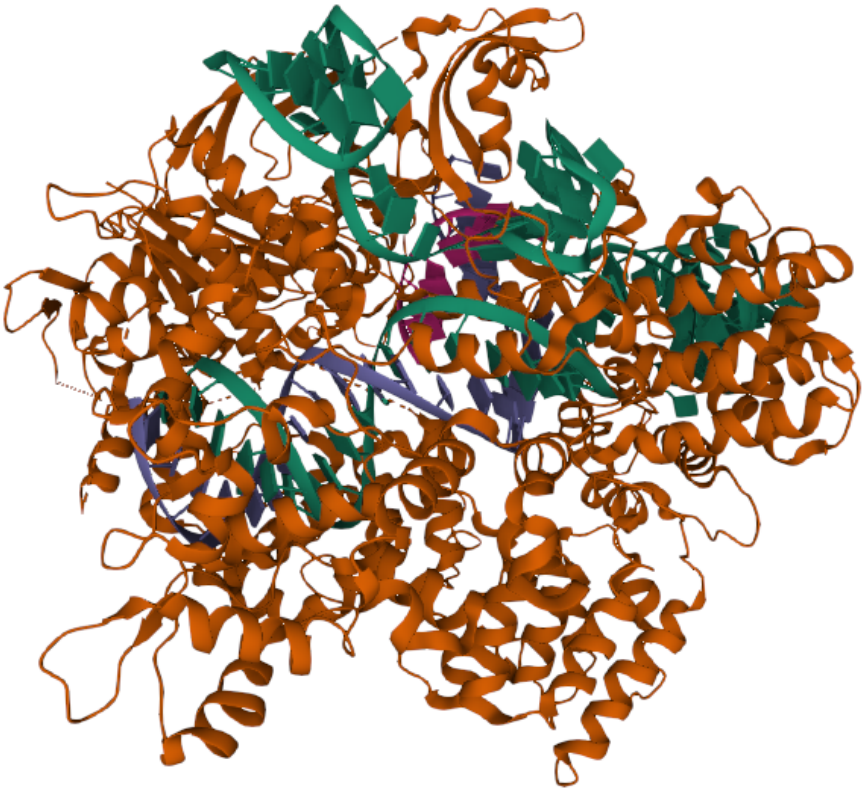
Polyhydroxyalkanoate production in bacteria, algae, plants, and yeast.

Can the production of polyhydroxyalkanoates be the solution to plastic pollution?



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SUMMARY

Plastic pollution is a serious global problem that has significant environmental and health consequences. There is a growing interest in developing sustainable alternatives like polyhydroxyalkanoates(PHAs). PHAs are a group of biodegradable and biocompatible polyesters produced by a range of microorganisms. In this research paper, bacteria, algae, plants, yeasts, and fungi will be studied for their ability to synthesize PHA. Bacteria show the highest PHA production of all microorganisms, especially when genetically modified. It can produce different types of PHA, such as short-chain length, medium-chain length, and occasionally long-chain length PHAs. As a result of high production costs, new ways of producing these polyesters are studied. One such is the usage of open cultures. No sterilization is needed which saves money. Furthermore, when combined in a mixed culture, the population becomes more adaptable and can utilize different waste food streams. Plants and algae use CO2 and light as their energy source, which is favorable for the reduction of global warming and decreasing production costs. In addition, plants can, when rightfully modified, accumulate PHA while still growing crops. Yeasts are able to produce PHA when genetically engineered. However, there are no clear favorable factors compared to other microorganisms. Consequently, Fungi were expected to produce PHA, but this was not the case. Nevertheless, Fungi can potentially degrade PHAs in a sufficient way due to their properties.

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INTRODUCTION

Plastic pollution is a global issue that has significant environmental and health consequences[1,2]. The widespread use of plastics has led to the accumulation of plastic waste in landfill, oceans, and other ecosystems. Plastics can persist for centuries and it harms wildlife and human health through food sources that get contaminated by plastic pollution[3]. Trillion pieces of macro- and micro-plastics roam in our oceans and million kg's enter the oceans annually[4]. Without proper waste management infrastructural improvements, the cumulative quantity of plastic waste available to enter the ocean from land is predicted to increase by an order of magnitude by 2025[5].

The first plastic, known as Parkesine, was produced in 1860 by Alexander Parkes[6]. Since then, the evolution of plastics has gone through many stages. Polymeric material is categorized based on its source into fossil-based or bio-based[7]. Fossil-based plastics, like petroleum-based plastics, have excellent properties. Versatility, low cost, formability, and light weight have made it the material of choice in a broad range of applications from smartphones to food packaging and 3D printing[5]. Unfortunately, the production of fossil-based plastics requires a large amount of oil[8] and is difficult to recycle or degrade. In addition, fossil-based plastic production increases non-cyclic atmospheric carbon dioxide concentration, resulting in climate change and ocean acidification[9]. Therefore, a more sustainable way of producing plastics should be developed.

To address the plastic pollution and increase in non-cyclic atmospheric carbon dioxide, there is a growing interest in developing sustainable alternatives to traditional petroleum-based plastics, so-called bioplastics. One such option is the use of polyhydroxyalkanoates (PHAs), a group of biodegradable and biocompatible polyesters produced by a range of microorganisms[10]. PHAs can be produced from renewable resources such as plant-based oils or agricultural waste, making them a more sustainable alternative. PHAs have several advantages over traditional plastics. For instance biodegradability, they can be broken down by natural processes into harmless compounds, such as water and carbon dioxide. Furthermore, PHAs are less toxic compared to fossil-based plastics and do not release harmful chemicals or microplastics into the environment. Bio-synthesized PHAs can be used for packaging, waste

management, medical devices, and agriculture applications[11].

The increasing interest in PHAs is expected to replace fossil-based plastics in the near future[12]. The production of PHAs can be achieved through microbial processes, where microorganisms such as bacteria, yeast, or algae convert organic matter, such as gasses, alcohols, alkanoic acids, alkanes, and saccharides, into PHAs[13]. The biodegradable polyesters are formed intracellularly as energy storage material by various microbes under poor growth conditions or synthesized at environmental stress (e.g., UV radiation, osmotic shock, high temperature)[14].

Yeasts like *Saccharomyces cerevisiae*, *Yarrowia lipolytica,* and *Pichia pastoris* are potentials hosts for the production of PHAs since they have been reported to be capable of producing short-chain length and medium-chain length PHAs from glucose[15,16,17]. They can utilize cheap substrates, have a high tolerance against high concentrations of sugars and organic acids, and are easy to genetically engineer. Although yeast shows a high potential as a productions organism, the yields of PHAs produced by yeasts[18,19,20,21,22] are unsatisfactory for large-scale production compared to microbes, which can accumulate PHAs up to 90% of their dry cell weight[23].

PHA can be produced in large fermenters by bacteria such as *Cupdrividus necator*[[24,25], and recombinant Escherichia coli [26], among others. However, for the cultivation processes, large amounts of organic carbon sources, such as glucose, and mineral salts, are responsible for approximately 50% of the total production costs [27]. Therefore, new methods are being developed. PHA production based on open mixed cultures has been proposed to lower production costs. No reactor sterilization is needed and the culture can adapt to various complex waste feedstock. The use of cellular biomass of prokaryotic microalgae, better known as cyanobacteria, can produce biomass using light and CO2 as the only source of energy[28]. PHA accumulation occurs naturally in photosynthetic organisms, but their yield can be increased under nutrient-deficient conditions in the presence of a carbon source. Therefore, obtaining PHA from microalgae can be characterized as an important tool to reduce the costs of obtaining the polymers. Microalgae are considered a promising source for producing PHAs and can provide greater competitiveness against synthetic polymers. Furthermore, the consumption of CO2 can also contribute to minimizing the greenhouse effect. In addition, the production of PHAs by microalgae can reduce the use of fossil resources and also reduce carbon dioxide emissions which is an important

environmental effect. Another major advantage of mixed culture PHA production is the opportunity to use real fermented wastes as feedstock for the production of PHA.

Plants, when bioengineered, can produce PHA[29]. Most productive plants in nature possess C4 photosynthetic pathways, these plants are attractive targets for engineering. PHAs, PHB in particular, in plants can potentially be produced at costs similar to starches and vegetable production. Poirier et al. (1992) reported the production of PHB in *Arabidopsis thaliana,* a well-studied model plant[30].

To use the produced PHAs, they must be extracted using different methods. However, the use of expensive solvents makes the process not suitable for industrial use. Different recovery methods, solvent-based, chemical, enzymatic, and mechanical recovery methods, are developed to reduce this cost[31]. One of the most popular extraction methods involves treating bacterial biomass with chemical solvents, such as chloroform or methylene chloride, to dissolve the PHAs. The PHA is separated from the biomass by filtration or centrifugation and precipitated by adding a non-solvent, like ethanol or isopropanol. The extraction method with chloroform can also be used for algae[32]. Enzymatic extraction uses enzymes like ligases that can break down the biomass and releases the PHAs. This method can be more selective for certain types of PHAs since it is less harsh[33]. Another way of extracting PHAs is by using a supercritical fluid, for instance, carbon dioxide. This method can be more environmentally friendly because it does not require toxic solvents[31]. In conclusion, the method used for extraction depends on the type of PHA, the scale of purity, and the desired purity of PHA.

Degradation of PHAs

PHAs can be fully degraded to water and carbon dioxide (under aerobic conditions) or water, carbon dioxide, and methane (under anaerobic conditions) by microbes in different environments, like soil, sewage, and seawater. Among the PHA-degrading microbes, bacteria are the most predominant group. However, fungi are potential degraders since they possess the ability to degrade effectively due to their fast surface growth rate and significant depolymerase activity[34].

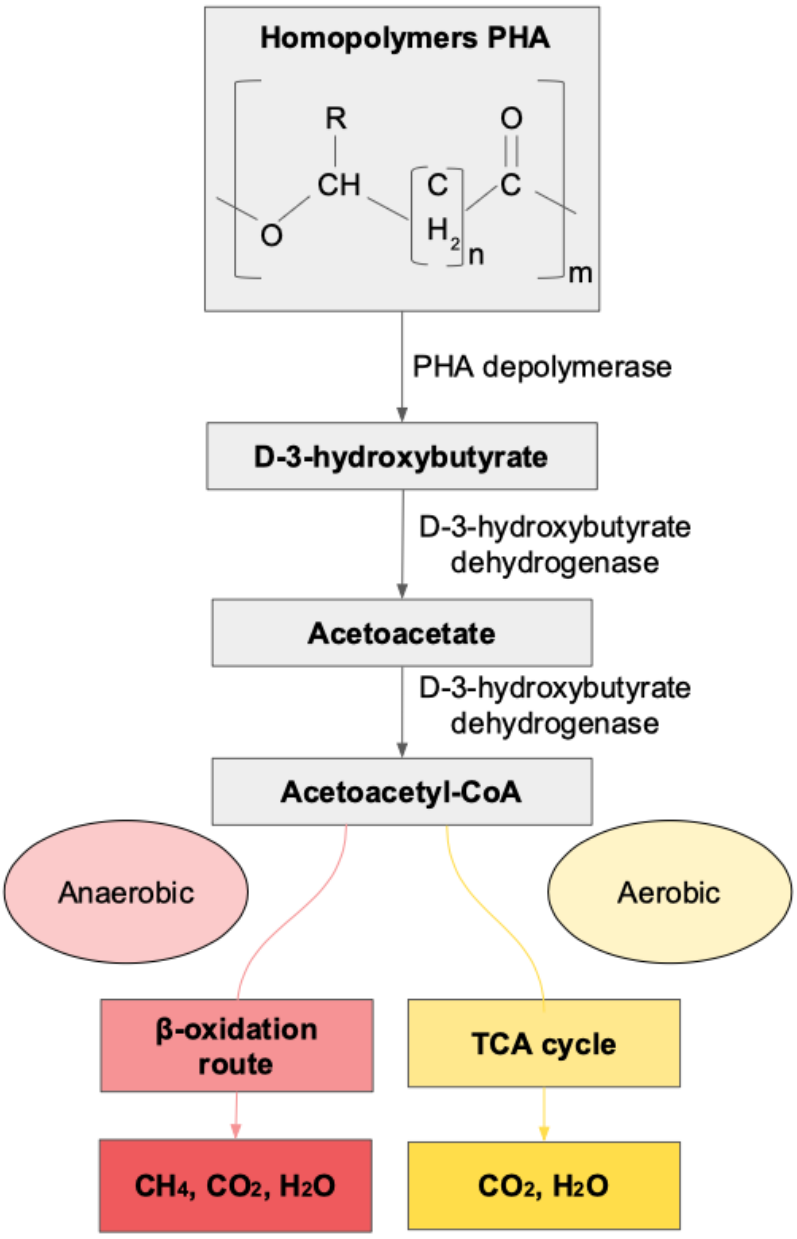
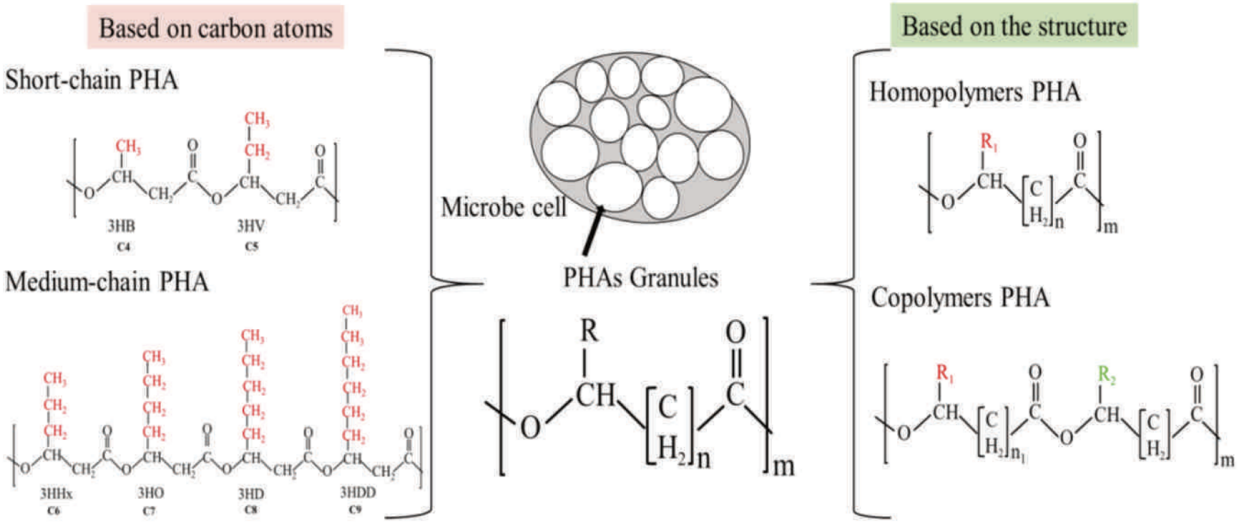


Figure 1. The simplified PHA degradation in anaerobic and aerobic conditions

Classification of PHAs

PHA molecules are typically classified into two main categories: short-chain-length (SCL) PHAs, which contain 3-4 carbon atoms, and medium-chain-length (MCL) PHAs, which contain 6-14 carbon atoms. Microbes rarely produce long-chain-length PHAs, which contain 15-20 carbon atoms. Furthermore, PHAs can be structurally classified as either homopolymers or copolymers, with copolymers further classified as random, block, or functional polymers[35]. The general structures can be seen in Figure 2.



(Zhou, W. 2023)

Figure 2. The chemical structures of PHAs include different categories based on carbon atoms and the PHA structure.

Currently, PHB, PHBV, P3HB4HB, and PHBHHX are the most common and commercialized PHAs in the world. They have already been used for packages, agriculture films, tissue engineering, drug carriers, and medical implants[35].

The accumulation of PHA is controlled by many different genes that encode a wide range of enzymes that are directly or indirectly involved in PHA synthesis[36,37,38,39]. The main pathway involves three key enzymes β-ketothiolase, NADPH-dependent acetoacetyl-CoA reductase, and PHA synthase, encoded by genes phaA, phaB, and phaC. These three genes are found together on an operon whose expression is relatively constant during PHA production [40]. During PHA production, the carbon

source is initially converted into coenzyme A thioesters of (R)-hydroxyalkanoic acid. β-Ketothiolase can then catalyze the condensation of two coenzyme A thioester monomers (such as an acetyl-CoA and a propionyl-CoA monomer). This is followed by an (R)-specific reduction to give (R)-3-hydroxybutyryl-CoA (or (R)-3-hydroxyvaleryl-CoA) (catalyzed by acetoacetyl-CoA reductase), which is then converted by PHA synthase into PHA [40], [41].

Knowing the enzymes involved in PHA synthesis, bioengineering becomes interesting to increase PHA production. This can be done by overexpression of the PHA synthase operon, inhibiting the PHA depolymerase(PhaZ) enzyme of the cell, increasing the available amount of NADH/NADPH for synthesis, Inhibiting beta-oxidation pathways to favor the PHA synthesis, enhancing cell size by modifying cell morphology, and suppressing pathways that compete with PHA synthesis[42]. Nevertheless, the most widely accepted method for enhancing PHA production is the overexpression of the involved enzymes. The combination of two or three enhancing ways in one recombinant strain can be combined to maximize the production

This paper is all about exploring the possibilities regarding PHA production. The research question goes as follows; Which organism is the most suitable for PHA production?

RESULTS

Bacteria can produce a wide range of PHAs

Plastic pollution has become a global environmental crisis with plastic waste polluting our oceans, rivers, and landfills. The traditional plastic materials we use in our daily lives are not biodegradable, and they can take hundreds of years to decompose, leading to severe environmental damage. Polyhydroxyalkanoates (PHAs) are a promising alternative to traditional plastics since they are biodegradable and can be produced from renewable resources. PHAs are produced by microbial producers such as bacteria, algae, and recombinant yeasts. There are more than 150 identified PHA monomers and this number is still increasing[43]. Promising bacterial PHA producers are *Cupriavidus necator*,[44,45], *Bacillus megaterium/subtilis*[46,47], and *Pseudomonas mendocina/resinovorans/aeroginosa/pseudoflava* [48,49,50,51]. These bacterial strains can utilize a wide range of carbon sources, such as glucose, fructose, glycerol, and fatty acids. The *C. necator* is well-studied for its ability to produce short-chain length (SCL) and medium-chain length (MCL) PHAs, making it a versatile producer. *Pseudomonas* strains are known to produce MCL PHAs with desirable properties. For instance a high molecular weight and a low polydispersity. The *Bacillus* family shows the incorporation of unique properties, such as high crystallinity and melting point, which can be used for different applications. Recent research (Sehgal & Gupta, 2020) shows that *C.necator H16*, when grown with 10g/L palm oil, and 0.54g/L urea in MM broth, gave a PHA content of 71wt%, 9.2 ± 1.6 g/L dry cell weight(DCW) and a PHA concentration of 8.1 ± 3.2 g/L[44].

Pseudomonas pseudoflava, under optimal conditions and synthetic wastewater as a carbon source, can have a PHA content of up to 57% w/w DCW and 20g/L PHA production. homo-polymer [poly-3-hydroxybutyrate (P3HB)] was produced when only acetate was used as a carbon source and it produced co-polymer [poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) P(3HB-co-3HV)] when co-substrate propionate was added[51]. The cultivation of *B. megaterium* and other *Bacillus* species obtain PHA yields of around 9%-12%[46,47] Even though, the yield isn't as high as other strains(*C. Necator H16*), *Bacillus* strains are known to be the most versatile PHA producers. Their ability to produce PHAs range from homopolymers to copolymers from

simple saccharides to complex industrial wastes[52]. Another organism used for PHA production can be the thermophilic strain *Schlegelella thermodepolymerans*[53]. When grown on 20g/L xylose, a carbon/nitrogen ratio of 100:1, an initial PH of 7, and at 50°C up to 80% of PHB on dry cell mass was accumulated. The biggest PHB granules were detected within 48 hours.

Different algae strains capable of PHA production

The production of PHA can also be utilized by algae. Different strains show the production of a variety of PHAs. The three most promising microalgae are cited by Costa, et. al, (2019)[54] and will be discussed in this section. The strain *Synechococcus sp. MA19*, grown autotrophically under phosphate-limited conditions at 50 °C produces PHB when the intracellular phosphate content was 0.043-0.076mmol per g of cellular biomass. *S. sp. MA19* accumulated 55% (w/w) PHB of the dry cells and the PHB content was 2.4g/L[55]. Bhati and Mallick(2010) were able to use *Nostoc muscorum* to produce PHA from milk and ice cream processing wastewater in a continuous mode reactor system. The PHA content in the PHA-rich biomass was around 43% of the sludge dry weight[5]. Another microalgae, *Aulosira fertillisima CCC 444,* supplemented with heavy metals showed enhancement in PHB accumulation up to 18.7% DCW in 1mg/L Ni-supplemented medium for 7 days, and 17.6% DCW in 1.5mg/L Cu-supplemented medium. NaCl stress, heat and chilling stresses, and deficiencies of potassium, calcium, and magnesium had negative effects on the PHB accumulation. However, supplemented with 0.5% acetate at the initiation of gas exchange limitation boosted the synthesis of PHB to 48.7% DCW for 14 days of incubation. Gas exchange limitation with carbon supplementation can increase PHB production with a value of ~7-fold higher compared to the control[56]. These results suggest that algae are viable PHA producers, but need further optimization to increase PHA content.

Production of PHA in yeast organisms

Yeast is also a competent PHA producer. For instance, strain *Pichia sp. TSLS24* isolated from Spratly Island in Vietnam showed its capability to grow and produce PHA efficiently in a wide range of temperatures from 15 to 45 °C, a wide pH range of pH 6–9, flexible carbon sources such as glucose, mannitose, lactose, starch, and sucrose as well as different nitrogen sources. The amount of PHA produced at different fermentation volumes was stable, around 52%. The extracted PHA was homologous

and had a purity of 91.4% which was measured using FTIR, NMR, and GC-MS analyses[4].

Mixed cultures have interesting advantages

Now we know that many different microbes can synthesize different PHAs dependent on their metabolic pathways of carbon sources. The major costs in PHA production are determined by the cost of substrates and extraction methods. Mixed Microbial cultures (MMC) can use mixed substrates since the population can adapt to changes in substrate. In addition, sterilization and sterile fermentation systems are prevented. PHA accumulation by MMC can reach up to 60% of the sludge dry weight (SDW). Even though it is less than 80% of CDW in pure cultures, it can be further optimized to succeed the conventional pure culture PHA production systems[57]. Wen Zhou (2023), created a PHA-producing MMC that was enriched by a feast-famine regime in an acetate-feeding bioreactor. The cells in the MMC contained an average of 50.4% DCW PHA when grown at an acetic acid concentration of 10g/L, a carbon/nitrogen(C/N) ratio of 10:1, PH of 7.0 and 30°C in a 2 L batch reactor. The PHA produced was identified as PHB. The dominant strain in this MMC was *Thauera aminoaromatica MZ1T,* with the PhaA, PhaB, PhaC, PhaR, PhaP and PhaZ genes[53].

Bioengineered organisms show improved PHA production

As discussed before, bacteria and algae are standard PHA producers. The accumulation of PHA in these microorganisms can be improved via bioengineering[46]. Furthermore, Organisms, such as plants[58] and yeasts[18] are potential PHA producers when genetically modified. There are different ways to improve PHA synthesis. This can be done by overexpression of the PHA synthase operon and inhibition of competing pathways to favor PHA synthesis. The most widely used method for enhancing the production is to overexpression of the concerned enzymes. This can be done by increasing the transcriptional levels of the PHA synthase enzyme. Zhao et al. (2019) showed that the integration of three promoters upstream of the phaC operon in Pseudomonas mendocino NK-01 resulted in enhanced transcriptional levels of phaC1 and phaC2. Furthermore, they deleted the phaZ gene, coding for PHA depolymerase. This led to higher production of PHA[59].

Another way to improve PHA production is by modifying cell morphology and increasing

cell size. The increase in the size of the bacterial cell increases PHA accumulation as well. Overexpression of genes such as sulA (inhibits cell division by interacting with FtsZ through GTP hydrolysis), ftsZ (responsible for Z ring formation and cytokinesis during bacterial cell division), mreB (maintains cell shape), minC and minD (inhibits FtsZ ring) can affect the PHA accumulation of the cell. The study conducted by Zhau et al. (2019), used a recombinant *Pseudomonas mendocino* with minCD knocked out and ftsZ, mreB and sulA genes overexpressed. The minCD knockout resulted in increased cell length and improved PHA yield from 0.28 g/L to 0.41 g/L (an increase of 45.62%). Overexpression of the mreB gene changed the morphology of the cell and increased the width. The ftsZ overexpression achieved multiple fission of the cell with an increase in PHA from 0.23 g/L to 0.37 g/L (an increase of 60.87%). Combining these methods increased the overall potential of the cell to produce a high amount of PHA[59].

Another research showed that E.coli, with the PHA synthesis genes from *Alcaligenes eutrophus* and overexpression of protein FtsZ, allowed the production of P(3HB) to a concentration of 104g/L, and a P(3HB) content of 70% in a defined medium[60]. This shows there are a lot of possibilities to improve the synthesis of PHAs[61].

Recombinant plants can produce PHA under the right conditions

Since the first plants were engineered to produce PHB, a lot of research has been done, improving the synthesis process significantly. Arabidopsis is a well-suited host to transform with a construct. Nawrath et al. (1994) showed that *Arabidopsis thaliana,* transformed with phbA, phbB, and phbC by agrobacterium-mediated transformation, accumulated PHB up to 14% of the dry weight within plastids. The PHB biosynthetic pathway in plastids had no obvious effect on the growth or fertility of transgenic plants[62]. Another promising research showed a 19.9% DWD PHB in single seeds of *Camelina sativa[60].* This is the highest percentage of Dry weight reported in single seeds to date. Even though plants can produce PHAs under controlled conditions(transformation, temperature, etc.), they can not compete with the fast and relatively easy production of PHAs in bacteria.

DISCUSSION

There is a lot of research done on the production of PHA, it becomes challenging to figure out which organism is the most viable for the accumulation of a certain type of PHA. Bacteria are the most studied organism that assembles PHA under stressful circumstances. It can produce many different types of PHA with unique characteristics. Bacteria have the highest PHA accumulation to this date. It would be an easy choice to use bacteria as a cell factory, but growing bacteria comes with a high cultivation cost. Substrates and sterilization are still a major part of the total production cost. More research is needed to lower the price of PHA synthesis to ensure completely replacing fossil-based plastics.

Open cultures are a good example of a substitute that does not need sterilization. Combined with mixed cultures, the population can be more adaptable to fluctuating circumstances like, different waste feedstock, increasing or decreasing PH, etc. This creates an opportunity for the accessible growth of PHA-accumulating bacteria or algae. Algae is an interesting choice since they can produce biomass using light and CO_2 as their only source of energy. Also, the CO_2 consumption of algae is favorable for the environment and this is a big advantage worldwide. Algae are already being utilized to remove CO_2 from the air in dense cities. Unfortunately, the PHA yield of algae is not as high compared to the yield of bacteria. PHA accumulation can be enhanced by nutrient-deficient conditions in the presence of a carbon source. If the production process of PHA by algae in a mixed open culture can be improved and lowered in cost, then I see this as the most promising future innovation for PHA production.

Since bioengineering has proved to be successful in enhancing the production of PHA, it could be interesting to combine it in a mixed culture. The population variety might be controlled so that the organisms can live in a symbiotic relationship, which could enhance the accumulation of PHA even more. Furthermore, when algae and bacteria can get modified into secreting the synthesized PHA, harvesting would become way more efficient compared to how it is done these days.

Plants and yeast are promising hosts, when modified with the right enzymes they show some good yields. However, still, too little research has been done to effectively use these organisms for industrial purposes. Plants and yeast can not compete with the

yield of bacteria yet, since there are more convenient and cost-friendly ways to produce PHA. Even though PHA production in plants is not efficient yet, the ability to grow crops and accumulate PHAs is an interesting feature. In addition, plants help lower the CO2 content in polluted air which will become an even more important factor in the future. This is why plants will be more interesting to study in the future than algae simply because plants can grow food at the same time. More research is needed to accomplish a significant PHA yield while still maintaining a high crop yield.

CONCLUSION

This study aimed to find out which organism(s) are the most suitable for PHA production. This research was done by using only literature that was already available. First, the general function and production of PHAs were studied. Followed by the production of PHAs in different microorganisms. It became clear that bacteria and algae are the most studied organisms for PHA accumulation. Plants and yeast were possible hosts when genetically engineered with the right enzymes. Fungi were thought to be a PHA producers as well, however after more investigation it became clear that fungi are useable for PHA degradation due to their fast surface growth and significant depolymerase activity. This research paper shows that bacteria are the most common PHA producers used for industrial production. Bacteria show a wide range of useful functions when producing PHAs. For instance, *Capriavidus necator* has the ability to produce short-chain length and medium-chain length PHAs, making it a versatile producer. *Pseudomonas* strains produce medium-chain length PHAs with desirable traits while the *Bacillus* family shows PHA production with unique properties. It ultimately depends on the desired product as to which bacterial strain is best suited. Still, alternative ways to synthesize PHA are being developed to reduce production costs. A rising alternative is using open cultures. No sterilization is needed and combined with a mixed culture can ensure high adaptability of the population. Mixed microbial cultures can accumulation up to 60% PHA of the sludge dry weight (SDW).

Algae are promising PHA producers due to their ability to utilize CO2 and light as their only source of energy. Photosynthetic organisms accumulate PHA naturally, but their yield can be increased under induced conditions like a nutrient deficiency in the presence of a carbon source. Furthermore, the consumption of CO2 can contribute to reducing the polluted air and scale down the greenhouse effect. Unfortunately, cultivation techniques and harvesting methods used with algae growth are too expensive to compensate for the sustainable properties. As a result, industrial production of PHA using these methods is not economically viable and is unlikely to be utilized soon.

Yeasts can be utilized as hosts when genetically modified with the right enzymes. However, there are no clear favorable factors compared to bacteria when used as a

PHA cell factory. Thus, the usage of yeasts is not common in the industrial industry. Nevertheless, genetic modification has proven to be successful in many cases. It can increase the production of PHAs in bacteria by up to 60%. With global warming becoming a bigger problem each day, plants become interesting host organisms in the near future due to their ability to grow crops and accumulate PHAs at the same time. Furthermore, plants can use light and CO2 as their only energy source, which is beneficial for decreasing global warming. The yield of plant-synthesized PHAs is too low to compete with the yield of bacteria.

FOREWORD & AFTERWORD

This bachelor's thesis was performed as part of my pre-master Biomolecular Sciences at the University of Groningen. Plastic pollution is a problem that everyone is aware of. Having spent some time reading and researching PHAs over the past year, I found the subject to be compelling enough to make it the topic of my bachelor's thesis. The idea of this study was to find out which organisms are most suitable for the production of polyhydroxyalkanoates. Given the abundance of information available on this subject, gaining a deeper understanding of the production of PHAs would be gratifying.

This thesis has a lot of useful information about PHAs and their producers that can help you choose the best organism for your needs and interests. I am happy to announce that I am pleased with the outcome, and I hope this thesis has been helpful to you too. My recommendation for future research is to study the PHA accumulation in plants while still growing crops. This is, in my opinion, the most favorable process possible.

As I conclude this thesis, I would like to express my gratitude to Dr. A. de Jong for supervising this bachelor's thesis and to Dr. D. Incarnato as my second supervisor. It has been a rewarding experience to gain a deeper understanding of this subject. I have enjoyed the process and I am excited about the potential implications of this knowledge for the future.

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