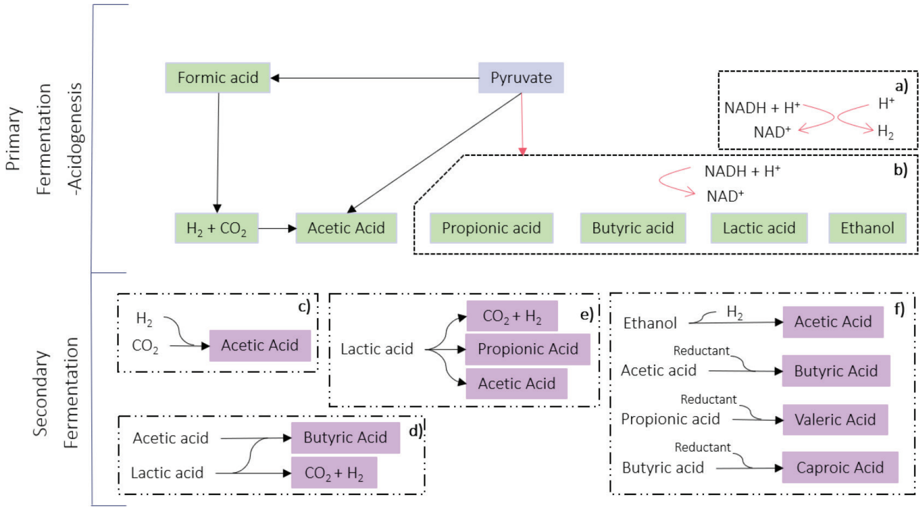


Figure 1.6. Biological pathways in the carboxylate platform. Subsequent conversions by primary and secondary fermentation reactions with undefined mixed cultures. Adapted from Agler ***et al.***, (2011).



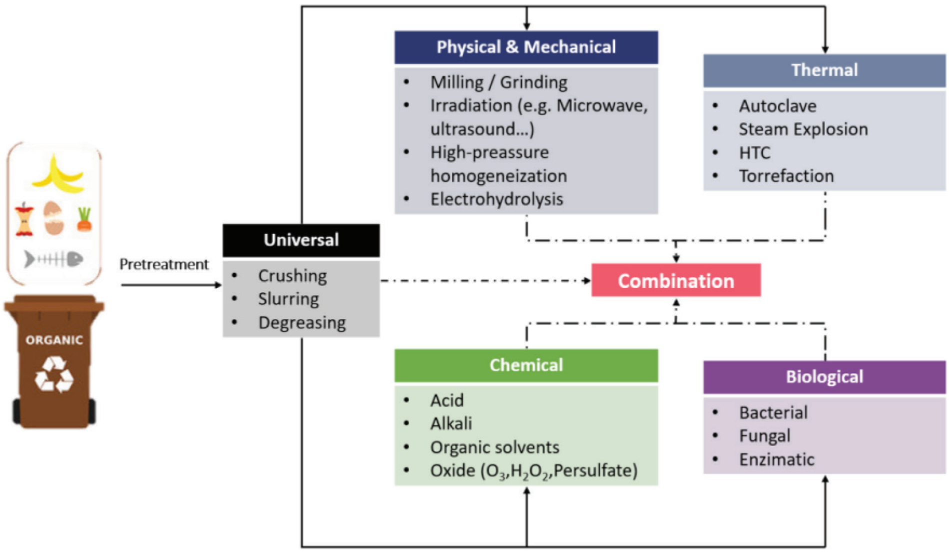
compounds. The anaerobic fermentation used on a carboxylate platform has three steps: hydrolysis, primary fermentation or acidogenesis, and secondary fermentation; however, in normal anaerobic digestion, it consists of four steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Kumar and Samadder, 2020). Therefore, the main difference between the carboxylate platform and the AD is that the disposal of electrons by methanogenesis is partially or entirely arrested.

The first two phases are the same for AD and acidogenic fermentation; firstly, in the hydrolysis phase, complex organic substances that bacteria cannot directly utilize are broken down into soluble compounds by the action of acidic hydrolytic enzymes. For example, proteins are transformed into amino acids, lipids into fatty acids, and polysaccharides into monomers and oligomers. Figure 1.6 shows the biochemical pathways linked to acidogenic fermentation. During the primary fermentation of sugars, substrates are converted to pyruvate, which results in the production of NADH and H^+. All equivalents must be re-oxidized via H^+ reduction by: (a) NADH oxidation; or (b) NADH oxidation via reduction of pyruvate or its oxidized organic derivatives, depending upon the hydrogen partial pressure. At increasing hydrogen partial pressures, the flow of electrons from NADH shifts from H_2, acetic acid, and CO_2 production towards the formation of increasingly reduced fermentation products (McInerney and Bryant, 1981). CO_2 and H_2 are produced in pyruvate oxidation or via dehydrogenation of formic acid. Figure 1.6 shows that the products of primary fermentation can react further through several secondary fermentation reactions: (c) autotrophic homoacetogenesis; (d) lactic acid oxidation to butyric acid (acetic acid and H+ as an electron acceptor); (e) lactic acid reduction to propionic acid (oxidation to acetic acid for energy conservation); (f) ethanol oxidation and chain elongation of carboxylates with a reductant (ethanol, lactic acid, hydrogen, etc).

The population of methanogenic archaea must be inhibited to promote the production of SCCA. The most widespread strategies to accomplish this are targeted inoculation, thermal pre-treatments, pH shocks, or inhibitor supplementation (Dahiya *et al.*, 2015; Xie *et al.*, 2014; Yu *et al.*, 2014). However, the most suitable strategy to reduce operating costs and obtain an effective start-up is to inhibit methanogenesis by overloading the methanogenic population. High organic loading rates (OLR) or shortened hydraulic retention time (HRT) cause imbalances between the methanogenic and acidogenic populations, accumulating SCCA in the reactor, consequently causing a decrease in pH and buffer capacity, which inhibits methanogenesis (Rajagopal *et al.*, 2013). Upon methanogenesis inhibition, the excess of reductants are released through SCCA production, lowering the energy state compared to other pathways (e.g., ethanol). In most anaerobic habitats, acetic, propionic, and butyric acid are the most commonly produced SCCA, but many other secondary routes are possible.

Different groups of anaerobic bacteria produce SCCA, and a thorough understanding of anaerobic metabolism is essential to maximize their potential and comprehend the diversity of SCCA obtained in the process. Most of these bacteria are chemotrophic and obtain energy through the oxidation of high-energy compounds (e.g., sugars) to lower energy compounds (e.g., acetic acid). To maintain the redox balance, electrons released in these oxidations are transferred enzymatically by electron carriers (e.g., NADH, NADPH or FADH), which donate electrons to electron acceptors (oxidants). The primary process is anaerobic catabolism, whose fundamental challenge is to exploit electron acceptors to capture energy while maintaining redox balance during the oxidation of substrates. Two processes are used for this challenge: fermentation, which uses organic electron acceptors, or anaerobic respiration, which uses inorganic electron acceptors (e.g., CO_2, NO_3, SO_4, Fe_3^+). Electron acceptors have a lower reduction potential than O_2, thus anaerobic bacteria have a lower energy yield for growth and higher retention of chemical energy in catabolic products than aerobic bacteria (which release CO_2 as the primary end product of metabolism). Predicting the catabolic products generated in a mixed anaerobic culture with heterogeneous substrates is highly challenging. It depends primarily on the structure of the microbial community and the identity and concentration of the donors and acceptors, which define the thermodynamics and kinetics of the metabolic reactions. These variables are the main drivers in determining which biochemical pathways are the most advantageous in individual anaerobic habitats.

Figure 1.7. Ilustration of the various pretreatment methods for organic solid waste. Adapted from Zhang ***et al.*** (2021).



1.3.2. Pretreatment

Pretreatment is a fundamental step in the acidogenic fermentation of solid organic wastes. The hydrolysis of complex organic matter is a rate-limiting step, especially in recalcitrant substrates such as lignocellulosic residues (Romero-Cedillo *et al.*, 2017). Hence, these pretreatments are necessary to speed up the initial hydrolysis step. Although studies on OFMSW pretreatment specifically focused on SCCA production are scarce some insights can be inferred from studies carried out in AD, even if they are not optimized for SCCA production. The different types of pretreatments can be classified into chemical, physical, and biochemical (or enzymatic). These pretreatments result in the acceleration of the hydrolysis stage and the yield of SCCA and hydrogen production. Overall, the drivers to select the appropriate pretreatments are operation costs, enhancement efficiencies, and desired final products.

As shown in Figure 1.7, after sorted OFMSW is crushed to reduce volume, and conventionally mechanically pretreated, other pretreatment methods are frequently used to improve the biological treatment effect. As an expensive but indispensable step in OFMSW resource utilization, appropriate pretreatment or even combined pretreatment methods should be selected from multiple perspectives such as biomass substrate composition, subsequent biological treatment methods, and economic feasibility. For example, chemical pretreatments can be effective, but have several disadvantages such as toxic compound generation. Moreover, they are not environmentally sound pretreatments and require recovery or neutralization treatments (Ramos-Suarez *et al.*, 2021). On the other hand, biological pretreatments are environmentally friendly, but their use on substrates of variable composition such as OFMSW is often discouraged (Plácido and Zhang, 2018). Hence, chemical and biological pretreatments are not very spread at the industrial level.

Among the physical pretreatments, both mechanical and thermal ones are the most studied and most technologically mature, but other emerging technologies are being implemented at both research and industrial levels, such as high-pressuring machines, microwaves, and ultrasound devices.

Mechanical pretreatment such as screening, meshing, or grinding improves the surface area, enhancing biodegradability and solubility, and they are typically used as a first step before thermal pretreatments. In AD, particle reduction accelerates the accumulation of SCCA (Romero-Cedillo *et al.*, 2017). In particular, it can increase the amount of acetic acid produced from food waste (FW) (Izumi *et al.*, 2010). This type of pretreatment allows particle reduction for better handling of waste in subsequent processes and has the advantage of requiring low energy and being simple to implement. The disadvantages are that it does not degrade lignin, present in lignocellulosic waste (LW), it does not remove pathogens or significantly increase the soluble chemical oxygen demand (SCOD) dissolution, and it has high maintenance requirements (Cesaro and Belgiorno, 2014).

Conventional hydrothermal pretreatment relies on temperature and reaction pressure control, although the latter is usually the endogenous pressure that increases with increasing temperature in a closed vessel like an autoclave. Hydrothermal pretreatment at a wide range of temperatures (55-200 °C) and times (5-60 min) has been considered, depending on the optimal pretreatment temperature on the type of substrate, use, etc. Earlier thermal pretreatment was used to improve the dewaterability and digestibility of sludge in the AD field. According to EU regulation EC1774/2002 organic, solid waste should be sterilized or pasteurized at least for 1 h at 70 °C before or after AD (Ariunbaatar *et al.*, 2015). Thermal pretreatment improves the AD process by solubilizing refractory particles (Ariunbaatar *et al.*, 2015), deflocculating macromolecules, improving dewaterability (Jin *et al.*, 2016), disinfecting by sterilization (Li and Jin, 2015), and reducing exogenous pollution. However, thermal pretreatment may also inactivate methanogenic archaea present in the feedstock. Generally, the effect of thermal pretreatment is measured in terms of soluble chemical oxygen demand (SCOD), SCCA, and biogas production. SCOD increases significantly due to the degradation and dissolution of insoluble organic compounds such as carbohydrates, lipids, and proteins (Ahmed *et al.*, 2021).

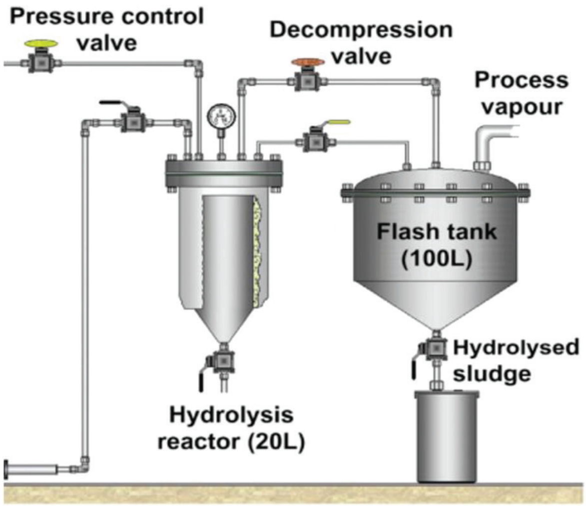
The effects of hydrothermal pretreatment have been studied on three main components of OFMSW such as kitchen waste, fruit and vegetable waste, and waste activated sludge, resulting in a considerable decrease of viscosity and an increase of SCOD, mainly sugars and soluble proteins (Liu *et al.*, 2012) at 175 °C and 60 min. A recent study on hydrothermal pretreatment of dewatered sewage sludge validated that a pretreatment at 170 °C achieved an efficiency of 0.59 gCODSCCA $gCOD^{-1}$ (Chen *et al.*, 2021). However, temperatures higher than 150°C promote the formation of refractory compounds via Maillard reactions, i.e., interactions between sugars and amino acids at high temperatures that can inhibit anaerobic processes (Tyagi *et al.*, 2018). In fact, temperatures as low as 100 °C are sufficient to improve the hydrogen production yield due to the effective suppression of methanogenic communities (Dong *et al.*, 2010). Therefore, evaluating physical pretreatment conditions is as vital as the waste composition analysis concerning the possible formation of not degradable by-products requiring proper monitoring systems.

Hydrothermal pretreatment is an environmentally friendly process because it does not use chemicals and has zero emissions. The integration of thermal pretreatment with acidogenic fermentation of OFMSW could have several potentially positive outcomes for sustainable production: increased process stability, increased specific SCCA and hydrogen yields, maximized substrate availability for the microbial community, reduced energy requirements during the fermentation process; reduced HRT, downsizing reactor volume and reduced remaining biosolids landfill use (Habarakada and Babel, 2020). One of the main drawbacks of this technology is the high pressure and high temperature, leading to energy-demanding and costly treatment. One strategy to overcome this problem is to use steam explosion, which is usual for lignocellulosic biomass and is becoming common for OFMSW, mainly in the presence of a nearby AD plant.

Steam explosion

Steam explosion is one of the few pretreatment technologies that have evolved to the point that pilot-scale and process equipment are commercially available. The most established commercial technology is the CambiTHP © process, and the schematic representation of the process is presented in Figure 1.8. Briefly, it consists of heating of the organic waste with saturated steam, followed by a sudden decompression of the pressurized system in a flash steam-heat recovery system and a standard size multi-reactor. This process requires a relatively low steam amount as it is recycled from the flash tank to the pulper where the feed is stored (Abu-Orf and Goss, 2021). It usually operates at 160 °C and 6 bar for 30 min and achieves high cell disintegration, where organic solids are dissolved in the water, such as proteins and carbohydrates, which are disintegrated into oligo- and monosaccharides

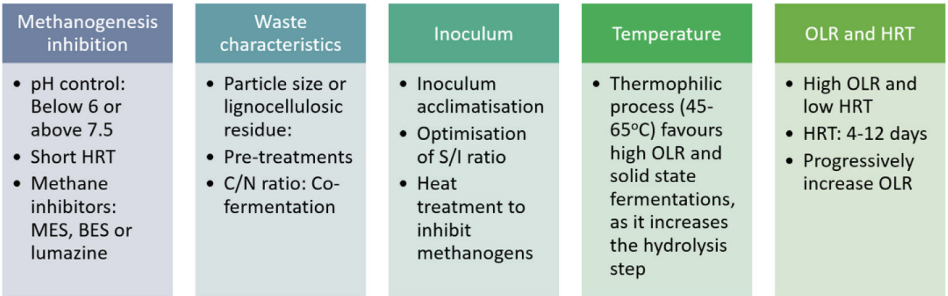
Figure 1.8. Schematic representation of a typical steam explosion reactor.



and amino acids. In addition, this feedstock is well sterilized, eliminating the risk of contamination by pathogens. Steam explosion is the most widely used chemical-physical pretreatment method for lignocellulosic biomass, but it has already been installed in food and sludge co-digestion plants in China, Norway, South Korea, and Sweden (Ahmed *et al.*, 2021). It has also been applied for source-separated OFMSW in Lillehammer, Oslo where 14,000 tonnes/y are treated and a 70% reduction of volatile solids (VS) is achieved (Barber, 2016).

The economic feasibility analysis of thermal pretreatment methods for OFMSW processing at the pilot-scale or full scales are limited in the literature. One of the keys to achieving a sustainable operation is the energy self-sufficiency of the process (Clauser *et al.*, 2021). Several pilot scale studies have already demonstrated a positive energy balance of steam explosion pretreatment with a CambiTHP © system combined with AD on several different organic wastes (Cano *et al.*, 2014; Díaz *et al.*, 2021). In any case, the energetic, economic and environmental feasibility of a hydrothermal pretreatment process can be improved by incorporating the use of renewable energies (e.g., solar), segregation of waste at source, co-digestion approach, and avoidance of high-temperature thermal pretreatment of carbohydrate and protein-rich substrates (Fan *et al.*, 2018). **An important task in this doctoral Thesis has been to study more in depth how hydrothermal pretreatment influences in the production of SCCA coupled with acidogenic fermentation, as well as a better understanding of how it affects each main element of the OFMSW to optimize time and temperature parameters.**

Figure 1.9. Workflow for optimization of operating parameters during acidogenic fermentation.



1.3.3. Optimizing SCCA production

The most important parameters to be optimized during acidogenic fermentation are the methanogenic inhibition, the waste characteristics, inoculum and microbial population, temperature, OLR and HRT. These factors affect both the yield and the type of SCCA produced. Figure 1.9 summarizes the strategies for their optimization, and briefly explained below:

a) Methanogenesis inhibition. The optimum pH for methanogens is 7.0 while acidogenic bacteria can handle wider pH ranges (5-11), which leaves room for acidic or alkaline fermentations. Slightly acidic fermentations improve hydrolysis due to increased activity of the hydrolytic bacteria, although alkaline fermentation can also be beneficial as they improve digestibility by dissolving lignin and can offer buffering capacity. However, alkaline addition can add high operational costs, whereas an acidic environment can be maintained by accumulating SCCA and providing a substrate close to pH neutrality. The growth of methanogens is slower than acidogens. Hence, a shorter HRT could also achieve the wash-out of methanogens, avoid consumption of SCCA and improve the treatment capacity (Yin, *et al.*, 2016).

b) Waste characteristics. Although the literature on acidogenic fermentation mainly focuses on optimizing hydrogen production, we can draw key conclusions for this process. For example, waste composition affects the overall yield. High-complex protein waste, such as FW, has the potential to produce higher concentrations of SCCA (Ramos-Suarez *et al.*, 2021). However, LW (between 50%-90% cellulose, hemicellulose, and lignin content) require pre-treatment to be digested properly (Jin *et al.*, 2016). The composition of the feedstock can be modified by mixing different substrates, in which case the process is known as co-fermentation. Co-fed substrates are known to improve performance due to synergistic effects such as helping to dilute toxic compounds present in the feedstocks and improving the nutrient balance, particularly the C/N ratio (Farmanbordar *et al.*, 2020; Soomro *et al.*, 2020). The presence of minerals in the fermentation medium is also very important to enhance microbial growth, although their effect on SCCA production is poorly studied (Zhang and Shen, 2006).

c) Inoculum. SCCA yield and distribution for a given substrate, are in essence a result of the microbial community and its activity. Microbial community studies can help understand the acidogenic fermentation process and the effect of fermentation conditions. Acidogenic species can be introduced through inoculation/seeding, sometimes from eutrophic waters, but most studies use AD digestate/sludge as the inoculum (Ramos-Suarez *et al.*, 2021). Nevertheless, AD sludge contains methanogens that convert the acetic acid into methane, therefore acclimatization of the inoculum for extended periods (Plácido and Zhang, 2018) or thermal pretreatment of the inoculum to inhibit methanogens (Blasco *et al.*, 2020) have been studied as possible strategies to improve the process. Regarding the process stability, sporulation must be controlled or avoided. During the process, sporulation decreases the substrate consumption rates and productivity and loses the microbes' autocatalytic capacity (Hawkes *et al.* 2002), increasing the dominance of bacteria populations that do not have sporulated (Hawkes *et al.*, 2002).

d) Temperature. This parameter has a significant effect on the yield and the SCCA produced. Typically, fermentation is classified according to the operating temperature as psychrophilic (<25 °C), mesophilic (25-45 °C), or thermophilic (>45 °C). In general, thermophilic conditions have a significantly higher accumulation of SCCA compared to mesophilic conditions (He *et al.*, 2012; Yin, *et al.*, 2016). In addition, thermophilic temperatures can enhance the hydrolysis of solid residues improving overall digestibility, but pH has a more significant influence on this than temperature (Garcia-Aguirre *et al.*, 2017). Temperature can also potentially influence the nature of SCCA produced. However, the findings are inconsistent at present (Zhang *et al.*, 2009), probably due to limited knowledge on variables interactions.

e) OLR and HRT. Low HRT (4 to 9 d) favors the production of acetic and butyric acids, promoting the dominance of acidogenic bacteria and prevents the growth of methanogenic microorganisms (Renaudie *et al.*, 2021). In contrast to HRT, high OLR values stop methane production and promote acidogenesis. Increasing OLR above the AD threshold (7 gVS L^{-1} d^{-1}) results in higher SCCA concentrations but lower yields. Therefore, a compromise between yield and concentration must be found. In addition, the optimal OLR will vary depending on the substrate, temperature, etc. In summary, the optimal process conditions must be studied in situ, depending on the substrate used, the cost analysis and the SCCA ratio to be obtained.

Bioreactor Configuration for acidogenic fermentation

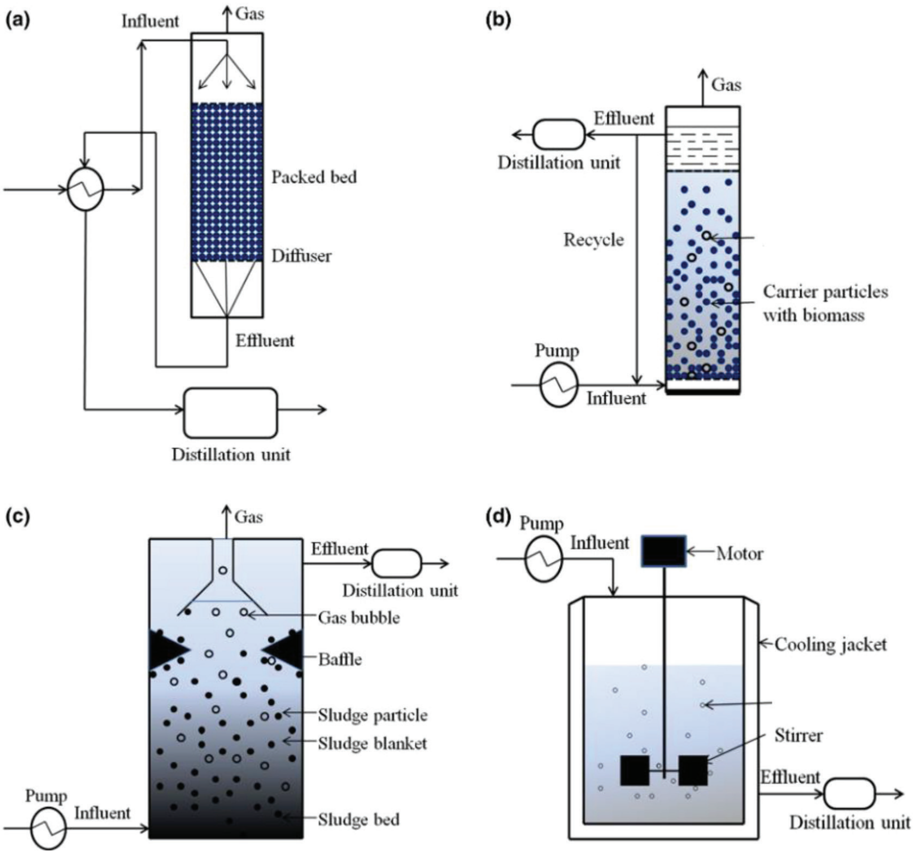
The bioreactor configuration influences the hydrodynamics and impacts the liquid-gas mass transfer phenomena, thus producing changes in the population of dominant microorganisms and enzymatic/metabolic changes. Figure 1.10 shows the most commonly used bioreactors for acidogenic fermentation. The two common technologies used in the anaerobic production of SCCA from waste are attached growth and suspended growth.

The two most used attached growth reactors are the packed and fluidized bed reactors. In packed bed reactors the biomass grows and adheres to porous packing material, such as alumina-based ceramic cubes and granular activated carbon (Beccari *et al.*, 2009), inserted into the reactor. This retains the biomass in the reactor, thus alleviating biomass washing. However, the packed bed reactor will become clogged with waste containing high concentrations of suspended solids. To avoid clogging, a fluidized bed reactor has been developed since in this type of reactor, the biomass grows attached to a small solid medium, such as sand, which remains in suspension by the upward movement of the fluid (Lee *et al.*, 2014). However, these types of reactors do not support high organic loadings (OLR) and have low volumetric activities, so suspended biomass reactors are more common.

Suspended growth technology allows the biomass to grow freely in suspension. Examples of reactors operated based on suspended growth technology are the upflow anaerobic sludge blanket (UASB) reactor and the continuous stirred-tank reactor (CSTR). The most common industrially used reactor is the UASB reactor, which consists of an upstream gas-liquid-solid separator and relies on the formation of dense and readily settleable biomass called granules (Wang *et al.*, 2007). These granules are retained in the reactor by sedimentation, forming a sludge blanket at the bottom of the reactor reducing the required reactor volume and increasing the efficiency of the process. The biggest drawback of the UASB reactor is a longer start-up period if the inoculum is not yet granulated (Jung *et al.*, 2011).

On the other hand, the operation of CSTR is the most straightforward, achieving complete mixing of waste and biomass, improving mass transfer (Show *et al.*, 2011). This can be approximately achieved by well-designed impellers, baffles and reactor shape. This mixture contributes to attaining saturation in the liquid phase, and the CO_2 and H_2 produced are transferred to the gas phase. Therefore, headspace clarification is also decisive for the chosen bioreactor since liquid saturation and the partial pressure of H_2 gas negatively affect the conversion reactions of NADH to H_2 by hydrogenases (Hallenbeck, 2005). A major problem with CSRT is the wash-out of biomass, but this problem can be fixed easily by a gravity settling clarifier used to separate and to recycle the biomass from the effluent. **When designed and**

Figure 1.10. Various types of reactors used in SCCA fermentation (Bhatia and Yang, 2017). a) Packed bed reactor, b) fluidized bed reactor, c) upflow anaerobic sludge blanket reactor (UASB), and d) continuous stirred tank reactor (CSTR). Workflow for optimization of operating parameters during acidogenic fermentation.



operated properly, a CSTR is ideal to mix waste and microbes thoroughly in the presence of suspended solids in the waste.

Parameter homogenization

In order to standardize and improve communication channels between the academy and industry, the units of the key variables must be well defined, as the disparity in literature prevents proper conclusions from being drawn. In AD processes it is common to use VS percentages for the characterization of substrate consumption, however in fermentation processes significantly less VS will be converted to CO_2 (and negligible amounts to CH_4) and instead, most are converted to SCCA. COD units are a better indicator to define SCCA yields and concentrations to compare these values with other soluble components present in the fermentation broth. SCCA composition is highly dependent on substrate and operational parameters, so standardization of product concentrations in terms of COD allows comparison between a range of variables. The proportions and yields of each SCCA present should also be provided, as this will allow a better understanding of the conversion and distribution of the product.

A common measure of fermentation performance is the **acidification extent**, which measures the equivalent COD of the SCCA over the SCOD in the broth. It is a good indicator of fermentation performance as it measures the purity within the aqueous solution obtained, which is extremely important in the downstream process. Whether this aqueous solution is used for further biological steps or recovery and purification of the SCCA obtained, the maximum possible acidification should be sought. However, it is not a complete overview of the fermentation yield; for example, the hydrolysis extent is another indicator that considers the ratio of SCOD increased in the reaction as well as the equivalent COD of the H_2 produced. It is also common to indicate the obtained SCCA concentrations which, although an interesting parameter, depends on the OLR or substrate concentration and therefore makes it difficult to compare between experiments.

1.3.4. Recovery and applications

The shortage of industrial SCCA-based processes is partly attributed to the difficulty in recovering the SCCA from the fermentate as well as low product yields. Individual SCCA are fundamental platform chemicals to produce esters, ketones, aldehydes, alcohols, and alkanes, therefore, the recovery process must selectively target the SCCA over other fermentation broth components including water, and increase their concentration in the product stream. Membrane extraction, electrodialysis and filtration have been the most researched recovery techniques (Ramos-Suarez *et al.*, 2021), but are still far from reaching industrial scale. The energy demand, and the number of unit operations required to achieve the desired product stream, and the fermentation broth conditions should be considered when designing the recovery process, which at the moment is not cost-effective (Greses *et al.*, 2020).

To bypass this bottleneck, direct use of the fermentation products in other bioprocesses is a promising alternative, although some physical processes like centrifugation to separate solid and liquid streams are necessary. The bioconversion of mixed organic acids entails hydrogen production, high added- value products like PHA, microbial protein, chain elongation products, or can allow nitrogen and phosphorus removal from wastewater (Ramos-Suarez *et al.*, 2021). **The implications of this platform and the different options**

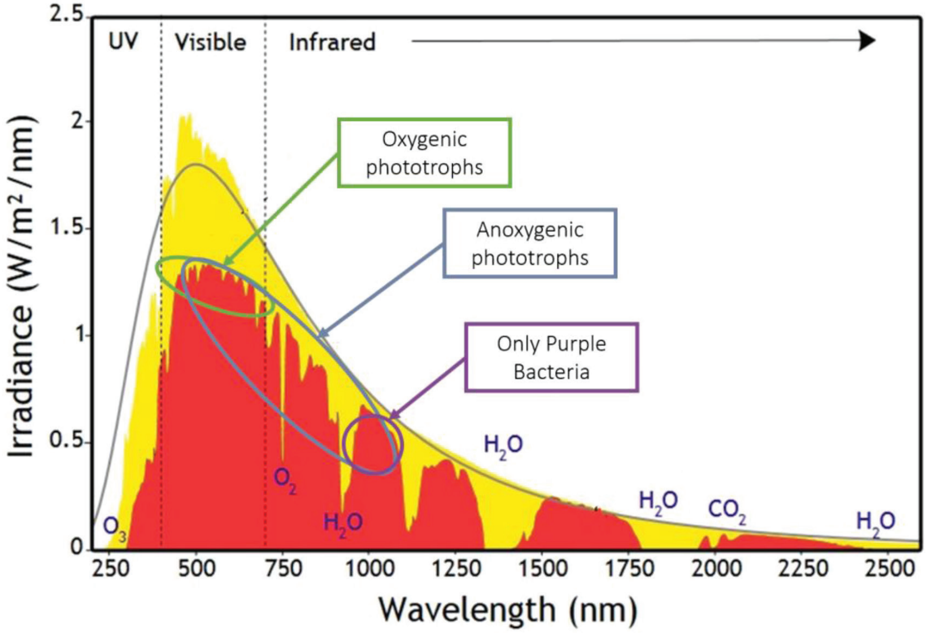
available will be discussed throughout this research. The production of bioplastics from PHA is one of the most attractive sectors in recent years, with considerable research into its production from waste (Rodriguez-Perez *et al.*, 2018). Nevertheless, this technology is still looking to advance to become economically viable, as PHA is not yet competitive with equivalent petrochemical plastics such as polyethylene (Ramos-Suarez *et al.*, 2021). Nevertheless, purple phototrophic bacteria (PPB) are attractive candidates for PHA production because, among other features, they can obtain energy from infrared light instead of oxygen, reducing operating costs.

1.4. Next step: The Photo-Biorefinery

Photosynthesis is the main supplier of carbon and energy required for the synthesis of the organic compounds that drive plant growth and development (Hussain *et al.*, 2021). Phototrophic microorganisms can be a powerful tool for the efficient conversion of the virtually unlimited supply of solar energy into bioenergy and renewable resources (Goh *et al.*, 2019; Tanvir *et al.*, 2021). The photosynthetic efficiency of these microorganisms (~10%) is much higher than that of terrestrial biomass (1.8-2.2%) (Ooms *et al.*, 2016). Among these microorganisms, two of the most studied are microalgae and cyanobacteria, which have 10 times the lipid content and require a quarter of the cultivation time of terrestrial plants (Ghosh *et al.*, 2016). In addition, they are highly adaptable to dynamic environmental conditions and do not compete for arable land (Zhu *et al.*, 2014). These microorganisms have the ability to feed on heterogeneous feedstocks such as some waste streams and accumulate high value-added bioproducts and bioenergy, which makes them excellent candidates to be the central element of a biorefinery using light as an energy source: a Photobiorefinery.

Phototrophic microorganisms capable of producing oxygen during the process of photosynthesis are known as oxygenic phototrophs (e.g., cyanobacteria, green algae), otherwise they are known as anoxygenic phototrophs (e.g., green and purple bacteria). Most oxygenic phototrophs absorb solar energy from the visible light region of the solar spectrum (400-700 nm), while anoxygenic phototrophs can photosynthesize with light from the visible and infrared regions, as shown in Figure 1.11. Oxygenic phototrophs require more input energy for photosynthesis (shorter wavelength light) compared to anoxygenic phototrophs (longer wavelength light) due to the inverse relationship between photon energy and wavelength (Chen and Blankenship, 2011). Anoxygenic phototrophs contain bacteriochlorophyll(s) instead of chlorophyll-like oxygenic phototrophs and require only a single type of photochemical reaction center (RCI or RCII) to carry out photosynthesis (Zeng *et al.*, 2015). Most species of the phyla *Chlorobi*, *Chloroflexi*, and *Acidobacteria* contain a unique light-harvesting pigment known as a chlorosome to absorb sunlight at 740-750 nm, while **purple phototrophic bacteria (PPB)** harvest sunlight from the infrared

Figure 1.11. Standard solar radiation spectrum. In yellow, the spectrum is high in the atmosphere. In red, is the spectrum at sea level.



region (800-1020 nm) of the light spectrum (Hanada, 2016). Furthermore, they are the only species capable of absorbing light beyond 1000 nm wavelength (Chen and Blankenship, 2011), which would give access to 19% additional photon flux compared with standard photosynthetically active radiation (PAR). Moreover, it allows them to grow without competing for the same light source, making them microorganisms with unique features. Therefore, **PPB has been the microorganism object of study in this research.**

1.5. Purple Phototrophic bacteria

PPB are a diverse group of anoxygenic, phototrophic, facultative anaerobes that inhabit aquatic and terrestrial environments. PPB have typically been classified according to their sulfur tolerance and utilization into purple sulfur and non-sulfur bacteria. Purple sulfur bacteria can oxidize sulfur to sulfur globules, which are stored intracellularly, while purple non-sulfur bacteria deposit them outside the cell (Hunter *et al.*, 2006). Overall, PPB are one of the most metabolically diverse types of microorganisms known on earth. Table 1.1 shows a summary of all possible metabolisms of PPB. In presence of light and an anaerobic environment, the two most common metabolisms are photoautotrophy and photoheterotrophy. Both use light as an energy source, while the former uses CO_2 as a carbon source, and the latter uses organic carbons (Capson-tojo *et al.*, 2020). Under these conditions, most catabolic energy comes from light absorbed by bacteriochlorophylls (BChl) and carotenoids (pigments required for light-harvesting and photosynthetic growth). In the presence of oxygen at high concentrations, suppression of BChl and carotenoids occurs (Yue *et al.,* 2015), which causes the loss of the usual color of PPB and shifts the metabolism towards chemotrophy using O_2 as an electron acceptor (Dubbs *et al.*, 2000). However, it is also possible that a mixed metabolism occurs where PPB obtain part of the energy from chemical catabolism and part from light in a mixed process of photo-fermentation. **This great metabolic flexibility allows PPB to be a perfect tool for treating a wide variety of heterogeneous wastes.**

In the last decade, there have been many studies on the use of PPB for wastewater treatment (Cao *et al.*, 2020). The abundant carbon (C), nitrogen (N), and phosphorus (P) in wastewater are highly dissipated resources. However, the current trend follows the circular economy in wastewater management, away from conventional treatments and focuses on resource recovery, where PPB is a perfect tool (Puyol *et al.*, 2017). PPB can tolerate wastewater with ample organic strength from different sources such as domestic wastewater (Hülsen *et al.*, 2016), poultry (Hülsen *et al.*, 2018), food processing (Chitapornpan *et al.*, 2012), brewery (Peng *et al.*, 2018) or slaughterhouse (De la Vega *et al.*, 2022) among others. PPB have also been used for soil remediation, polyphosphate accumulation, or monitoring environmental stress (Capson-tojo *et al.*, 2020). Nevertheless, the processes have not yet reached an industrial scale.

Compared to the usual chemoheterotroph technologies, the most significant advantage of PPB's technology is their ability to generate energy via photophosphorylation growing through anaerobic phototrophy. This results in:

1. Increased biomass yields. Since there is no need for ATP generation from chemicals, PPB biomass yields on simple substrates can reach values up to 1.0 gCOD $gCOD_{removed}^{-1}$ (Puyol *et al.*, 2017), which is better compared to the yields commonly achieved in activated sludge systems (0.5 gCOD $gCOD_{removed}^{-1}$, Henze *et al.*, 1997). Moreover, when the substrate is more reduced than the biomass, yields higher than 1 gC gC^{-1} are possible by simultaneous CO_2 fixation.

2. No aeration is needed. In this case, the energy comes from light, which can be supplied cost-free by solar illumination. The economic efficiency of artificial lighting is still a matter of debate. According to estimates in a current review article from Capson-tojo *et al.* (2020), even using NIR LED lamps (800-1000 nm), the energy cost of biomass production would be

Table 1.1. Main metabolic modes of PPB. Adapted from Puyol et al. (2019).

Metabolism	Process	e^- donor	e^- acceptor	Energy source	Carbon source	Redox conditions
Photo-organo-heterotrophy	Heterotrophic photosynthesis	Organic	CO_2 (fixation), H^+ (H_2 production)	Infrared light	Volatile fatty acids, alcohols, sugars	Anaerobic
Photo-litho-heterotrophy		H_2	CO_2 (fixation)			
Photo-litho-autotrophy	Autotrophic photosynthesis	$S^x/S_2O_3^2$	CO_2 (fixation)		CO_2	
	Photoanaerobic Fe^{2+} oxidation	Fe^{2+}				
	Photoanaerobic H_2 oxidation	H_2				
Chemo-organo-heterotrophy	Fermentation	Organic	Organic	Chemical	Organic	
	Denitrification		NO_3^-		Organic	Anoxic
	Aerobic oxidation		O_2		Volatile fatty acids, alcohols, sugars, proteins	Aerobic
Chemo-litho-autotrophy	Nitrification	NH_4^+	O_2		CO_2	
	Halophilic S^{2-} oxidation	s^{2-}				

pretty high (1.7 € $kg_{biomass}^{-1}$). This cost could be reduced by increasing the biomass concentration in the bioreactors through biomass recirculation techniques and optimizing the volumetric irradiance.

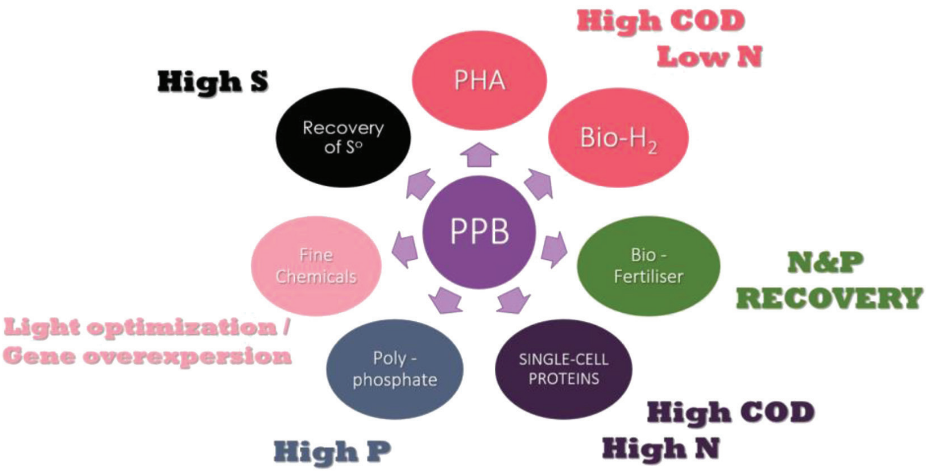
3. Effective selection of mixed PPB cultures in non-sterile environments for heterogeneous waste treatment. As discussed in previous sections, PPB absorbs at wavelengths between 805 and 1035 nm, with no competition in that spectrum from other microorganisms. These features provide this technology with clear advantages for its application, but the optimal high value-added products must fit the economic balances when scaling up. **The key bottlenecks of the scale-up of the photoheterotrophic process will be discussed in this Doctoral Thesis.**

1.5.1. High added-value products

In addition to the main growth metabolic pathways, PPB can undertake accumulation or side processes that allow them to better survive in stressful environments, such as the discontinuous presence of carbon or nutrients. Figure 1.12 summarises the products that can be extracted from PPB and some strategies for increasing their production. In excess of organic matter and absence of essential nutrients, PPB can accumulate carbon mainly as polyhydroxyalkanoates (PHA) (Fradinho *et al.*, 2016). However, they can also accumulate glycogen (Fülop *et al.*, 2012) or even produce extracellular polymers (EPS) in aggregated growing mode (Stegman *et al.*, 2021). PPB can also accumulate the excess of critical nutrients like P and S as polyphosphates form (Liang *et al.*, 2010) or as intracellular globules after sulfide oxidation (Weissgerber *et al.,* 2014), respectively. PHA, polyphosphate, and S globules all have in common that they serve as sinks for reducing redox potential and PHA and S can be used also as alternative electron donors. Another key mechanism of PPB is the release of excess electrons as H_2 to maintain redox homeostasis via nitrogenase in a low ammonium environment (Hadicke *et al.*, 2011). H_2 production by PPB using waste as substrate in photo fermentation processes has already been extensively studied (Ghosh, *et al.*, 2017), even by coupling acidogenic fermentation with photofermentation to maximize H_2 production (Rai and Singh, 2016). Although it is a promising technology as a stand-alone process, there is not yet a widespread scale-up of the technology in outdoor reactors (Sagir and Alipour, 2021). Bottlenecks such as slow productivity and low light conversion efficiency in the cells remain critical problems for the scaling-up of this technology (Tiang *et al.*, 2020). What is certain is that the scale-up of these products will have to consider all these mechanisms and possible production synergies in a PPB biorefinery.

Other high-value products can be obtained from PPB according to different cultivation strategies. If the substrate contains high levels of nutrients

Figure 1.12. Possible strategies for producing different high value-added products using the metabolic versatility of PPB. Adapted from Puyol *et al.* (2019).



(especially N, P, and K), PPB biomass can serve as an organic fertilizer or biofertilizer exhibiting a high capability to recover and recycle C and N (Zarezadeh *et al.*, 2019). With high C and N substrates, PPB cultivation can be oriented to protein production. PPB can accumulate high amounts of protein (>70%) with high-quality amino/fatty acid profiles (Alloul *et al.*, 2021). Finally, PPB can be used to extract fine chemicals such as carotenoids (Kuo *et al.*, 2012), 5-aminolevulinic acid (5-ALA) (Kars and Alparslan, 2013), or coenzyme Q10 (He *et al.*, 2021). Carotenoids are pigments used in cosmetics and as food coloring agent formulation. 5-ALA is a non-protein amino acid that can be used as an herbicide or an active component for the medical industry. The production of both products can be optimized by increasing the light intensity in PPB cultivation (Yu *et al.*, 2021). Coenzyme Q10 is a type of ubiquinone mainly used in the medical and cosmetic fields. Several attempts have been made to optimize its production via gene editing in pure PPB cultures (Lu *et al.*, 2013). The major problem for the commercialization of these products is the high cost and complexity of their extraction (Bogacz-Radomska and Harasym, 2018), which usually involves using environmentally toxic solvents.

1.6. Polyhydroxyalkanoates (PHA): A biodegradable plastic

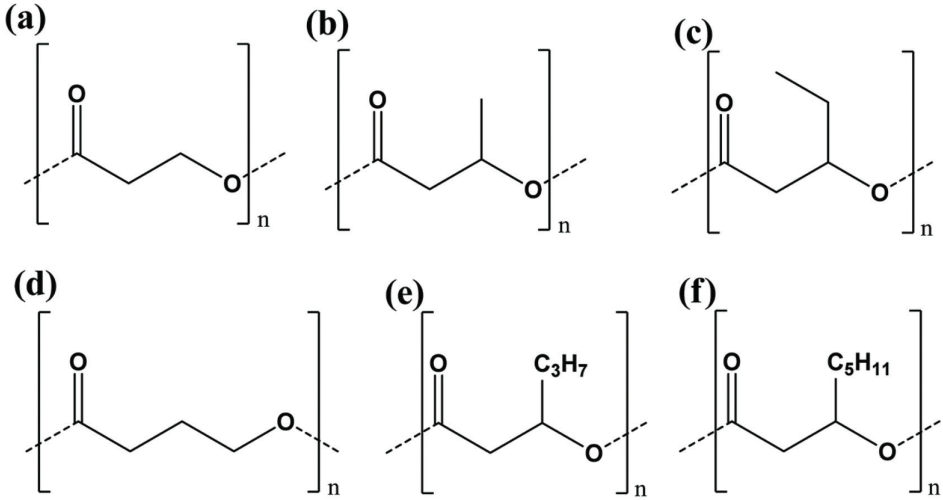
In recent years the research on PPB is shifting from water treatment to resource recovery, mainly PHA. The main reason is that plastics are nowadays widely used as synthetic polymers, mainly due to their resistance to chemical and physical degradation. However, at the end of their life cycle, the synthetic plastics' resistance to degradation has caused one of the world's major environmental problems: plastic pollution (Heidbreder *et al.*, 2019). Replacing petroleum-based polymers with bio-based polymers is a potential solution that produces significantly lower carbon emissions and energy production requirements (Gironi and Piemonte, 2011). Bio-based polymers can be subdivided into three types, plant-based (TPS), polymerized bio-monomers (i.e., PLA, polyimides, polyurethanes, poly(butylene succinate) (PBS), bio-PE, among others), and extracted bio-polymers (PHA) (Meereboer *et al.*, 2020). PLA and PHA are biodegradable (de Castro *et al.*, 2021), specifically, PLA is compostable but not marine biodegradable like PHA (Meereboer *et al.*, 2020). Biodegradability is essential if we want to move towards circular economy practices. Moving towards bio-based biodegradable polymers allows for a more sustainable option by implementing a cradle-to-cradle approach (Braungart *et al.*, 2007), where the output of biodegradation becomes the production input for the same polymer in a reasonable time frame within the biological cycle.

PHA are becoming important due to their many advantages such as their biodegradability, biocompatibility (Chen and Wu, 2005), controllable thermal and mechanical properties (Laycock *et al.*, 2014), as well as PHA molecular weight diversity ranging from several tens of thousands to several millions Daltons (Rodriguez-Perez *et al.*, 2018). PHA are accumulated intracellularly and assembled into hydrophobic spherical inclusions and have the biological function of carbon and energy storage (Reddy *et al.,* 2003). Many types of PHA are known, being the most well-known poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV). Both are short-chain PHA and represent the most basic forms commercially available (Leong *et al.*, 2014). However, many other types of PHA can be classified by their chain length:(i) a short chain length of 3-5 carbons; (ii) a medium chain length of 6-14 carbons; and (iii) a long chain length of 15+ carbons. Examples of the most common medium-chain PHA are polyhydroxyhexanoate (PHH) or polyhydroxyoctanoate (PHO) (Meereboer *et al.*, 2020). The properties of PHA depend on the chain length and combination of monomers present. In general, copolymers decrease the degree of crystallinity and melting temperature and increase the extension at break. However, in particular, the combination of short-chain PHA (PHB and PHV) with medium-chain PHA, such as polyhydroxyhexanoate (PHH), lead to polymers with elastic properties that increase their value in the industry, as they can be used as additives for medical and pharmaceutical applications (Pereira *et al.*, 2019).

1.6.1. Current industrial PHA production

While it is challenging to estimate the current volume of PHA produced industrially, according to the Global Bioplastics Market Report 2020, annual PHA production exceeded 10,000 t annually, with a 20–30% yearly growth rate (Bhola *et al.*, 2021). Industrially, PHA is synthesized through fermentation,

Figure 1.12. Different PHA functional components. (a) 3-Hydroxypropionate (HP), (b) 3-hydroxybutyrate (HB), (c) 3-hydroxyvalerate (HV), (d) 4-hydroxybutyrate (4HB), (e) 3-hydroxyhexanoate (Hx), and (f) 3-hydroxyoctonaote (HO) chemical structures.



using pure and engineered aerobic heterotrophic microorganisms fed with relatively pure plant-derived substrates like sugars and starch, among others, in batch reactors. The typical strategy is dynamic feeding in two steps: a selection reactor (feast phase) and a storage reactor (famine phase). Industrial-scale PHA are manufactured by various companies worldwide, for example, BioMatera (Toronto, Canada), Metaboli (Woburn, USA), Procter and Gamble Co., Ltd. (Cincinnati, USA), Tianjin Green Bioscience Co., Ltd. (Tianjin, China) Bio-on (Italy), Biocycle PHB Industrial SA (Serrano, SP, Brazil) and Goodfellow Cambridge, Ltd. (UK) (Palmeiro-Sánchez *et al.*, 2022). However, the price of PHA remains relatively high (1.7 - 3.5 € kg PHA^{-1}) and is 2 to 4 times more expensive than petroleum-based plastics (Tan *et al.*, 2021). In any case, the EU under the umbrella of the Green Deal and more specifically in the Directive on single-use plastics (Directive (Eu) 2019/904 of the European Parliament and of the Council of 5 June 2019 on the Reduction of the Impact of Certain Plastic Products on the Environment, 2019) and the Packaging Waste Directive (DIRECTIVE (EU) 2018/852 on Packaging and Packaging Waste, 2018) considers bioplastics indispensable for the achievement of climate targets, as they can be collected along with the organic waste and treated in a circular and environmentally sustainable way.

The main bottlenecks in industrial PHA production must be firstly identified to reduce production costs. The main one is the substrate cost and the sterilization required in the use of pure cultures, which can account for more than 50% of the production cost (Kourmentza *et al.*, 2017). The other is the cost of the downstream process: both the cost of extracting and purifying the PHA, which can account for up to 30% of the total cost (Fernández-Dacosta *et al.*, 2015). The most commonly used strategies to reduce these costs are using mixed microbial communities (MMC) as biocatalysts for PHA generation under non-sterile conditions and applying a variety of low-value substrates, such as industrial and municipal waste and by-products (Novelli *et al.*, 2021). These processes, mainly with aerobic microorganisms, achieve high PHA dry mass contents (0.5 - 0.9 g_{PHA} $g_{DryBiomass}^{-1}$) and substrate conversion yields up to 32% (Koller, 2018). **Nevertheless, the production of PHA with mixed PPB cultures is gaining great attention in the last years** (Monroy and Buitrón, 2020) **and shows several features that make this strategy very exciting and promising.**

PHA accumulation routes via PPB are complex and vary depending on various parameters, including bacteria strain, carbon substrate, and metabolic pathway. Photoheterotrophic production of PHA is the predominant route from which attractive advantages over aerobic microorganisms are foremost:

1. PPB can deliver up to 90% PHA yields on substrates, almost three times higher than aerobic yields (Fradinho *et al.*, 2019).

2. PPB does not require aeration, as they obtain their energy through IR illumination.

3. PPB can accumulate PHA while growing, and nutrient availability drives the accumulation process (Fradinho *et al.*, 2016). Thereby, PPB can accumulate PHA in a permanent carbon feast regime, eliminating standby production times and simplifying the operation to a single reactor, as opposed to the commonly used feast/famine regime that involves two stages: In the first stage, an abundant carbon source is provided to favor the production of biomass, and in the second stage, the carbon source is limited to stimulate PHA accumulation (Fradinho *et al.*, 2019).

However, the outstanding performance of PPB have not yet been fully exploited, and further optimization of PHA accumulation is part of the research of this Doctoral Thesis with the purpose of helping scaling up and commercializing this technology.

1.6.2. PHA applications

As indicated, depending on the monomeric composition, different properties of the designed PHA can be obtained. The polymer can be hard and crystalline or elastic and rubbery. These properties will designate the future application of the polymer. As an example, pure PHB is highly crystalline with a melting temperature of 180°C making it brittle and stiff (Koller, 2018). In contrast, the different copolymers that can be formed and medium-chain PHA are materials with more elastic properties, high elongation at break, low crystallinity and glass transition temperatures, less mechanical resistance, and low melting points; hence, they are more versatile materials and more desirable for industrial applications (Shahid *et al.*, 2021). PHA has unique properties that give it an edge over other biopolymers in this field. It has improved barrier properties (sufficient oxygen and water transmission rates) and higher mechanical strength than PLA (Mathuriya and Yakhmi, 2017). It also has lower acidity and reduced bioactivity, as well as greater proliferation, stronger calcium deposition, and fibrillar collagen synthesis than PLA or polyglycolic acid (PGA) (Ali and Jamil, 2016). PHA have applications in the following sectors:

- Medical and farmaceutical sector. PHA can be used as artificial heart valves, blood vessels, cartilage or tendons, nerve conduits, esophagus replacements, bone replacement, surgical sutures, porous microspherical implant-scaffolds for microsurgery, and many other applications (El-malek *et al.*, 2020; Moradali and Rehm, 2020; Muneer *et al.*, 2020).

- Packaging industry. It can be used for the production of bottles, cosmetic containers, bags, and utensils, for example (Mozejko-Ciesielska *et al.*, 2019; Muneer *et al.*, 2020). It can also be converted into biofilms for the food industry, as it has the necessary properties (Topuz and Uyar, 2020).

- Agricultural sector. PPB with PHA are proposed as bacterial inoculants to improve nitrogen fixation in plants and also can be used to make soil friendly compostable greenhouse films, grow bags, and protection nets (Muneer *et al.*, 2020).

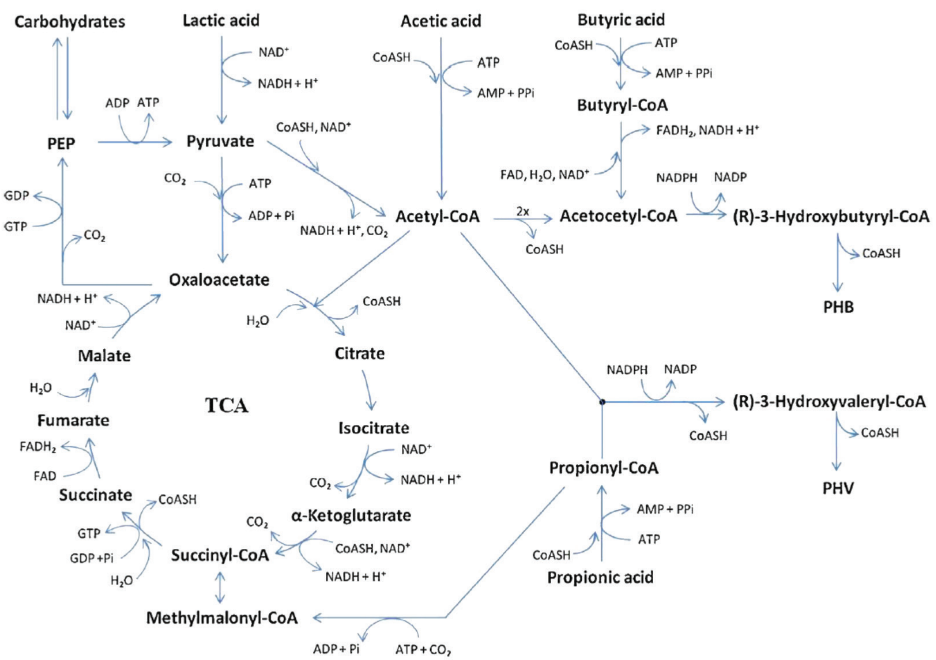
- Other sectors. PHBV copolymer is also used to synthesize a polymer gel electrolyte for use in high-density lithium batteries (Dall'Asta *et al.*, 2017). It also can be used as a biofuel precursor (Montiel-Corona and Buitrón, 2021).

1.6.3. Mechanisms of PHA synthesis by PPB

The PPB preference for acetic acid as a substrate for PHA production has been repeatedly reported due to its easy assimilation into the metabolic pathway for Acetyl-CoA synthesis, a precursor to PHA (Fradinho *et al.*, 2014). PPB uses different metabolic pathways to assimilate acetic acid, such as glyoxylate, citramalate, and ethyl malonyl-CoA pathways. Figure 1.13 depicts a summary of these metabolic pathways. Some studies have observed higher PHA yields with butyric acid (Carlozzi *et al.*, 2018), although Fradinho *et al.* noted that the presence of acetic acid accelerates the assimilation of propionic and butyric acid (Fradinho *et al.*, 2019). Also, the sole presence of acetic acid entails the accumulation of PHB only, and PHV accumulation is only possible in the presence of precursors such as propionic or valeric acid. Thus, propionate is converted to propionyl-CoA and condensates with acetyl-CoA to form PHV (Mukhopadhyay *et al.*, 2005). In addition, there are few studies on the production of polymers other than PHB or PHV with PPB, though C6 and C7 monomers (PHH among others) were detected in *Rhodospirillum rubrum sp.* (Brandl *et al.*, 1989). In general, the diversity of SCCA in the substrate increases the chances of PPB to accumulate longer-chain PHA, which is beneficial in the context of heterogeneous waste treatment. However, the presence of carbohydrates decreases PHA accumulation, shifting PPB metabolism towards photo-fermentative processes (Almeida *et al.*, 2021).

Other metabolic pathways that limit PHA accumulation are the diversion of electrons to H_2 production or the allocation of carbon to other storage products. During photofermentation, PPB evolves H_2 through nitrogenase catalysis with adenosine triphosphate (ATP) consumption and electrons from reduced

Figure 1.13. PPB assimilation and PHA accumulation pathways (Fradinho *et al.*, 2014).



NADH. Nitrogenase activity is crucial for H_2 production by photosynthetic bacteria and is strongly inhibited by high ammonia concentration (Koku *et al.*, 2002). H_2 synthesis can occur in parallel with PHA accumulation on high COD and low ammonium substrates (Padovani *et al.*, 2016). **However, a more comprehensive investigation of this competition in heterogeneous substrates and mixed PPB cultures is still missing. Hence, a comprehensive examination of the mechanism of carbon allocation between different types of storage products such as glycogen and EPS has been addressed in this research**. Likewise, rather than eliminating the possibility of one route occurring by giving preference to others, synergies increase the value of bioconversion of PPB wastes into alternative energy sources and bioproducts. This information is crucial to better understand and optimize PHA production with PPB.

1.6.4. PHA optimization with PPB

Although PHA accumulation with mixed PPB cultures has not been optimized, critical parameters like pH, irradiance, strain type, feed regime, and COD/nutrient ratio have been studied. Table 1.2. summarizes the studies found on PHA production with mixed phototrophic cultures. Studies on PPB mixed cultures for PHA production are still limited, however, it can be observed the trend that, in recent years, waste has increasingly been studied as a substrate, replacing the use of synthetic substrates. **During the development of this Thesis, the use of OFMSW hydrolysates and fermentates for PPB growth and PHA accumulation was studied.**

Irradiance is one of the most important parameters in PPB culture, typically using monochromatic IR LED light sources. The optimal wavelength of 850 nm for COD and ammonia removal, as well as biomass growth, was determined on *Rhodopseudomonas sphaeroides sp.* (Qi *et al.*, 2017). However, this aspect is unexplored for PHA production. As for light intensity, it depends heavily on cell concentration. Light penetration controls the amount of light reaching the biomass. Higher cell growth reduces the light intensity reaching the cells and the overall productivity (Fradinho *et al.*, 2016). Mixed PPB cultures illuminated with a light intensity of 5.6-6.7 W g^{-1} (80 W m^{-2}) got the highest PHA content so far (60% on a dry basis) (Fradinho *et al.*, 2014). Thus, one of the major challenges in designing PPB accumulation bioreactors is the irradiance distribution at high biomass concentrations. Another lighting strategy studied is light/dark cycling to avoid photoinhibition problems (Montiel *et al.*, 2017).

PPB requires a higher proportion of nutrients than chemoheterotrophic systems, as their catabolic energy source arises from light, which directs a higher proportion of carbon to growth. The nutrients required can be related to the COD consumed, and a COD/N/P ratio of 100/7.1/1.8 is the ideal ratio for biomass growth in PPB (Puyol *et al.*, 2017). Presumably, values for PHA accumulation should be below this ratio. N limitation links to PHA accumulation (Sali and Mackey, 2021); however, a distinction between nitrogen sources used must be made. Ammonia availability is related to biomass growth due to nitrogenase inhibition, while the presence of sufficient glutamate as a nitrogen source enabled PHA accumulation in the environment (Carlozzi, *et al.*, 2019). Limitation of other nutrients such as P and S also allows PHA accumulation and is advantageous as that limitation does not imply production of H_2, a metabolic pathway that competes with PHA accumulation, as discussed above. An S-free environment links to higher PHA accumulation (Mukhopadhyay *et al.*, 2005). However, studies with P limitations have yielded opposite conclusions (Sali and Mackey, 2021), which may be due to the role of polyphosphate in PHA accumulation, a topic not yet addressed in the literature.

When working with mixed cultures, community analysis is an indispensable tool for optimizing bioprocesses. It is therefore essential to know which are the highest PHA-accumulating species. For example, *Rhodobacter sphaeroides* can accumulate up to 90% of PHA (Sangkharak and Prasertsan, 2007), whereas *Rhodobacter capsulatus*, *Rhodospirillum rubrum* and *Rhodopseudomonas palustris* achieved accumulations of 24%, 45%, and 53%, respectively (Carlozzi, *et al.*, 2019; Hustede *et al.*, 1993). A lesser-known species, *Rubrivivax sp.*, accumulated 85% PHA (Ramana *et al.*, 2006). So far, with mixed PPB cultures, lower but encouraging values have been achieved, such as 60% using acetic acid as substrate (Fradinho *et al.*, 2016) or 31% using fermented domestic water (Almeida *et al.*, 2021). **Strategies to shift communities towards species capable of higher PHA accumulation are still a topic to be explored that has been studied in this research.**

Bioreactor designs could also affect the final output of PHA as light distribution and penetration, and biomass retention can be key factors for the cultivation system. **A** large surface area or surface-to-volume ratio is often preferred as it allows higher light distribution into the media. The most studied geometries include flat-panel, tubular, raceways and CSRT bioreactors:

a) Flat-sheet reactor offers a high surface-to-volume ratio and light exposure as it has a small thickness (Adessi and De Philippis, 2014), furthermore, In flat-panel bioreactors the membrane is easier to assemble into a membrane photobioreactor (MPBR) making volumes smaller and easier to scale up (Hülsen *et al.*, 2018). Hülsen *et al.* (2020) also explored a cylinder pipe-over flow-type photobioreactor to research to investigate the cultivation of PPB in suspended biomass and in biofilm, concluding that the latter is an interesting system to explore.

b) Tubular systems are tubes that can be arranged under different orientations such as vertical, horizontal, or serpentine. They provide high light

Table 1.2. Summary of studies of PHA production on PPB mixed cultures. Adapted from (Sali and Mackey, 2021).

Substrate	Light source	PHA (%wt)	PHA yield	PHA composition PHB: PHV	Refs
Acetic acid	Halogen lamps 16,000 lx	60%	0.45 $g_{PHA}\ g_{COD}^{-1}$	100:0	(Fradinho *et al.*, 2016)
Acetic acid	Halogen lamps 19,000 lx	20%	0.46 $g_{PHA}\ g_{COD}^{-1}$	100:0	(Fradinho *et al.*, 2013)
Propionic acid	Halogen lamps 19,000 lx	-	0.33 $g_{PHA}\ g_{COD}^{-1}$	51:49	(Fradinho *et al.*, 2014)
Acetic and butyric acid	LED light	44%	-	-	(Guerra- Blanco *et al.*, 2018)
Winery wastewater	LED light 4000 lx	-	0.001 $g_{PHA}\ g_{COD}^{-1}$	100:0	(Policastro *et al.*, 2020)
MSW fementate	Fluorescent lamps 4000 lx	-	0.55 $g_{PHA}\ g_{COD}^{-1}$	100:0	(Luongo *et al.*, 2017)
Acidogenic fermentation effluent	Fluorescent lamps 4000 lx	24%	0.212 $g_{PHA}\ g_{COD}^{-1}$	100:0	(Ghimire *et al.*, 2016)
Cheese whey	Halogen lamps 19,000 lx	20%	0.55 $g_{PHA}\ g_{COD}^{-1}$	88:12	(Fradinho *et al.*, 2019)
Fermented domestic wastewater	Halogen lamp 315 $W.m^{-2}$	31%	-	85:15	(Almeida *et al.*, 2021)

penetration and avoid short-circuiting risks of flat-panel reactors, but have high energy requirements for pumping (Sirohi *et al.*, 2022). This type of reactor is mainly used for microalgae cultivation, although some studies have been done with PPB (Carlozzi *et al.*, 2019).

c) Raceway reactors are the most studied and applied in microalgae cultivation due to their low investment and operating costs (Sirohi *et al.*, 2022). However, they have many drawbacks such as control of temperature and water level, high chances of contamination etc. This type of reactor has been studied for the cultivation of PPB for protein production (Alloul *et al.*, 2021), and is currently being studied for the production of PHA.

d) CSTR-type photobioreactors are limited by the low area/volume ratio, which prevents uniform light distribution (Adessi and de Philippis, 2014). However, it provides easy scale-up, higher control of the process and better biomass mixing to ensure cells receive uniform exposure to light. Furthermore, the irradiation problem can be overcome through optical fibers or with IR-LED illuminator tubes that have been developed to overcome this disadvantage (Hülsen *et al.*, 2020).

A CSTR with internal illumination and a hollow fiber membrane was the type of bioreactor chosen for the continuous study of this Thesis. A novel MPBR configuration that has never been studied before.

1.7. Integration of PHA into Photo-biorefinery process

The global PHA production is expected to expand to meet the increasing market demand. However, the low PHA global productivity and overall process yield have limited the full-scale implementation of PHA production using microbial mixed cultures (MMC). The joint optimization of all the necessary steps (pretreatment, fermentation, accumulation) is key to determining the overall economic viability. Efforts on PHA production by MMC in the pilot-scale should focus on its integration with existing processes in waste plants in order to reduce production costs by exploiting the available infrastructure.

Several European projects are developing aerobic MMC projects for PHA production on a pilot scale. For example, the PHARIO project, developed in the Netherlands, has produced PHA for more than 10 months from activated sludge biomass and fermented streams rich in SCCA, resulting in high-quality PHA (Werker *et al.*, 2018). The RES-URBIS project developed a biorefinery in Treviso, Italy, where biogas and PHA were co-produced from the liquid fraction of OFMSW and WAS, achieving a yield of 7.6% PHA over the initial TS (Moretto, *et al.*, 2020). Other projects such as YPACK aim to optimize PHA applications for food packaging or VOLATILE the integration of PHA production in existing biogas plants. The performance of these pilot-scale plants is lower than those reported in laboratory-scale experiments, and further improvements are needed to foster the scale-up of the processes and reduce the overall production costs and environmental impacts.

Process integration becomes even more critical for energy integration, a key parameter in determining the technology's economic viability. In a study in the Lombardy region, pretreatment and separation of OFMSW into two phases (solid and liquid) for separated treatment for the production of PHA and biogas via AD produced a 30% higher energy balance than just AD (Papa *et al.*, 2022). Integrating PHA production into processes such as wastewater or MSW treatment plants, hydrogen production, or biodiesel plants could reduce production costs. In the future, the production of PHA from waste streams will require flexible processes, which could be adapted to different seasonal waste streams by adjusting operational variables. Various studies emphasize the potential and value of the double output in the context of industrial advantage presented by a stable, optimized simultaneous production system culminating from the bioprocesses aimed at a zero-waste strategy (Patel *et al.*, 2021; Thulasidharan *et al.*, 2021). However, scale-up studies of PHA production in PPB photobiorefineries are almost non-existent.

As discussed above, PPB has several advantages in producing PHA and has a metabolism capable of adapting to co-produce other high value-added products by adapting to the substrate and operating conditions seasonally. A recent life cycle assessment (LCA) analyzed the protein production with PPB from food waste (LaTurner *et al.*, 2020). This study inferred the importance of co-production with other high value-added products, e.g. with carotenoids. However, studies are still needed to determine the operating conditions that increase the PHA concentration and production rate by PPB using wastes. The production of PHA with PPB is still in its infancy, but it has entered a new phase. For example, the INCOVER proyect (https://incover-project.eu/) presents research solutions to recover energy (biomethane) and bioproducts (bioplastics, organic acids, biofertiliser, biochar, irrigation water) from municipal, industrial and agricultural wastewater using PPB. Another relevant project is DEEP PURPLE (https://www.deep-purple.eu/) which is establishing the first pilot-scale PPB-based photobiorefinery in Europe. The DEEP PURPLE concept relies upon a versatile, integrated and flexible Multi-Platform Biorefinery, based on the metabolism of PPB to extract and recover high added- value compounds such as PHA, ectoine and cellulose from urban waste (e.g. OFMSW). **The results obtained in this Thesis have provided the foundation for the development of the production of PHA in the DEEP PURPLE project.**

1.8. References

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