UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI BIOLOGIA

Corso di Laurea in Biotecnologie

ELABORATO DI LAUREA

AN ALTERNATIVE TO PLASTICS: PHA AND THEIR PRODUCTION

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ANNO ACCADEMICO 2021/2022

Index

1. **Introduction**
    - 1.1. Synthetic plastics
    - 1.2. Bioplastics: an alternative to synthetic plastics
    - 1.3. Polyhydroxyalkanoates (PHA)
        - 1.3.1. Definition and properties
        - 1.3.2. Biosynthesis
        - 1.3.3. Biodegradation
        - 1.3.4. Applications
2. **Discussion**
    - 2.1. Engineering *Escherichia coli*
        - 2.1.1.Using *Escherichia coli* for the biosynthesis of PHA
        - 2.1.2. Biosynthesis of scl-PHAs, mcl-PHAs and scl-mcl PHAs
        - 2.1.3. Waste products as substrates
        - 2.1.4. Extraction and purification
    - 2.2.Algae
        - 2.2.1. Algae-based bioplastics production
        - 2.2.2. Production of PHA using Microalgae
        - 2.2.3.Production of PHA using Cyanobacteria
        - 2.2.4.Genetic engineering of algae to improve PHA production

References

Abstract

Plastic products became necessary in our society. However, the current fossil-based method of manufacturing plastics results in significant emissions of greenhouse gases. Furthermore, synthetic plastics, because of their durability and toughness, are accumulating as waste in our environment, causing a serious threat. For these reasons, bio-based and biodegradable plastics are currently being used as a substitute. Among these, polyhydroxyalkanoates (PHAs) are one kind of biodegradable plastics which are gaining attraction. PHAs serve as an intracellular carbon and/or energy storage reserve in various microorganisms under unfavorable growth conditions. They can be applied in various fields, since they are thermoplastic, biocompatible and non-toxic.

The aim of this literature research is to focus on PHA production methods which are sustainable both environmentally and economically. It enlightens the recent application of engineered *Escherichia coli* as a host for the production of PHAs. In particular, it outlines the use of waste products as substrates for PHA production. Another production method is by using Algae, which can be used as a potential biomass source. An outline on the strategies of using Microalgae and Cyanobacteria is done, also adding a focus on genetic engineering to improve PHA production.

1. Introduction

1.1. Synthetic plastics

Synthetic plastics have been a widely used resource for many years in our society, since the invention of the first synthetic polymer in 1869 by John Wesley Hyatt. Synthetic plastics became essential during World War II, in particular nylon and poly-methyl methacrylate (PMMA) were produced in large demand (Sudesh and Iwata, 2008). At first, plastics were seen as a revolution, due to the vast application fields, from food packaging and clothes to communication, transportation, construction, and health care. However, during the 1960s plastics started to be seen less positively. People started to recognize that plastics caused accumulation of waste and pollution.

Nowadays, around the world, 78 million tons of plastic packaging are manufactured annually; of these, only 2% comes from closed-loop recycling. Also, 32% of the plastic products produced remain in drainage ditches and streams flowing out in the oceans (Filiciotto and Rothenberg, 2021). Because of its durability, toughness, and resistance to natural degradation, these amounts of waste are a serious threat for the environment. The accumulated plastics in the environment can be decomposed into microplastics and nanoplastics, increasing their distribution and finally endangering the survival of all forms of life on earth (Lebreton and Andrady, 2019).

Other than waste disposal problems, the manufacturing method itself is polluting. Currently almost all the plastics used are produced from petroleum and synthetic resources leading to significant emissions of greenhouse gases, which are one of the principal reasons for global warming and climate change. In synthesis, traditional synthetic plastic products and their production are not sustainable for the environment anymore.

1.2. Bioplastics: an alternative to plastics

A more sustainable alternative was proposed with the development of bioplastics. The term "bio-plastics" refers to polymers which are bio-based, biodegradable, or both (Moshood, Nawanir and Mahmud, 2022). Bio-based means that a material is derived from biomass, that is, organic material of biological origin or that the feedstock used for producing a material derives from any form of organic waste. On the other hand, biodegradable means that the material can be converted into environmentally sound substances under natural environment conditions after use. It can be decomposed by microbes existing in nature to materials which can be integrated into the natural ecosystem. Biodegradability may vary depending on humidity, temperature, and other conditions (Nazareth et al,. 2019). Not all bio-based polymers are biodegradable and vice versa not all biodegradable polymers are bio-based. Poly lactic acid (PLA), for instance, is a crystalline biobased polymer which is not biodegradable. On the other hand, some petroleum-based plastics are biodegradable.

Biodegradable plastics present a major benefit regarding the waste disposal problem. In theory, they can potentially replace most of the conventional plastics, particularly plastic packaging. Many polymers have been investigated by now, but their production level is still very low. This is mainly due to economic factors (Moshood, Nawanir and Mahmud, 2022): biodegradable plastics are much more expensive than conventional plastics. Also, the production of plastics is too large in the world to be replaced in a short period of time. In addition, the criteria that a polymer has to fulfil to be suitable are many, including suitable strength, flexibility, non-toxicity, impermeability to oxygen or other gases, good moisture resistance, stability during storage and low cost starting materials and production process. These and other challenges must be overcome to replace conventional plastics and solve plastic pollution problem. Key factors for doing this come from public environmental awareness and political and financial measures.

The methods to produce biobased and biodegradable polymers can be classified into three main groups based on the processes involved and also the types of the polymers (Balal and Farid, 2016) The first method is via chemical polymerization of monomers derived from biological processes, PLA for instance. Another is the direct biosynthesis of polymers in microorganisms, and polyhydroxyalkanoates (PHAs) are the most prominent example. Lastly, they can be produced from the modification of natural polymers such as starch and cellulose.

1.3. Polyhydroxyalkanoates (PHA)

1.3.1. Definition and properties

One of the most notable biobased and biodegradable plastics are polyhydroxyalkanoates (PHA). They were first studied and characterized in the 1920s and started to receive serious attention during the petroleum crisis in the 1970s. PHA are a class of linear polyesters synthesized by numerous bacteria as insoluble carbon and energy storage compounds. They are biosynthesized only during times of environmental stress conditions, when there is excess of carbon and limiting concentration of essential nutrients such as nitrogen, magnesium, phosphorus and oxygen (Możejko-Ciesielska and Kiewisz, 2016). They consist of hydroxy acid monomers (HA) connected by an ester bond between the carboxylic group of a monomer with the hydroxyl group of a neighboring one (Figure 1). Their structure and features depend on the carbon compound supplied as the growth substrate, the bacterial host, and the fermentation conditions used towards their production.

They can be classified into three groups according to the length of the side chain (Raza et al., 2018): short-chain length PHAs (scl-PHAs) consisting of C2-C5 atoms, medium-chain length PHAs (mcl-PHAs) consisting of C6-C14 atoms and long-chain length PHAs (lcl-PHAs). Scl-PHAs are stereoregular polyesters and therefore their structure is highly crystalline. They have high melting and low glass transition temperatures. The high crystallinity makes them relatively stiff and brittle. Scl-copolymers such as P(3HB-co-3HV) are known as more desirable than scl-homopolymers because their melting point is much lower, and they are less crystalline, easier to mold, and tougher. Mcl-PHAs act as elastomers within a very narrow temperature range due to their low melting temperature. They are more flexible and elastic materials than scl-PHAs. Another group of PHAs were demonstrated to be synthesized by a limited number of bacteria; it consists of monomer units from C3 to C14 forming scl-mcl PHAs copolymers. These have properties between the scl state and the mcl state, depending on the ratio of the monomers. Chemical modifications of the PHAs side chains can be used to introduce the desired functional group into natural PHAs influencing the material properties of the polymers to a great extent.

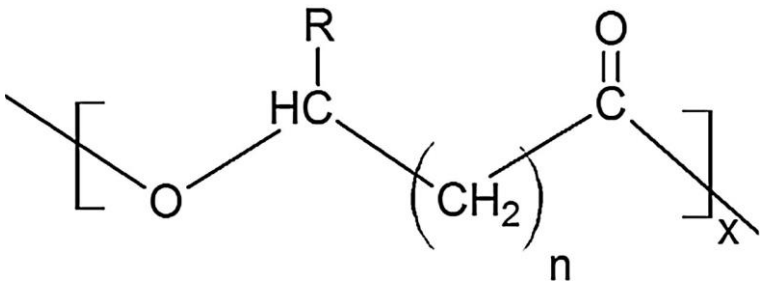

Figure 1: Structure of polyhydroxyalkanoates; n varies from 1 to 4, x varies from 100 to 300 000, R is the alkyl side chain. Based on Możejko-Ciesielska & Kiewisz, 2016.

1.3.2. Biosynthesis

The natural metabolic production of PHAs can be classified into two groups. The first one is represented by *Ralstonia eutropha* and the process is initiated by the condensation of acetoacetyl-CoA and ends with the synthesis of polyhydroxybutyrate (PHB). The second group is represented by *Pseudomonas aeruginosa* and is synthesised by fatty acid de novo biosynthesis or β-oxidation (Steinbuchel and Lutke-Eversloh, 2003). Biosynthesis pathways are shown in Figure 2.

The biosynthetic pathway of *R. eutropha* involves three key enzymes, phbA, phbB, and phbC and has been known for many years (Schubert et al., 1988). The first one is a β-ketothiolase which catalyzes the condensation of two acetyl-CoA molecules into acetoacetyl-CoA. PhB, an NADPH–dependent acetoacetyl-CoA reductase, then reduces it to (R)-3-hydroxybutyryl-CoA. The production of PHB is catalyzed by the PHB synthase phbC. This pathway leads to the production of scl-PHAs. Various *Pseudomonas*, on the other hand, are capable of accumulating mcl-PHAs in large amounts (Steinbuchel et al.,1992). The production follows two different metabolic routes: the degradation of fatty acids β-oxidation and de novo fatty acid biosynthesis. Enoyl-CoA, an intermediate from β-oxidation cycle, is converted to R-3-hydroxyacyl-CoA by R-3-hydroxyacyl-CoA hydratase (Pha J). The resultant R-3-hydroxyacyl-CoA is then transformed by mcl PHA synthase into mcl-PHA. R-3-hydroxyacyl-CoA can also come from R-3-hydroxyacyl-ACP which is an intermediate product of fatty acid de novo synthesis.

In recent times, scientists have investigated the possibility to develop novel PHAs with enhanced properties using metabolically engineered organisms. Specifically, native PHA-producing bacteria can be genetically engineered to increase their polymer production. *Cupriavidus necator* for instance can accumulate PHAs in satisfactory amounts (Valappil et al., 2007; Chanprateep, 2010). On the other hand, non-native bacteria can be modified to produce PHA. *E. coli* is the most promising organism for the large-scale production of PHA (Horng et al., 2010).

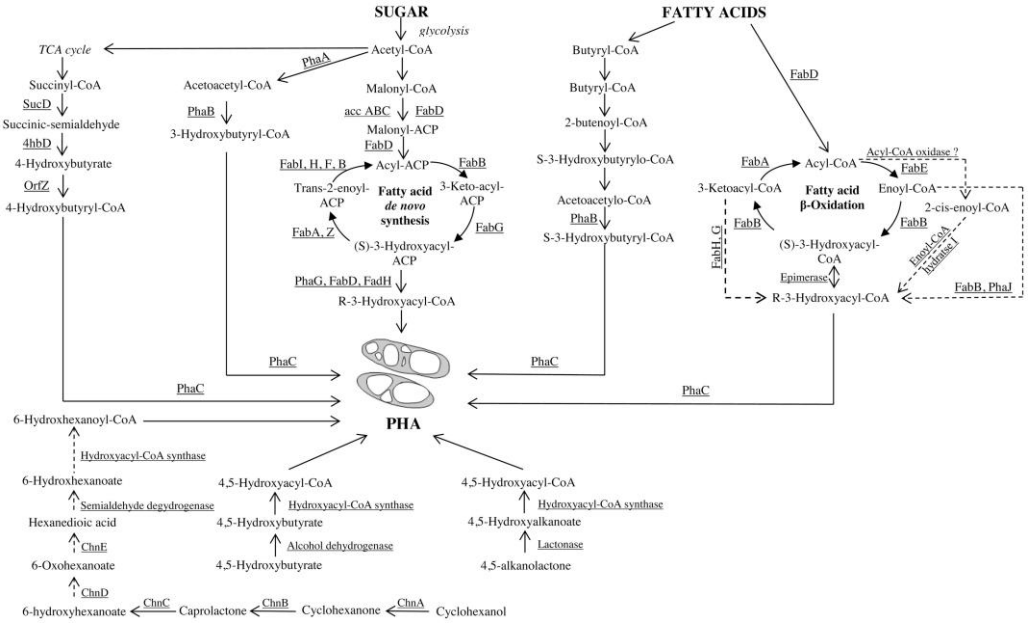

Figure 2: PHAs biosynthesis pathways, based on Możejko-Ciesielska and Kiewisz, 2016

1.3.3. Biodegradation

PHAs degradation consists in their assimilation by bacteria and/or fungi to give environmentally friendly products. PHAs have been found to be able to degrade in environments with high microbial activity, such as soil, lake and marine waters and even sewage sludge (Mergaert et al., 1993). Microorganisms able to degrade PHAs possess intracellular or extracellular PHA depolymerases, which vary in their molecular organization and substrate specificity. These enzymes hydrolyze water-insoluble PHAs into water-soluble forms so that it can be utilized by microorganisms. The rate of biodegradation depends on several environmental factors such as temperature, microbial population, nutrient supply, pH, moisture level as well as the conditions of the PHA materials including the surface area of the PHA, its composition and crystallinity (Doi, 1990).

PHA depolymerases consist of a catalytic domain and a substrate-binding domain connected by a linker domain. The process of degradation begins with the substrate domain which binds to the PHA material; then, the catalytic domain starts to cleave the polymeric chain. The intracellular PHA depolymerase (PhaZ) is involved in the degradation of intracellular PHA, which is stored as insoluble inclusion bodies (Jendrossek and Handrick, 2002). Degradation takes place when the carbon source is exhausted. Accumulated PHA granules in the cells are hydrolyzed as carbon and energy sources (Luengo et al., 2003). From there, PHA is broken down to 3-hydroxyalkanoic acid. If the PHA consists of only one type of monomer, for example, 3-hydroxybutyrate, the resulting PHA is called poly(3-hydroxybutyrate) [P(3HB)] homopolymer. P(3HB) is the most common type of PHA synthesized by most bacteria naturally. The intracellular degradation of P(3HB) results in the liberation of 3-hydroxybutyric acid which is then oxidized by a dehydrogenase to acetoacetylCoA, which is finally converted into acetyl-CoA by β-

ketothiolase. The breakdown products of PHA are naturally found in animals, therefore they are not toxic but biocompatible.

Extracellular depolymerases were found to possess the ability to hydrolyze partially crystallized P(3HB) (Eggers and Steinbüchel, 2013). Besides PHA depolymerase, several lipases are capable of hydrolyzing poly(ω-hydroxyalkanoates) such as poly(6-hydroxyhexanoate) [P(6HHx)] and poly(4-hydroxybutyrate) [P(4HB)] and are specifically active towards polymers with no side chains in the carbon backbone (Jaeger et al., 1995). There are few parameters such as monomeric composition and crystallinity of the PHA that can be included to evaluate the degradation rate of PHA.

1.3.4. Applications

PHAs are considered a good replacement for petrochemical polymers because of their biodegradability, thermoplasticity, biocompatibility and non-toxicity. Moreover, they are insoluble in water, enantiomerically pure and display a high degree of polymerization. These properties make them suitable for applications in various areas (Możejko-Ciesielska and Kiewisz, 2016). They can be used for packaging, biomedical, and agricultural applications. They can act as carriers for long term release of herbicides or insecticides.
PHA are considered optimal polymers for biomedical applications. The homopolymer P(3HB) and the copolyester P(3HB-co-3HV) are the most studied. In recent years they have been considered as materials in the fabrication of cardiovascular products, in drug delivery systems, in wound management, and orthopedics.

2. Discussion

2.1. Engineering *Escherichia coli*

2.1.1. Using *Escherichia coli* for the biosynthesis of PHA

As previously explained, wild type and recombinant bacteria can be used to produce PHAs through fermentation processes. Of all bacteria, one of the most used is *Escherichia coli,* but it does not normally produce PHAs. Schubert et al. (1988) genetically engineered the first metabolic pathway to enable the synthesis of scl-PHAs was carried; they cloned the whole phb gene operon into *E. coli.* After that, many researchers identified and functionally expressed PHA biosynthesis genes in *E. coli* (Li et al., 2007). Experiments reported that genetically modified *E. coli* is able to accumulate up to 90% of P(3HB) (Wang et al., 2009). Genes responsible for PHA biosynthesis from several microorganisms, such as *Cupriavidus necator* (Vandamme and Coenye, 2004; Horng et al., 2010); *Pseudomonas aeruginosa* (Lagenbachet al., 1997); *Alcaligenes latus* (Choi et al., 1998; Seo et al., 2004); *Thiocapsa pfennigii* (Liu and Steinbüchel, 2000) and *Streptomyces aureofaciens* (Mahishi et al., 2003), have been introduced into *E. coli*.

E. coli is an ideal host for the biosynthesis of PHA, as it is vastly known and there is proper experience to culture it. Its genome can be easily modified and it can use a vast range of substrates. Furthermore, it is capable of accumulating PHA polymers to a high content possibly leading to large-scale production of PHA. It can be recovered efficiently and easily by inexpensive chemicals. *E. coli* grows quickly at high temperatures and it can be easily lysed for cost-effective PHA granules purification.

2.1.2. Biosynthesis of scl-PHAs, mcl-PHAs and scl-mcl PHAs

The pathways of biosynthesis of PHAs can be classified into different groups, depending on their structure and chain length. For scl-PHAs biosynthesis, metabolically engineered *E. coli* has been proven as the most promising host microorganism. The biosynthesis of PHB in *R. eutropha,* the natural-producing bacteria, has been described above (1.3.2). The two key genes phbA and phbB are involved in the formation of 3-hydroxyacyl-CoA with short side chain. To reproduce this metabolic pathway in *E. coli*, both of the genes together with phbC, which encodes PHB synthase, have to be functionally expressed in the host. In metabolically engineered *E. coli*, the presence of NADPH and acetyl-CoA is crucial for the production of P(3HB). Anaerobic fermentation is a low-cost production method for the P(3HB) biosynthesis. Higher P(3HB) conversion rates, reliable control methods, a simpler reactor design and mild operating conditions are the key causes of this. Low oxygen levels cause *E. coli* to undergo mixed acid fermentation, which results in the production of byproducts such lactate, acetate, formate, succinate, and ethanol (or H2 and CO2). To reduce the formation of by-products, the ldhA, poxB, ackA-pta, pflB and adhE genes have been knocked out from *E. coli BW25113*. Because of the deletion of these genes, most of the recombinant strains consume less glucose, while acetate formation drops by 90% and other by-products are not detected.

Mcl-PHA, on the other hand, are synthesized and accumulated in large amounts by a variety of *Pseudomonas* (Steinbuchel et al., 1992). The co-monomer composition of mcl-PHA depends mainly on the carbon source, the cultivation conditions, and the metabolic routes leading to PHA formation (Steinbuchel et al., 1995). The first successful metabolic production of mcl-PHA in *E. coli* was accomplished by the employment of a fatty acid oxidation deficient strain, *E. coli* fadB mutant (Langenbach et al., 1997). Up until 1997, the Steinbuchel group used the fadB mutant with a partially inhibited fatty acid oxidation pathway to successfully produce mcl-PHA in *E. coli.* Later, by cloning phaC1 onto the host cell genome, Prieto et al. created a stable mcl-PHA producer (Prieto et al., 1999). The enzymes responsible for channeling the β-oxidation intermediates to mcl-PHA biosynthesis in the *E. coli* fadB mutant were discovered to be partially encoded by yfcX and maoC (Park and Yup Lee, 2004). More recent applications were the synthesis of mcl-PHA in recombinant *E. coli* from non-fatty acid feedstock such as glycerol and glucose, with the result of mcl-PHA production up to 0.4 g/L (Wang et al., 2012b). *E. coli* does not utilize fatty acids at all because of carbon catabolite repression (CCR). CCR is a phenomenon closely related to the sugar

phosphotransferase system (PTS) where *E. coli* chooses to consume substrate that can provide the highest growth rate first in a mixture of carbon sources. To overcome this drawback, an *E. coli* strain with disrupted PTS was constructed to synthesize mcl-PHA efficiently from a substrate mixture which consists of various carbon sources. Recombinant *E. coli* LR1110 harboring the phaC1 gene from *Pseudomonas aeruginosa* successfully utilizes fatty acids (decanoate) and glucose simultaneously producing mcl-PHA with cell density up to 1.6 g/L.

Co-polymers known as short-chain-length-medium-chain-length (scl-mcl) PHAs combine the mechanical and thermal properties of scl-PHAs and mcl-PHAs, making them appropriate for a variety of commercial applications (Li et al., 2011). One example is the copolymer P(3HB-co-3HHx) which contains less than 20% mole fraction of 3HHx (Lu et al., 2003, 2004). Using decanoate as a carbon source, recombinant *E. coli* was able to produce scl-mcl PHA copolymers with a 3HHx up to 63 mol% composition. To further boost the mole fraction of 3HB and because it can provide additional acetyl-CoAs, sodium gluconate is added. As a result, the carbon sources in scl-mcl PHAs can be changed to alter the monomer composition (Park and Lee, 2004a). Adding glucose not only boosted the 3HB mole percentage up to 83.4%, but it also had a favorable impact on the biomass and PHA content (Li et al., 2011). PHA synthases from *A. caviae, Aeromonas hydrophila, Pseudomonas sp. 61-3, P. stuzteri* and *N. corallina* are able to incorporate 3-hydroxyacyl-CoA with both short and medium side chain (Hall et al., 1998; Matsusaki et al., 1998; Chen et al., 2004). Metabolic pathways resulting in the creation of these precursors were developed to offer both types of precursors for co-polyester biosynthesis in *E. coli*. Recombinant *E. coli* LS5218 produces scl-mcl PHA copolymers with a concentration of 0.118 g/l when fabG from either *Pseudomonas sp. 61-3* or *E. coli* with a PHA synthase gene is coexpressed with it (Nomura et al., 2008). Aiming to synthesize scl-mcl PHA copolymers in recombinant *E. coli* from unrelated carbon sources (e.g., glucose), coexpression of both PHA synthases and 3HB-CoA monomer supplying enzymes with different mutant fabH genes have been realized (Nomura et al., 2004). Scl-mclPHA copolymers of concentration up to 1.435 g/L with 3HHx and 3HO of mole fraction up to 0.6% and 0.1%, respectively, have been effectively synthesized by recombinant *E. coli* JM109. The ability of fabG, encoding a 3-ketoacyl-ACP reductase, to convert 3-ketoacyl-CoA to 3-hydroxyacyl-CoA with a wide range of substrates was demonstrated. When this gene was coexpressed with phaC in *E. coli*, 0.041 g/L of scl-mcl PHA copolymer with compositions of 3HHx, 3HO, 3HD, and 3HDD up to 8.3%, 2%, 1.3%, and 1.2%, respectively, was produced (Nomura et al., 2005). Both works agreed that both fabH and fabG play an important role in promoting PHA yield**.** Later, the production of scl-mcl PHA copolymers from recombinant *E. coli* LS5218 instead of JM109, harboring the same genes and using the same carbon source, was investigated. This resulted in a yield up to 0.406 g/L and composition of 3HHx, 3HO, 3HD and 3HDD up to 13.3%, 0.8%, 0.9% and 0.6% respectively (Nomura et al., 2008). These protein engineering techniques are useful in producing scl-mcl PHA copolymer with desired monomer composition and increase the flexibility in carbon source utilization.

2.1.3. Waste products as substrates

PHA production rate relies on the type of carbon source, nutrient concentration, and the metabolic pathways of fermenting microorganisms (Ganesh Saratale et al., 2021). The use of carbon substrates is the main expense in the pathway for PHA production. This can be attributed to the fact that PHA accumulation is mainly an aerobic process, leading to the waste of carbon by intracellular respiration (Hermann-Krauss et al., 2013). Availability of affordable and sustainable carbon sources is therefore very crucial, making waste streams perfect carbon sources for PHA production (Khatami et al., 2021).
PHA can be produced from a wide range of substrates, for instance:

1. renewable sources such as cellulose, hemicellulose, sucrose, glucose, starch, and triglycerides;
2. organic acids such as 4-hydroxybutyric acid and propionic acid;
3. alcohols such as methanol, n-amyl alcohol, 1,3-propandiol, and glycerol;
4. waste substrates such as molasses, corn steep liquor, rice bran, and fatty acids;
5. wastes such as waste water, activated sludge effluents, and kitchen waste.

To be optimal substrates, they must follow basic requirements: they must be available in sufficient amounts and constant quality, as invariable as possible, stable, resistant against microbial spoilage (Koller et al., 2017).

Molasses

About 4 tons of molasses and 10 tons of sucrose are produced during the extraction of 100 tons of sugarcane. As a waste stream, the sugar factories produce about 50 million tons of molasses annually (Ganesh Saratale et al., 2021). Molasses consists of sucrose and a lesser amount of glucose and fructose with a small concentration of vitamins and trace elements (Raza et al., 2018). Yeast fermentation using molasses as a potential substrate has been widely used to produce liquid biofuels and value-added biochemicals (Saxena et al., 2009). To utilize molasses efficiently as a carbon source (sucrose) there is a crucial requisite to eliminate the contaminants, for instance, polyphenols and inorganic salts, which can suppress the growth of microorganisms. This can be possible by employing various membrane filtration techniques (Sjolin ¨ et al., 2020).

Waste oils

Plant oils as a carbon feedstock have many advantages over other conventional carbon sources, sugars for instance, including price competitiveness and significantly higher yields of PHA. Triacylglycerols (TAG) are the main constituents of plant oil. The microorganisms must be able to secrete lipases that hydrolyze TAG into fatty acids, which are then transformed to PHA via the -oxidation pathway in order to use them as a source for PHA production (Talan et al., 2020). Significant amounts of lipid-rich waste are produced by a variety of food processing companies, the processing of edible oils, slaughterhouses, and oil mills

(Jiang et al., 2016). The management of such oil waste is a significant concern because its presence in soil ecosystems and water bodies results in significant environmental contamination, water deoxygenation, and adverse effects on the ecosystem. Therefore, using cheap oily wastes as a growth substrate for PHA production might be a workable solution to reduce production costs and make the process environmentally friendly. Oil can be thought of as a more effective source of PHA than carbohydrates because oil produces PHA at a rate of 0.6-0.8 g/g compared to 0.3-0.4 g/g of glucose in sugar (Cruz et al., 2016).

Whey

Whey is one of the by-products of producing cheese and is mostly made of lactose, proteins, and lactic acid. According to estimates, wastewater treatment plants discard close to 50% of the whey (Pescuma et al., 2015). One benefit of using cheese whey as a feedstock is that no complicated pretreatment is required before acidogenic fermentation, which is advantageous from an economic standpoint. Recombinant *Escherichia coli* is frequently used in studies that employ whey as the carbon source because many conventional PHA-producing bacteria are unable to directly metabolize whey due to a lack of β-galactosidase activity. For instance, Ahn et al. (2001) obtained PHB concentrations and PHB contents of 168 g/L and 87%, respectively, using a recombinant strain of *E. coli* carrying *Alcaligenes latus* PHA producing genes.

Biodiesel industry waste (Glycerol, methanol)

Crude glycerol, which is produced in significant quantities by the transesterification of oils and fats with biodiesel (approximately 10%), is a key by-product. Glycerol can be viewed as an effective carbon source for PHA production because of the fewer carbon atoms present in its structure, in comparison to carbohydrates. A significant amount of glycerol is produced as a waste stream as a result of the enormous amounts of biodiesel that are produced globally. This strategy could be a helpful means of offsetting the cost of producing biodiesel (Koller and Braunegg, 2018). However, there are a few more chemical components found in crude glycerol as impurities, such as biodiesel residues, free fatty acids, soap, methanol, and salts. Methanol, which inhibits bacterial growth and fermentation, is found in crude glycerol. Removal of methanol can be achieved by applying vacuum-assisted evaporation, or advanced phase separation practices.

2.1.4. Extraction and purification

The extraction and purification of PHA from bacterial cell biomass is time consuming, requires harsh chemicals and causes an increase in the total PHA production cost. Downstream processing contributes about 50% of the overall production expenses; thus, it is a crucial aspect to define the PHAs biomanufacturing costs (Li and Wilkins, 2020). This procedure is crucial because it establishes the polymers' quality, which in turn determines their final application and market worth. Different factors, such as the content and type of biopolymers,

the producer of PHAs, the purity of the product, costs, and environmental considerations must be considered when choosing a suitable recovery strategy. *E. coli*, being a recombinant producer, has a thinner and more fragile cell membrane compared to wild type PHA accumulators (Mohammadi et al., 2012), making the purification process easier. In general, recovery methods can be categorized in two groups (Jacquel et al., 2008):

1. Digestion methods where the biomass is dissolved to separate the PHA granules
2. Direct extraction of PHA from the biomass using proper solvents.

The digestion can be carried out through chemical or enzymatic treatments (Raza et al., 2018). The most prevalent chemical procedure for the recovery of PHA is solvent extraction, in which solvent increases the bacterial cell membrane permeability and solubilize the PHA. Normally, it consists in the immersion of the PHA-containing biomass into a proper solvent or mixture of solvents to dissolve the granules, followed by addition of a precipitating agent to retrieve the polymers in the crystal form (Kosseva and Rusbandi, 2018). Solvent extraction is usually used when high purity polymers are desired (Jacquel et al., 2008). Some of these solvents, nevertheless, are unsustainable and pose grave risks to human health and the environment. For more environmentally friendly alternatives, anisole, cyclohexanone, and phenetole have been suggested (Rosengart et al., 2015). Samor et al. (2015) examined a unique method of PHA extraction that used dimethylcarbonate (DMC) as a green alternative to traditional chlorinated solvents. Without any loss in purity or molecular weight, polymer recoveries comparable to dichloromethane use were attained. Novel extraction techniques have been proposed by using switchable anionic surfactants (SAS) as a sustainable substitute for surfactants which are more economical and less solvent intensive (Mannina et al., 2019).

2.2. Algae

Algae are divided into microalgae and macroalgae based on their size and morphology. They can grow in different habitats such as freshwater or marine environments that contribute to their diversity, namely freshwater microalgae or marine macroalgae (Dang et al., 2022). They can grow under polluted conditions such as CO2-rich gases or nitrogen and phosphorus-containing wastewater.

2.2.1. Algae-based bioplastics production

Algae are becoming more and more popular as a potential new biomass source for making bioplastics. In the modern era of bio-based plastic production, phycological approach surpasses the other traditional methods which rely on terrestrial crop plants as the feedstock (Chew et al., 2017). Indeed, algae do not compete with food production for human consumption. Furthermore, they tolerate harsh environmental conditions, can remediate wastewater, and utilize carbon dioxide as a nutrient source for biomass production. In conclusion, algal-

based bioplastics provide a viable and non-toxic alternative that can lessen the consumption of fossil fuels, improve the quality of plastics, and limit the harm to the environment caused by the excessive use of plastics derived from petroleum.

Algal-based bioplastics can be produced through a few approaches, such as direct use of microalgae biomass, blending with other materials, inclusion of biorefinery concept and enhancement of bioplastics production through genetic means and manipulation of growth conditions as well.
The algal biomass comprises protein and carbohydrate-based polymers which can be utilized as one of the bioplastics components. Currently, some of the components from algal biomass utilized to create biodegradable plastics include starch, cellulose, PHA, PHB, PLA, and protein-based polymers (Mal et al., 2022). PHA is the polymer that is most frequently suggested for use in the production of bioplastics since it can be broken down by enzymatic action. In addition, PHB recently has emerged as a new polymer to produce bioplastics because of its good oxygen barrier properties.

The steps for algal-based bioplastics production, characterization and commercial use are the following (Mal et al., 2022) (Figure 3):

1. Strain selection
2. Large scale cultivation. The nutrient regime, other growth factors and conditions, and the choice of appropriate strain should be considered in order to improve the yield. Large scale algal cultivation mainly relies on both open and closed systems; however, there are contradictions regarding the establishment of a standard system. Open cultivation systems cannot eliminate the risk of contamination; in contrast, this risk can be managed fairly well in closed systems, such as photobioreactors (PBRs).
3. Biomass harvesting
    a. Centrifugation: the algal biomass is sedimented under a huge centrifugal force in addition to gravitation and the biomass recovery depends on the range of the sedimentation along with its time and temperature (Mal et al., 2021). But huge maintenance, installation and construction cost makes it unsuitable for large scale cultivation.
    b. Flocculation: it relies on the lumping of the algal biomass using flocculants or manipulating the cultivation condition, for example through pH change. This method is very well-defined for freshwater algae, but not for their marine counterparts. Several polyelectrolytes of aluminum and iron are mostly sound flocculants, but their addition may harm further down-stream processing. (Satpati and Pal. 2018).
    c. Filtration: Borowitzka (2005) claims that dead-end filtration, in which the diluted cell suspension is passed over a membrane filled with filters, is the most straightforward method for harvesting algae. However, employment of this method is limited by the rheological traits of the grown strain, which readily obstruct the

filtration pathway by covering it with a compressible algal mat. Both biomass and metabolites are separated using ultrafiltration, a more advanced form of membrane filtration.

d. Floatation: algal biomasses are collected as scum after being allowed to froth over the medium during flotation. Dissolved air flotation (DAF) and froth flotation are two popular flotation methods. In the first approach, the culture media are ozonated before being treated with polyelectrolyte salts, creating tiny bubbles that induce the floccules to float over the surface. The second technique produces algal foam by adjusting pH and bubbling via air columns (Satpati and Pal, 2018).

4. Drying of biomass
5. Bioplastics production: the approach relies either on the direct synthesis of intracellular polymer or fermentation of algal biomass for biopolymer production (Cinar et al. 2020).
6. Characterization
7. Downstream processing and plastic film casting
8. Commercial usage

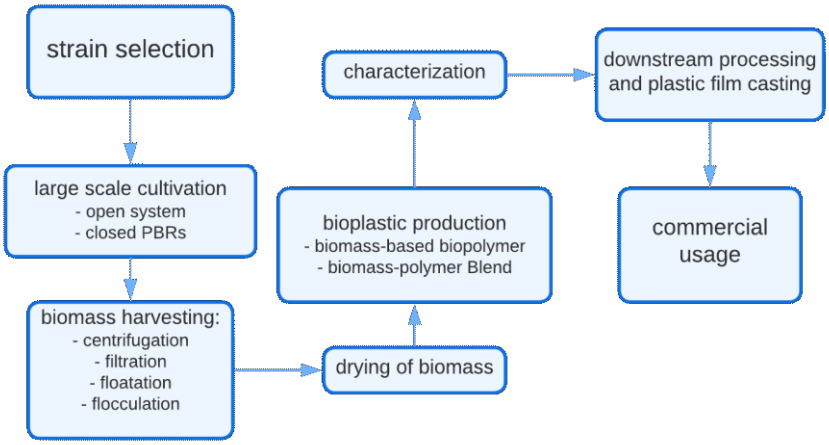

Figure 3: Synthesis of algae based bio-polymers, characterization and commercial use

2.2.2. Production of PHA using Microalgae

Most intracellular biopolymer production methods have laid emphasis on PHB because it is naturally synthesized by various microorganisms. Several studies have proposed that the production of PHAs takes place when microalgae grow under a condition of nutrient deficiency. The nutrient-deficient condition diverts microalgal metabolic pathway to forming carbon-rich compounds (e.g., PHAs) (Mendhulkar and Shetye 2017), likely attributed to preferential degradation of one or more macromolecules contained in microalgae responding to the nutrient-limited conditions (Coelho et al. 2015).

Microalgae are able to accumulate PHB intracellularly when growing with nitrogen or phosphorus deficiency. This is favored due to the high NADPH pool, when ATP

production, electron transfer and protein synthesis are downregulated (Nanda & Bharadvaja, 2022). The PHA product is accumulated intracellularly in the cytoplasm of microalgae, so extraction of the accumulated PHA from the microalgal cells is required after the cultivation of microalgae. The PHA product can be recovered mostly by chemical methods, using solvents such as acetone, chloroform, ethyl acetate, isoamyl alcohol, methylene chloride, methyl isobutyl ketone, and propylene carbonate (Costa et al. 2018b) or biological methods, through the hydrolysis of cells using proteases, lysozymes, and nucleases (Suzuki et al. 2008). After the recovery of the PHA product, precipitation or sedimentation is conducted to terminate the process (Ghatnekar et al. 2002). PHA accumulation in microalgae is highly associated with the amount of acetate supplied, nutrient conditions, and light/dark conditions (Monshupanee et al. 2016). *Calothrix scytonemicola, Neochloris oleoabundans, Scenedesmus almeriensis* (Johnsson and Steuer 2018), and *Microcystis aeruginosa* (Abdo and Ali 2019) were found to be suitable microalgae species for the intracellular production of PHA because they are rich in PHA or starch.

Microalgae are very small in size and are highly sensitive to moisture. So, mixing them with materials that complement their properties makes them cheaper, highly processable and widely applicable. Acetone and sodium sulfite are used for washing the biomass before the blending process (Cinar et al. 2020).

2.2.3 Production of PHA using Cyanobacteria

Cyanobacteria are unicellular organisms found in fresh, marine, and brackish water. They accumulate the homopolymer of PHB as intracellular granules under stressful environments. Out of 137 screened cyanobacterial strains from 88 species, 63 of them showed extraordinary PHB accumulation, when subjected to nitrogen, phosphorus and potassium deficiency (Nanda & Bharadvaja, 2022). Furthermore, Cyanobacteria do not require as much sugar as heterotrophic microorganisms for PHB production, resulting in less impact on agricultural activity. They reserve PHB as a surplus carbon and energy source to be metabolized later under unfavorable conditions. They are a preferred host system due to their ability of photoautotrophy and survival in minimal nutrient supply. They are powerful CO2 scavengers and significantly reduce greenhouse gas emissions (Sharma and Mallick 2005). Acetate supplementation in the beginning along with nitrogen depletion supplies 44–48% of the total carbon required for PHB synthesis, doubling the accumulated PHB amount. (Dutt and Srivastava 2018). Hein et al. found that *Synechocystis sp.* PCC6803 has inherent PHB synthase activity and is a well-suited PHB expression system due to ease in genetic modification and spontaneous transformability. *Spirulina* species is a Cyanobacterium found commonly in highly alkaline freshwater, which possesses 46% to 63% protein content.

Typically, the PHB content in Cyanobacteria is less than 10%, one order of magnitude lower than that of heterotrophic bacteria (up to 87%) (Lane and Benton, 2015). However, both phototrophic and heterotrophic conditions can

stimulate PHB accumulation, depending on the Cyanobacteria strains, such as genera: *Synechocystis, Synechococcus, Arthrospira (Spirulina), Nostoc*, and others. Using thermophilic Cyanobacterium, *Synechococcus sp. MA19PHB* can spike up to 55% (w/w) of PHB under phosphate-limited culturing conditions (Nishioka et al., 2001). S*ynechocystis PCC6803* under heterotrophic conditions can produce 38% (w/w) of PHB in 10 days (Panda and Mallick, 2007). *Nostoc muscorum sp.* yielded 35% (w/w) of dry cells when cells supplemented with 0.2% acetate were subjected to dark incubation for 7 days (Sharma and Mallick, 2005). PHB extraction from Cyanobacteria can be achieved by using sodium hypochlorite, methanol, and hot chloroform.

2.2.4 Genetic engineering of algae to improve PHA production

Genetic engineering is easy to perform on simple, single-celled phototrophs like microalgae and cyanobacteria due to no cell differentiation as in complex plants (Nanda & Bharadvaja, 2022). It has been successfully reported in PHA synthesizing *C. reinhardtii, Synechococcus sp.* and *Synechocystis sp.* Cyanobacteria *Synechocystis sp. PCC 6803* is widely researched for enhancement of PHA production via the genetic engineering route. The reasons behind it are its well-studied metabolic pathways, optimized growth conditions and proper characterization.

Cyanobacteria and microalgae can be genetically modified in various ways. Random mutagenesis method uses physical mutagens like UV radiations or chemical mutagens like ethyl-methanesulfonate and ethidium bromide (EtBr) which lead to a transversion in the algal genome when exposed (Kamravamanesh et al. 2018b). Metabolic engineering of algal and cyanobacterial strains has been attempted to introduce PHB production pathways, enhance the PHB yield, produce new PHBs and to utilize cheaper and a variety of substrates to achieve economies of scale. Metabolic engineering is the science of exploring metabolic pathways and understanding the role of enzymes and molecules involved in them. Chaogang et al. (2010) have paid attention to one chemolithotrophic bacterium, *Ralstonia eutropha*, an effcient PHB producer under nutrient deficiency. They isolated the phbB and phbC genes from this organism and incorporated this gene within *C. reinhardtii* to enhance the intracellular PHA accumulation, which results in the accumulation of copious amounts (6 mg/g dry cell weight) of intracellular PHA. Furthermore, heterologous transformation of cyanobacteria with PHB synthetic genes (3-ketothiolase, acetoacetyl-CoA reductase and PHB synthase) of *R. eutropha* has been reported (Balaji et al. 2013). *Synechococcus sp. PCC7942,* a cyanobacterium that does not produce PHB indigenously, has been transformed with the above genes, creating a recombinant strain. Moreover, PHB productivity was enhanced by the incorporation of a stronger promoter and supplementation of acetate. Lau et al. (2014) designed the PHA biosynthetic cassette with PHA synthase from *Chromobacterium sp. USM2* (phaCcs), acetoacetyl-CoA reductase (phaBcn) from *C. necator* and acetoacetyl-CoA synthase (nphT7ss) from *Streptomyces sp.* and emplaced the entire cassette within *Synechocystis sp. PCC 6803*. The highest PHA accumulation in recombinant strain has been reported in

the presence of acetate and limited gaseous exchange. Combination of molecular microbiology and genetic engineering can help us understand the qualities of bioplastics through analysis of genetic and metabolic blueprints.

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