Industrial Production of Poly-β-hydroxybutyrate from CO$_2$: Can Cyanobacteria Meet this Challenge?

Carpine, R.; Olivieri, G.; Hellingwerf, K.J.; Pollio, A.; Marzocchella, A.

DOI
10.3390/pr8030323

Publication date
2020

Document Version
Final published version

Published in
Processes

License
CC BY

Citation for published version (APA):
Carpine, R., Olivieri, G., Hellingwerf, K. J., Pollio, A., & Marzocchella, A. (2020). Industrial Production of Poly-β-hydroxybutyrate from CO$_2$: Can Cyanobacteria Meet this Challenge? Processes, 8(3), Article 323. https://doi.org/10.3390/pr8030323

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

UvA-DARE is a service provided by the library of the University of Amsterdam (https://dare.uva.nl)
Industrial Production of Poly-\(\beta\)-hydroxybutyrate from \(\text{CO}_2\): Can Cyanobacteria Meet this Challenge?

Roberta Carpine\textsuperscript{1,2,*}, Giuseppe Olivieri\textsuperscript{1,3,*}, Klaas J. Hellingwerf\textsuperscript{4*}, Antonino Pollio\textsuperscript{5*} and Antonio Marzocchella\textsuperscript{1}

\textsuperscript{1} Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale, Università degli Studi di Napoli “Federico II”, P.le Tecchio 80, 80125 Napoli, Italy; marzocch@unina.it
\textsuperscript{2} Department of Organic Chemistry, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland
\textsuperscript{3} Bioprocess Engineering Group, Wageningen University & Research, Droevendaalsesteeg 1, 6708PB Wageningen, The Netherlands
\textsuperscript{4} Molecular Microbial Physiology Group, Swammerdam Institute for Life Sciences—Science Park, 904 1098 XH Amsterdam, The Netherlands; K.J.Hellingwerf@uva.nl
\textsuperscript{5} Biology Department, Università degli Studi di Napoli “Federico II”, Via Foria 223, 80139, Napoli, Italy; anpollio@unina.it

* Correspondence: roberta.carpine@uzh.ch (R.C.); giuseppe.olivieri@wur.nl (G.O.)

Received: 8 February 2020; Accepted: 5 March 2020; Published: 10 March 2020

Abstract: The increasing impact of plastic materials on the environment is a growing global concern. In regards to this circumstance, it is a major challenge to find new sources for the production of bioplastics. Poly-\(\beta\)-hydroxybutyrate (PHB) is characterized by interesting features that draw attention for research and commercial ventures. Indeed, PHB is eco-friendly, biodegradable, and biocompatible. Bacterial fermentation processes are a known route to produce PHB. However, the production of PHB through the chemoheterotrophic bacterial system is very expensive due to the high costs of the carbon source for the growth of the organism. On the contrary, the production of PHB through the photoautotrophic cyanobacterium system is considered an attractive alternative for a low-cost PHB production because of the inexpensive feedstock (\(\text{CO}_2\) and light). This paper regards the evaluation of four independent strategies to improve the PHB production by cyanobacteria: (i) the design of the medium; (ii) the genetic engineering to improve the PHB accumulation; (iii) the development of robust models as a tool to identify the bottleneck(s) of the PHB production to maximize the production; and (iv) the continuous operation mode in a photobioreactor for PHB production. The synergic effect of these strategies could address the design of the optimal PHB production process by cyanobacteria. A further limitation for the commercial production of PHB via the biotechnological route are the high costs related to the recovery of PHB granules. Therefore, a further challenge is to select a low-cost and environmentally friendly process to recover PHB from cyanobacteria.

Keywords: biopolymer; genetic engineering; kinetic model; medium optimization; PHB recovery

1. Introduction

Nowadays, plastic materials are widely used and essential components of product packaging, cars, household/office appliances, computer equipment, and medical devices [1]. Conventional plastics accumulate in the environment because they are not biologically degradable, producing a large spectrum of pollutants [1]. Circa 140 million tons of plastic are consumed/produced every year all around the world. Plastic production requires the processing of approximately 150 million tons of fossil fuels and a huge amount of waste is produced whose depolymerization can take thousands of years [2]. In May 2018, the European Commission proposed new European rules aimed at reducing...
plastic pollution in cities and oceans by banning several plastic products. EU officials noted that the single-use plastic objects and fishing gear accounts for 70 percent of trash in the ocean and that the move towards “innovative alternatives” could create around 30,000 jobs. EU First Vice President Frans Timmermans hailed the draft directive, saying: “Plastic waste is undeniably a big issue and Europeans need to act together to tackle this problem, because plastic waste ends up in our air, our soil, our oceans, and in our food” (http://europa.eu/rapid/press-release_IP-18-3927_en.htm).

The methods used for the disposal of plastic materials are challenging. Indeed, in landfills, the rates of degradation are tremendously low. Incineration generates toxic by-products. Recycling can be done, but it is a very time-consuming process and also causes changes in the properties of plastic materials [3].

To reduce world pollution, researchers have been focused on biodegradable plastics as alternative to conventional fossil-derived plastics. Plastics are biodegradable at a tunable rate depending on the surrounding environmental conditions (e.g., location or temperature) and on the material (http://www.european-bioplastics.org). The biodegradable plastics can be classified into four categories, as reported in Table 1 [4].

| Types of Biodegradable Plastics | Examples |
|--------------------------------|----------|
| Bioplastics that are biobased (biomass products). | This group includes polysaccharides, such as starches or animal protein (e.g., casein, whey and gelatin). |
| Bioplastics that are biobased (from microorganisms). | This group includes biodegradable biopolymer extracted from microorganisms (e.g., polyhydroxyalkanoates (PHA), polyhydroxybutyrate (PHB), poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV)). |
| Bioplastics that are biobased (from biotechnology). | This group includes biodegradable biopolymer produced by a conventional synthesis of bio derived monomers (e.g., polylactic acid (PLA)). |
| Bioplastics that are fossil-based. | This group includes bioplastics from petrolchemical products obtained from conventional synthesis of synthetic monomers (e.g., polycaprolactone (PCL), poly(butylene adipate-co-terephthalate) (PBAT)). |

1 after Singh et al. [4].

Currently, 359 million tons of plastic are annually produced of which bioplastics represent about 1% of the whole market (http://www.european-bioplastics.org). However, the bioplastic market is growing by about 20% to 100% per year to fulfill the rising demand and the request for more sophisticated materials, applications, and emerging products. According to the latest market data compiled by European Bioplastics in cooperation with the Research Institute Nova-Institute, the global bioplastics production capacity is set to increase from around 2.11 million tons in 2019 to approximately 2.43 million tons in 2024 (http://www.european-bioplastics.org).

Biodegradable plastics altogether, including polylactic acid (PLA), polyhydroxyalkanoates (PHA), starch blends, and others, account for over 55.5 percent (over 1 million tons) of global bioplastics production capacities. The production of biodegradable plastics is expected to increase to 133 million in 2024, especially due to PHA’s significant growth rates. Indeed, in the current scenario of increasing environmental awareness, PHAs are becoming potential substitutes for non-biodegradable polymers [1]. Poly-β-hydroxybutyrate (PHB) is a well-known and characterized PHA found in bacteria. The production capacity of PHB has been growing steadily during the last few years.

The present review reports main issues regarding the PHB production and the challenging issues to be investigated/developed. The PHB structure as well as its formation/accumulation in the cyanobacteria is presented. The biotechnological route to produce the PHB is presented with the potential solution to improve the productivity and reduce the cost of the production. The effects of the PHB structure in the cell on the recovery performance is discussed.
2. Poly-β-hydroxybutyrate (PHB) Structure

Polyhydroxyalkanoates (PHAs) are the main blocks of biodegradable plastics. They are polyesters produced by various microorganisms, such as cyanobacteria. Due to their chemical and physical features, PHAs are the most investigated biodegradable polymers.

The PHAs are divided into three groups: short-chain-length-PHAs (SCL-PHAs), medium-chain-length-PHAs (MCL-PHAs), and long-chain-length-PHA (LCL-PHA) [5–7]. The polyhydroxybutyrate (PHB) (Figure 1) is the most common of the SCL-PHAs. PHBs have been characterized with respect to the following interesting features: thermoplastic processability, hydrophobicity, biodegradability, biocompatibility, and optical purity [8]. Key physical features of PHBs are a melting temperature of about 177 °C, glass transition temperature of about 2 °C, crystallinity of about 60%, tensile strength of about 43 MPa, and extension to break of about 5% [9].

![Figure 1. General structure of poly-β-hydroxybutyrate.](image)

3. Commercial Status of PHB

The current bioplastic production plants (Figure 2) are characterized on a small scale when compared to plant petroleum-based plastics. One example is China’s TianAn PHA plant that produces approximately 2000 metric tons of PHA per year. This production is quite small if compared to the standards. However, since bioplastics represent an interesting product that can be used in various sectors, some of the producers became leaders in the biopolymer field production all over the world [4,10].

![Figure 2. PHB production plants: (A) Bio-On in Italy that produces Minerva; (B) PHB industrial in Brazil that produces Biocycle; (C) Meredian in the USA; (D) Metabolix in the USA.](image)
PHA bioplastic businesses are largely used in North America, Europe, Asia-Pacific, etc. [2]. Companies such as Metabolix Inc. (USA), Shenzhen Ecomann Technology Co. Ltd. (China), Tianjin GreenBio Materials Co. Ltd. (China), Meredian Inc. (USA), Mango Materials (USA), Newlight (USA), and Biomer (Germany) are the main producers of PHA [2] (Table 2).

### Table 2. Global manufacturers of PHB/PHA (http://www.european-bioplastics.org; [4]).

| Name of the Company         | City/Country     | Production (kt Year⁻¹) | Raw Material | Brand Name      |
|-----------------------------|------------------|------------------------|--------------|-----------------|
| Biomer                      | Schwalbach am    | -                      | PHB          | Biomer          |
|                             | Taunus/Germany   |                        |              |                 |
| Biomateria                  | Toronto/Canada   | -                      | PHA          | Biomateria      |
| Bio-On                      | Bologna/Italy    | 10                     | PHA          | Minerv          |
| Kaneka                      | Osaka/Japan      | 10                     | PHB          |                 |
| Tianjin Green-Bio          | Taijin/China     | 10                     | PHA          | Green Bio       |
| Imperial Chemical Industries (ICI) | London/UK       | 0.3                    | PHB          |                 |
| Danimer Scientific         | Georgia/USA      | 0.3                    | PHA          |                 |
| PHB Industrial              | Serrana/Brazil   | 0.1                    | PHB          | Biocycle        |
| TEPHA                      | Massachusetts/USA | -                      | PHA          | Tephaflex/TephElas |
| Tianan                     | Zhejiang/China   | 10                     | PHB          | Enmat           |
| SIRIM                      | Selangor/Malaysia | 2                      | PHA          |                 |
| Shenzhen Ecomann            | Shandong/China   | 5                      | PHA          | AmBio           |

In 2006, Metabolix and Archer Daniel Midland (ADM) developed a joint company called Telles that became the major PHA producer in the USA. Unfortunately, this company ended production in 2012 [11].

Another company in the USA called Meridian Inc. produces around 15,000 tons of PHA bioplastics per year obtained from the fermentation of bacteria grown on oils (fatty acids). The obtained products are used for films, non-woven fabrics, and food-contact packaging. The full capacity of the plant is able to produce more than 300,000 tons of PHA per year [2,11].

The Mango Materials, founded in the USA in 2010, focused its production on PHB-based bioplastics using a new technology based on intellectual property licensed from Stanford University. In this case, the PHB is produced by methanotrophs bacteria that grow using the methane contained in wastewaters. The PHB is extracted from the cellular biomass, transformed into bioplastic, and finally converted into products of interest [11].

The Dutch company DSM and the Chinese company Tianjin Green Bio-Science Co. created a joint venture to produce bioplastics. The company is now producing PHA resin (10,000 metric tons per year) [2,11].

Of note, the Japanese company Kaneka produced 50,000 metric tons annually of PHB in 2010 [2,11].

### 4. Conventional Production System of PHB

The commercial PHB production is based on cultures of microorganisms, such as *Cupriavidus necator*, *Alcaligenes*, *Azotobacter*, *Bacillus*, *Nocardia*, *Pseudomonas*, *Rhizobium* [12,13]. Under optimal conditions using fructose as carbon source, bacteria—e.g., *Ralstonia eutropha* (also known as *Cupriavidus necator*)—can produce PHB up to 80% of the cell dry weight [14]. Table 3 reports an overview of bacterial strains used to produce PHB, the feedstock used as carbon source, and the chemical structure of the produced copolymers.
Table 3. Overview of bacterial strains used for the production of PHB.

| Bacterial Strain                  | Carbon Source                          | Polymer (s)        | PHB % | References |
|-----------------------------------|----------------------------------------|--------------------|-------|------------|
| Aeromonas hydrophila 4AK4 mutant  | Lauric acid, oleic acid                | MCL-PHA 1          | 64    | [15]       |
| Alcaligenes latus                 | Malt, soy waste, milk waste            | PHB 2              | 70    | [16]       |
| Azotobacter chroococcum H23       | Waste water from olive oil mills       | PHB 2              | 80    | [17]       |
| Bacillus cereus UW85              | Glucose                                | PHB 2              | 9     | [18]       |
| Bacillus megaterium ATCC 6748     | Corn steep liquor and molasses         | PHB 2              | 43    | [19]       |
| Bacillus spp. 87I                 | Glucose                                | PHB 2              | 67    | [20]       |
| Burkholderia sacchari sp. IPT101  | Glucose                                | PHB 2, PHBV 3      | 68    | [21]       |
| Burkholderia cepacia IPT 048      | bagasse                                | PHB 2              | 62    | [22]       |
| Caulobacter crescentus DSM 4727   | Glucose                                | PHB 2              | 18    | [23]       |
| Cupriavidus necator DSM 545       | Corn syrup                             | PHB 2              | 30    | [24]       |
| Cupriavidus necator DSM 545       | waste glycerol                         | PHB 2              | 62    | [25]       |
| Enterobacter aerogenes 12Bi       | wastewater                             | PHB 2              | 43    | [26]       |
| Escherichia coli mutants          | Xylose, starch, hydrolsate, maltose, maltotetraose, and maltohexaose | PHB 2              | 56    | [28]       |
| Halomonas boliviensis LC1         | MCL-PHA 1                              | 16                 |       | [29]       |
| Legionella pneumophila 74/81      | Nutrient broth                         | PHB 2              | 51    | [30]       |
| Methylocystis sp. GB 25 DSM 7674  | Methane                                | PHB 2              | 30    | [31]       |
| Microlunatus phosphovorus DSM 10555 | Glucose, acetate                      | PHB 2              | 90    | [32]       |
| Mixed Microbial Culture           | Lactate                                | PHB 2              | 63    | [33]       |
| Pandoraea sp.                     | Crude glycerol                         | PHB 2              | 66    | [34]       |
| Pseudomonas aeruginosa NCIB 40045 | Agro-industrial oily wastes            | MCL-PHA 1          | 21    | [35]       |
| Pseudomonas hydrogenovora DSM 1749 | Dairy whey                            | MCL-PHA 1          | 30    | [36]       |
| Pseudomonas putida CA-3           | Petrochemical plastic waste            | PHB 2              | 70    | [37]       |
| Pseudomonas fluorescens A2a5      | Sugar cane liquor                      | PHB 2              | 34    | [38]       |
| Rhodopseudomonas palustris SP5212 | Acetate                                | PHB 2              | 10    | [39]       |
| Raistonia picketti 61A6           | Sugar cane liquor                      | PHB 2              | 70    | [40,41]    |

1 MCL-PHA: medium-chain-length-polyhydroxyalkanoates  2 PHB: polyhydroxybutyrate  3 PHBV: poly(3-hydroxybutyrate-co-3-hydroxyvalerate).

The high cost of the raw materials (which represent 30–40% of the total production costs) for PHB production by bacterial fermentation makes this process not economically competitive (about 2.6 U.S.D./kg as reported by Lopez-Arenas et al. [39]). Therefore, low cost photosynthetic production systems have been proposed as alternative [40,41]. However, the use of transgenic plants compete directly with subsistence crops for agricultural acreage and have led to ethical concerns because the dissemination of transgenic plants could be difficult to regulate [2].

5. Cyanobacteria and PHB

Cyanobacteria display all the advantages of bacteria when compared to the chemoheterotrophs. In addition, cyanobacteria are characterized by the potential to exploit sunlight energy and to convert CO₂-abundant and not competitive carbon-based feedstock into a product of interest, such as PHB. Two further advantages of the cyanobacterial system are that (i) they do not compete with the agro-food market for resources [42–44] and (ii) they contribute to capture and to use the CO₂, decreasing the pressure of the greenhouse gas release into the atmosphere.

Cyanobacteria—or blue-green algae—are prokaryotic organisms that have proved to be interesting because of their potential contribution to the sustainable economy. They can perform oxygenic
photosynthesis capturing free energy from (sun) light for the synthesis of ATP and NADPH [42]. Valuable products from CO$_2$, sunlight, and water can be produced by photosynthetic metabolism [44].

The ancestors of cyanobacteria were responsible for the oxygen enrichment in the—originally anoxic—atmosphere of the earth more than 3.2 billion years ago. This event had an enormous impact on life on earth resulting in favorable conditions for aerobic organisms. Subsequently, the thylakoid membranes of the ‘primordial cyanobacteria’ emerged from oxygenic photosynthesis evolution [45] leading to the development of plants.

The cyanobacteria evolution succeeded due to their unique metabolic pathways that can carry out oxygenic photosynthesis and respiration simultaneously in the same cell compartment. Furthermore, many cyanobacterial species can fix nitrogen and survive under many environmental conditions [46].

Photosynthesis and respiration involve electron transport pathways that are catalyzed by protein complexes in membranes. The oxygenic photosynthesis is characterized by the conversion of CO$_2$ and water to sugars fixing the energy from sunlight. This process is the reverse of respiration, which is the conversion of sugars to CO$_2$ and water releasing energy. The respiratory electron transport chain is in the cytoplasmic membrane—the membrane that separates the cytoplasm and the periplasm—or can occur in thylakoids. Instead, the photosynthetic electron transport in most cyanobacteria occurs only in thylakoids. A schematic representation of the respiratory and photosynthetic electron transport chains in cyanobacterial thylakoid membranes is shown in Figure 3.

![Figure 3. Schematic representation of the intersecting photosynthetic and respiratory electron transport pathways of the cyanobacterium Synechocystis sp. PCC 6803.](image)

The spectrum of potential applications of cyanobacteria or of their components is very large. Cyanobacteria have been used as food and feed sources. Strains of *Spirulina* are rich in vitamins and proteins and are used as food supplement. One of the largest industrial applications of cyanobacteria is their capability of being effective fertilizers. Because cyanobacteria can fix nitrogen, they are able to provide support for plant growth in rice fields [47]. Cyanobacteria were also investigated to produce anti-fungal, anti-bacterial, and anti-cancer agents. The cyanophycin polymer, built from aspartate and arginine, can replace polycrylate (which is derived from propylene and thus from petroleum) in paints and surface coatings. Cyanobacteria are capable to produce a protein–pigment complex called phycocyanin, which is used as a food colorant in cosmetics and diagnostics [48].

Under photoautotrophic conditions and minimal nutrient conditions, such as phosphate, nitrate, magnesium, and calcium, as well as macro-minerals, ferrous, manganese, zinc, cobalt, and copper [9],
cyanobacteria may accumulate the homopolymer of PHB [49]. Indeed, PHB can be used by cyanobacteria as a specialized biomass for carbon and energy storages [50].

The cyanobacterial strain *Synechocystis* sp. PCC 6803 is the most used and well characterized strain because its growth is quite fast (minimal doubling time of less than seven hours) and it does not have specific nutritional demands [51].

6. Improvement of PHB Production in Cyanobacteria

6.1. Optimization of Growth Conditions

A deficiency or limitation of nutrients, such as nitrogen and phosphate, stimulates the accumulation of PHB in cyanobacteria under photoautotrophic growth conditions [52–54] (Table 4). A PHB production fraction in cyanobacteria as high as 71% (of dcw) may be produced in *Nostoc muscorum Agardh* cultures carried out in a medium supplemented with glucose, acetate, and valerate coupled with nitrogen and phosphate limitation [52]. The PHB pool in *Spirulina* sp. LEB 18 reached 31% (of dcw) under photoheterotrophic conditions in a medium supplemented with sodium bicarbonate and deficiency of nitrogen and phosphorus [55]. *Aulosira fertilissima* CCC 444 accumulated 77% of PHB—with respect to the dry cell weight—for growth in a medium that contained fructose and valerate and under phosphorus deficiency [53]. For *Synechocystis* sp. PCC 6803, PHB accumulation of 38% (of dcw) under nitrogen depletion with fructose and acetate supplementation was reported [56]. The same cyanobacterium accumulated PHB: (i) up to 4%—with respect to the dry cell weight—under photoautotrophic growth conditions and nitrogen depletion [50]; and (ii) up to 8% under photoautotrophic conditions—CO₂ concentration in the gas stream 2% (v/v)—and nitrate concentration reduced to half of the optimal cell growth concentration (BG1/2 medium) [57]. Haase et al. [58] reported that *Nostoc muscorum* CCAP 1453/9 reached a PHB content of 22% under photoautotrophic conditions and deficiency of nitrogen. *Calothrix scytonemicola* TISTR 8095 accumulated 25% of PHB—with respect to the dry cell weight—under photoautotrophic growth conditions and deficiency of nitrogen.

The comments above and the data summarized in Table 4 highlight the necessity to grow cells under the unbalanced feeding of nitrogen and phosphorus in order to activate PHB production [2]. The high accumulation of PHB under limited nitrogen conditions can be rationalized by considering that when photosynthesis continued to fix CO₂ the absence of nitrogen did not support the synthesis of proteins. Therefore, cyanobacteria accumulate the carbon energy as glycogen and/or PHB [59].

Bhati and Mallick [52], Panda and Mallick [56], and Samantary and Mallick [53] measured the maximum PHB content for cultures carried out under heterotrophic growth conditions. Although the PHB production may be very interesting, this PHB production requires the supply of a reduced carbon source (like a sugar). Coelho et al. [55], Haase et al. [58], and Kaewbai-ngam et al. [60] reported interesting results for cultures carried out under autotrophic conditions in shake flasks. They cultivated *Calothrix scytonemicola* TISTR 8095, *Nostoc muscorum* CCAP 1453/9, and *Spirulina* sp. LEB 18, respectively. However, these strains are biofilm forming strains [61] and their performance may be strongly impaired when cultivated in photobioreactors, the culture system that preserve the high quality of the product. *Synechocystis* PCC6803 was characterized by high PHB productivity under heterotrophic growth conditions [62]. However, its performance was drastically reduced when cells were grown under autotrophic conditions [50]. Indeed, the PHB fraction reported by Wu et al. [50] (4% of PHB) was half the value reported by Carpine et al. [57].
Table 4. Overview of cyanobacteria strains that produce PHB.

| Cyanobacteria Strain                        | Culture Conditions                        | Polymer          | Polymer Content (%) | References |
|---------------------------------------------|-------------------------------------------|------------------|---------------------|------------|
| *Arthrospira (Spirulina) platensis*         | Photoautotrophic                          | PHB              | 6                   | [63]       |
| *Anabaena cylindrica* 10C                   | Propionate                                 | P(3HB-co-3HV)    | 2                   | [64]       |
| *Aulosira fertilissima* CCC 444             | Citrate + acetate and K$_2$HPO$_4$         | PHB              | 85                  | [65]       |
| *Aulosira fertilissima* CCC 444             | Fructose + valerate                        | P(3HB-co-3HV)    | 77                  | [26]       |
| *Gloeothecce sp.* PCC 6909                  | Acetate                                    | PHB              | 9                   | [66]       |
| *Nostoc muscorum Agardh*                    | Nitrogen deficiency + acetate + glucose + valerate | P(3HB-co-3HV)  | 71                  | [25]       |
| *Nostoc muscorum* CCAP 1453/9               | CO$_2$                                     | PHB              | 22                  | [31]       |
| *Scytonema geitleri Bharatia*               | Acetate 30 mM                              | PHB              | 7                   | [67]       |
| *Synechococcus sp.* PCC 7942                | Acetate + nitrogen deficiency CO$_2$; Nitrate concentration is the half of the optimal concentration | PHB              | 26                  | [27]       |
| *Synechocystis sp.* PCC 6803                |                                            | PHB              | 8                   | [30]       |
| *Synechocystis sp.* PCC 6803                | CO$_2$; Nitrate deficiency                 | PHB              | 4                   | [23]       |
| *Synechocystis sp.* PCC 6803                | Phosphate-deficiency + gas exchange limitation + acetate sodium bicarbonate + nitrogen and phosphorus deficiency | PHB              | 38                  | [35]       |
| *Spirulina sp.* LEB 18                     |                                            | PHB              | 31                  | [28]       |
| *Spirulina platensis* UMACC 161             | Acetate and CO$_2$                         | PHB              | 10                  | [68]       |

6.2. Genetic Engineering Approach

Genetic engineering of photosynthetic microbes—cyanobacteria—may enhance the conversion of CO$_2$ into chemical commodities [44,69]. The development and application of engineered cyanobacteria is a very promising approach to develop renewable and carbon neutral processes to provide chemicals typically produced by fossil sources and alternative energy vectors [70].

Several cyanobacterial strains can be employed to transfer exogenous DNA across the cell membrane into the host organism by a yet unresolved mechanism [70] due to their natural competence. *Synechocystis* sp. PCC 6803, *Synechococcus elongatus* PCC 7942, and *Synechococcus* sp. PCC 7002 are naturally transformable and highly suitable for genetic engineering applications [71].

Cyanobacteria can be considered the cell factories of the future. For example, introducing an exogenous biosynthetic pathway in a cyanobacterial cell may improve a certain product fabrication (a chemical, a biofuel, or a precursor of a bioplastic) obtained from the conversion of CO$_2$ (Figure 4) [72].

Cyanobacteria, genetically engineered with foreign genes, can produce two main products: (i) proteins and (ii) small-molecule chemicals. Depending on the targeted product, cyanobacteria can be engineered with the addition of a single gene or of an entire new pathway. The central carbon metabolism of cyanobacteria is a highly complex network which includes the CBB cycle, glycolysis, the pentose phosphate (PP) pathway and the TCA cycle [72].
Figure 4. Schematic representation of a cyanobacterial cell factory. (A) A non-modified cyanobacterial cell is investing carbon (in form of CO₂) and energy sources into growth and the generation of biomass. (B) A modified cyanobacterial (here: through the introduction of an alternative biosynthetic pathway) cell factory is investing the majority of the incoming carbon and energy sources into a (excreted) product (here: carbon containing products), allowing only the replacement of dying cells and the maintenance of the cells to be fueled by the native metabolism.

Cyanobacteria may accumulate considerable amounts of PHB as product of the wild metabolism and as results of the genetic engineering of strains by heterologous transformation with genes involved in the PHB pathway of *R. eutropha* [73]. In many cyanobacteria, PHB is a native polymer that stores carbon, produced via the polyhydroxyalkanoate (PHA) biosynthetic pathway [74]. The pathway from acetyl-CoA to PHB includes the following three catalytic steps:

- The condensation of two molecules of acetyl-CoA into acetoacetyl-CoA by PHA-specific beta-ketothiolase (*phaA*) [75];
- The reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase (*phaB*);
- The (R)-3-hydroxybutyryl-CoA condensation into PHB by PHA synthase (*phaC/phaE*) [75] (Figure 5).

Genetic and metabolic engineering together with recombinant DNA technology have shown the ability to increase the PHB accumulation to produce a considerable amount of biomass [4]. Table 5 lists the numerous genetic engineering approaches described in the literature and that have been applied to increase the PHB content in cyanobacteria. The degree of success of those approaches depends on several issues. Several studies have evaluated the PHB accumulation under mixotrophic growth conditions [54,76,77] in mutants of *Synechocystis* and *Synechococcus*. The researchers have investigated the effect of supplementation of the growth medium with acetate. The acetate, which can be converted into acetyl-CoA via the activity of acetyl-CoA synthetase, may support many metabolic pathways. Those pathways are the TCA cycle for cell growth, glycogen synthesis, and fatty acid metabolism relevant for biofuel production (such as butanol, propanol, and isopropanol [72]) and PHB biosynthesis [75]. The heterologous expression of the PHA-synthesizing gene operon from the bacterium *R. eutropha* in *Synechocystis* sp. PCC 6803 has been reported to increase the PHB content from 7% to 11% of dcw when grown in a N-deprived medium supplemented with 10 mM acetate [76]. Similar results have been obtained for the cyanobacterium *Synechococcus* sp. PCC 7942 in which the entire *A. eutrophus* PHB-synthesizing apparatus was heterologously expressed. These recombinant *Synechococcus* cells accumulated 25% PHB of dcw [75] for cultures carried out supplementing 10 mM acetate and under N-starvation [33]. The PHB content of the phaAB-carrying strain obtained by Khetkorn et al. [75] increased to 35% of dcw for cultures carried out supplementing 4 mM acetate under N-starvation conditions. The accumulation was very high when compared with the 13% PHB of dcw measured for the wild type cultures carried out under the same operating conditions.
Table 5. PHB fraction in engineered cyanobacteria under different cultivation conditions.

| Cyanobacterial Strain | Engineered Genes                                                                 | Carbon Source         | Culture Conditions | Reactor | Polymer Fraction (% dcw) | References |
|-----------------------|----------------------------------------------------------------------------------|-----------------------|--------------------|---------|--------------------------|------------|
| Synechocystis sp. PCC 6803 mutant strain | The agp gene was inserted                                                       | CO₂ 0.035%, Acetate 5 mM | Nitrogen deprived | flask   | 18.6                     | [43]       |
| Synechococcus sp. PCC7942 mutant strain | A PHB synthesizing enzyme from A. eutrophus was expressed from a plasmid        | CO₂ 5%, Acetate 10 mM | Nitrogen deprived | flask   | 25                        | [27]       |
| Synechocystis sp. PCC 6803 mutant strain | PHA synthase from C. necator was expressed from a plasmid                       | CO₂ 1%, Acetate 10 mM | Nitrogen deprived | flask   | 11                        | [42]       |
| Synechocystis sp. PCC 6803 mutant strain | The native sigE was expressed from the chromosome                              | CO₂ 1%                | Nitrogen deprived | flask   | 1.4                       | [45]       |
| Synechocystis sp. PCC 6803 mutant strain | A PHA biosynthetic operon from M. aeruginosa was expressed from a plasmid      | CO₂ 2%                | Nitrogen deprived | flask   | 7                         | [44]       |
| Synechocystis sp. PCC 6803 mutant strain | The native phaAB was overexpressed from the chromosome                         | CO₂ 0.035%, Acetate 4 mM | Nitrogen deprived | flask   | 35                        | [41]       |
| Synechocystis sp. PCC 6803 mutant strain | xfpk from B. breve was expressed in a double pta and ach knock out.            | CO₂ 2%                | BG11 PBR           |         | 12                        | [46]       |

Figure 5. PHB biosynthetic pathway.

The PHB content reported for cultures carried out under photoautotrophic growth conditions using just CO₂ as carbon source was lower [78,79] than that measured for cultures with other carbon sources. The overexpression of the native sigE gene (encoding of the minor sigma factors of the organism) provided the integration of the latter in the Synechocystis chromosome, and increased the PHB content from 0.6% to 1.4% [74] when cells were grown in a N-deprived medium. Hondo et al. [78] transformed Synechocystis cells with the vector pAM461c, harboring a PHA biosynthetic operon from Microcystis aeruginosa NIES-843, and reached a PHB content of about 7% in the N-deprived
medium [74]. In a recent publication of our group, Carpine et al. [74] measured a PHB content larger than 12% (of dcw) by overexpression of phosphoketolase (xfpk), combined with the double deletion of phosphotransacetylase (pta) and acetyl-CoA hydrolase (ach) (under balanced growth conditions, i.e., using BG11 [80] as the growth medium). This result was almost two-fold higher than previously reported under photoautotrophic growth conditions [30].

6.3. Continuous PHB Production

A possible way to implement the process of PHB production by a genetically modified strain of *Synechocystis* PCC6803 is to carry out the process under a continuous mode. As comprehensively described in the literature, PHB production includes two distinct phases for most production strains. During the first phase, enough concentration of active biomass with low PHB accumulation is produced in a nutritionally balanced growth medium. During the second phase, a nutritional stress is induced by restricting the supply of a growth-essential nutrient (e.g., nitrogen, phosphate, etc.). This implies PHB accumulation due to the deviation of the carbon flux from biomass production. In this second phase, PHB accumulation is much higher than biomass formation [81]. A potential reactor configuration could be the serial arrangement of two sequential reactors: (i) reactor I for biomass production under optimal growth conditions; (ii) reactor II for PHB accumulation under nitrate-starvation conditions. The advantages of this configuration are

- The possibility to separate the biomass growth phase from the PHB production phase. The two phases are characterized by different optimal operating conditions;
- Minimization of equipment downtime and time loss due to the lag phase of the microbial cultures [82];
- The continuous cultivation can guarantee the growth of microorganisms and their long-term genetic stability under defined nutrient limitations for prolonged time periods, resulting in both high productivities and constant product quality [82];
- The possibility to harvest biomass at a desired PHB-mass fraction;
- The applied dilution rate D may significantly influence the molar mass of PHB. The PHB productivity/concentration may be affected by the dilution; indeed, alteration of the dilution rate could affect both utilization of the substrate and the growth of cells [83].

6.4. Mathematical Modeling

The researchers and industries involved in the blue biotechnology have focused on the potential replacement of conventional plastics by PHBs. The improvement of large-scale productivity and biochemical/genetic properties of producing strains requires mathematical modeling and process/strain optimization procedures [84]. In any case, the development of industrial processes asks for mathematical models of cell growth and PHB production.

Models available in literature to describe cell growth and the PHB production rate depend on the PHB production modality. Three classes of PHB producing microorganisms can be identified:

- Strains characterized by distinct phases: biomass growth phase and PHA production phase, typically induced by N or P limitation (prototype organisms: *Pseudomonas* sp. 2E, *Methylmonas extorquens*) [8];
- Strains characterized by the accumulation of PHB during cell growth phase under balanced nutritional conditions and by the increase of the PHB fraction under the non-growth phase (usually induced by N or P limitation) (prototype organism: *Cupriavidus necator*) [85];
- Strains characterized by remarkable PHB formation during the (exponential) growth phase without limitation of any essential growth components [86,87] (prototype organisms: *Azohydromonas lata* DSM 1122, or *Pseudomonas putida* GPo1 ATTC 29347).
The mathematical models reported in literature that describe the PHB production from microorganisms can be classified as follows: (i) models that are based on enzyme and/or microbial kinetics (kinetic models); (ii) models that are based on the characteristics of metabolic networks that are time invariant (dynamic models); models that are based on cellular metabolism (metabolic models); (iii) models that are based on microbial processes and on the metabolic regulation by the cybernetic framework (cybernetic models); and (iv) hybrid models (that combine at least two of the types of model properties mentioned before) [85].

A number of metabolic and bioreactor models have been proposed in literature for PHB production by bacteria (Table 6). Mozumder et al. [88] reported the autotrophic PHB production in *Cupriavidus necator* by using two-phase processes: biomass growth and subsequent PHB production. This study simulated the two distinct phases to achieve a high overall PHB production rate and PHB content. Faccin et al. [89] presented a model that aimed at the kinetics growth of *Bacillus megaterium* for the production of P(3HB). Khanna and Srivastava [90] proposed a structured unsegregated model for PHB accumulation in *Ralstonia eutropha* based on exhaustive investigations on substrate limitation and inhibition by the carbon, nitrogen, and phosphorus sources. Our group [57] proposed the first model to describe photoautotrophic PHB production by *Synechocystis* PCC6803 using a light/dark cycle (18 h light/6 h dark) for cyanobacterial growth. This model, presented in a publication by Carpine et al. [57], was based on mass balances. The effect of significant factors and nutrients on the cyanobacterial system was considered. In particular, the proposed model included the nitrate and phosphate utilization rate, the PHB production rate, the cellular growth and the rate of lysis of the cells. One of the innovations of this model was that the cells were considered to be in the cultures in two different statuses: the growing cells (X) that were able to grow, and the cells that were able to produce PHB (X_{PHB}). The specific velocity (kT) of X_{PHB} formation was considered dependent on the initial concentration of nitrate [57].
Table 6. Mathematical models for PHB production. Classical Monod, Haldane, Droop, and Pirt sub-models (with associated kinetic and stoichiometric parameters) represent the main blocks of the complete models.

| Microorganism          | Growth Rate | Production Rate | $\frac{dX}{dt}$ | $\frac{dPHB}{dt}$ | References |
|------------------------|-------------|-----------------|------------------|--------------------|------------|
| *Bacillus megaterium*  | $\mu_1 = \mu_{\text{max}} \left( \frac{N}{N_{\text{max}}} \right)$ | $\frac{dX}{dt} \cdot (\mu - k_1) \cdot X$ | $-\frac{\mu X}{\Phi}$ | $-K_1 \mu + k_2 \cdot X$ | [56]       |
|                        | $\mu_2 = \mu_{\text{max}} \left( \frac{N}{N_{\text{max}}} \right)$ | $-\frac{\mu X}{\Phi}$ | $-\frac{\mu X}{\Phi}$ |                     |            |
|                        | $\mu_3 = \mu_{\text{max}} \left( \frac{1}{(1 + \frac{n}{k_2} + k_3 \cdot N)} \right)$ | $-\frac{\mu X}{\Phi}$ | $-\frac{\mu X}{\Phi}$ |                     |            |
|                        | $\mu_4 = \mu_{\text{max}} \left( \frac{1}{(1 + \frac{k_1}{N_{\text{max}}} + k_2 \cdot N)} \right)$ | $-\frac{\mu X}{\Phi}$ | $-\frac{\mu X}{\Phi}$ |                     |            |
| *Cupriavidus necator*  | $\mu_{\text{KS}} = \mu_{\text{max}} \left( \frac{N_{\text{max}}}{C_0 + k_{N_{\text{max}}}} \right)$ | $\frac{dX}{dt} \cdot (\mu_{\text{KS}} - X)$ | $-\frac{\mu X}{\Phi}$ |                     |            |
|                        | $\mu_{\text{PHB}} = \mu_{\text{max}} \left( \frac{N_{\text{max}}}{C_0 + k_{N_{\text{max}}}} \right)$ | $\frac{dX}{dt} \cdot (\mu_{\text{PHB}} - X)$ | $-\frac{\mu X}{\Phi}$ |                     |            |
|                        | $\mu_{\text{max}} \left( \frac{1}{(1 + \frac{n}{k_2} + k_3 \cdot N)} \right)$ | $k_1 \cdot X \cdot (C_{\text{CO}_2} - C_{\text{CO}_2}) - \frac{F_{\text{PHB}}}{V_{\text{PHB}}} \cdot X$ | $-\frac{\mu X}{\Phi}$ |                     | [55]       |
| *Ralstonia eutropha*   | $\mu = \mu_{\text{max}} \left( \frac{N_{\text{max}}}{(C_{\text{N}} + k_{N_{\text{max}}})^n \cdot (C_{\text{P}} + k_{C_{\text{P}}})^n} \right)$ | $\frac{dX}{dt} \cdot \left( \frac{\mu}{\Phi} \cdot X \cdot C_1 - \frac{\mu}{\Phi} \cdot C_3 \cdot \left( \frac{X}{N} + m_1 \right) \cdot X + \frac{\mu}{\Phi} \cdot m_3 \cdot X - \frac{\mu}{\Phi} \cdot S_2 + \frac{\mu}{\Phi} \cdot N \cdot X \right)$ | $-\frac{\mu X}{\Phi}$ |                     | [57]       |
| *Synechocystis*        | $\mu_{\text{max}} \left( \frac{C_{\text{N}}}{k_{N_{\text{max}}} + C_{\text{N}}} \right) \left( 1 - \frac{C_{\text{P}}}{k_{C_{\text{P}}} + C_{\text{P}}} \right)$ | $\frac{dX}{dt} \cdot (\mu_{\text{PHB}} - X)$ | $-\frac{\mu X}{\Phi}$ |                     | [30]       |

For PHB models, $\rho_{\text{PHB}} = k_{\text{PHB}} \cdot C_{\text{PHB}} \cdot X_{\text{PHB}}$, and $\rho_{\text{PHB}} = \frac{\rho_{\text{PHB}}}{\Phi}$.

References:

- [30]
- [55]
- [56]
- [57]
7. PHB Recovery

The market penetration of PHB has the limitation of high production cost. The economic bottleneck is the cost of the fermentation and the recovery/purification technologies. The recovery/purification technologies issue significantly affects the overall process economics. The recovery of PHB granules from bacterial cytoplasm significantly increases total processing costs [91]. The process characterized by the lowest recovery cost was with the sodium hydroxide and sulphuric acid treatments (1.02 € kg\(^{-1}\) and 1.11 € kg\(^{-1}\), respectively) [92]. However, an efficient, economical, and environmentally friendly extraction of PHB from cells is required for a sustainable process from a cost point of view and from a cost-effective industrial production point of view.

The PHB recovery requires more details regarding its formation/accumulation mechanism in the cell to address the more appropriate recovery technique. PHB is a lipid-like compound synthesized by many microorganisms as storage material. The PHB is accumulated intracellularly under the form of inclusion bodies (PHB granules). PHB granules are wrapped by a considerable number of proteins: the granule structure suggests that they are supramolecular complexes with specific functions rather than being only simple sacks rich in carbon and energy [93] (Figure 6). The formation of PHB granules in the cells is currently under intense discussion. Three models of PHB granule formation were developed based on theoretical considerations and experimental data obtained from Ralstonia eutropha cultures. The first model—the Micelle model—assumes that soluble (cytoplasmic) PHB synthase molecules (phaC) start to produce the hydrophobic PHB molecule only when the concentration of the substrate (3-hydroxybutyryl-CoA) is sufficiently high [94]. The initial polymer chains of PHB form aggregates characterized by hydrophobicity and low solubility in an aqueous environment. The aggregates form the micelle structures in the cytoplasm of the cells. As a consequence of the Micelle model, the PHB granules can be randomly localized in the cell [93,95]. The second model—the Budding model—assumes that the PHB synthase is located in the cytoplasmic membrane and that the newly formed PHB chains are realized into the bilayer of the membrane [96]. The third model—the Scaffold model—assumes that PHB synthase of nascent PHB granules is—or becomes—attached to a yet unknown scaffold molecule within the cell [93]. The granules-scaffold complexes are bound to the bacterial nucleoid and constitute the PHB granule initiation complexes. In this case, subcellular localization of PHB granules would depend on the nature and localization of the scaffold of the PHB-accumulating cell [93].

**Figure 6.** PHB granules inside the cyanobacteria cells: (A) Synechococcus sp. PCC7942 [27]; (B) Synechococcus sp. PCC7942 [97]; (C) Synechocystis PCC6803 [23]; (D) Cyanothece sp. strain PCC 7822 [98]; (E) Synechococcus sp. MA19 [72].
Whatever the PHB formation/accumulation mechanism, the recovery of the PHB granules requires the rupture of the bacterial cell and the removal of the protein layer wrapping the PHB granules. A possible alternative is the selective dissolution of the PHB granules by means of suitable solvents and successive precipitation of the dissolved PHB.

Solvent extraction is the most widely adopted method to recuperate PHB from the cellular biomass. This method is simple and rapid and allows the assessment of the PHB fraction accumulated in the cells [99]. The two main steps of the PHB extraction are the modification of permeability of the cell membrane to enhance the release and solubilization of PHB and the PHB non-solvent precipitation [100]. Extraction of PHB with solvents such as chlorinated hydrocarbons—e.g., chloroform, 1,2-dichloroethane [101]—and cyclic carbonates—e.g., ethylene carbonate and 1,2-propylene carbonate—is very common. Precipitation of PHB is commonly induced by a non-solvent, such as methanol and ethanol [101]. Solvent extraction has undoubtedly advantages over the other extraction methods of PHB in terms of efficiency. Furthermore, this method can remove bacterial endotoxin and cause insignificant degradation of the formed polymers [100]. Therefore, it is possible to obtain very pure PHB with a high molecular weight. Unfortunately, a large-scale application of solvent extraction is considered not environmentally friendly.

The digestion technique to recover the PHB requires the solubilization of the cellular material wrapping the PHB granules. The digestion techniques are well established approaches developed as an alternative to solvent extraction.

Table 7 reports information about the main PHB recovery techniques. Results regarding the PHB purity and the yield are listed as well as reported in the reviewed paper.

A simple, practical, efficient, and cost-effective system of PHB recovery using non-halogenated solvents should be developed. Different strategies for PHB recovery are reported hereinafter:

- Recovery of PHB accumulated in cyanobacteria using ionic liquids to dissolve cyanobacteria and retain PHB [102]. Ionic liquids (ILs) have emerged as a potential solvent for several applications. ILs are characterized by a melting point below 100 °C. They are characterized by interesting properties—e.g., high ionic conductivity and high thermal stability—which are difficult to achieve in general organic solvents [102]. Furthermore, ILs are characterized by negligible vapor pressure. They are gaining attention as novel green solvents;

- Spontaneous liberation of intracellular PHB granules using a genetic engineering approach. Jung et al. [103] manipulated the initial inoculum size and the composition of the medium and obtained an Escherichia coli strain that was able to produce PHB at a very high fraction [103]. After spontaneous cell lysis, PHB granules were released into the medium;

- Extraction of PHB using non-chlorinated solvents (e.g., anisole) [104] or non-halogenated solvents (e.g., butyl acetate) [105];

- Extraction of PHB with a solvent-free approach using enzyme digestion in an aqueous medium [106]. Martino et al. [106] used biomass of Cupriavidus necator DSM 428 grown on used cooking oil (UCO) for extraction of the PHB granules using sodium dodecyl sulphate (SDS), ethylenediaminetetraacetic acid (EDTA), and the enzyme alcalase in an aqueous medium. The recovered PHB granules showed >90% purity and no crystallization.
Table 7. PHB recovery methods reported in literature.

| Extraction Method | Materials | Strain | Results | References |
|------------------|-----------|--------|---------|------------|
| **Solvent extraction** | Chloroform | *Bacillus cereus* SPV | Purity: 92%; Yield: 31% | [107] |
| | Chloroform | *Cupriavidus necator* | Purity: 95%; Yield: 96% | [108] |
| | 1,2-Propylene carbonate | *Cupriavidus necator* DSM 545 | Purity: 84%; Yield: 95% | [108] |
| | Methyl tert-butyl ether | *Pseudomonas putida* KT2440 | Yield: 80–85% | [109] |
| | Butyl acetate | *C. necator* | Purity: 99; Yield: 96 | [91] |
| | Non halogenated acetone/ ethanol/propylene carbonate | *C. necator* | Purity: 93%; Yield: 92% | [110] |
| | Acetone | *P. putida* GPO1 | Yield: 94% | [111] |

| Digestion Method | Surfactant | SDS | Recombinant *Escherichia coli* | Purity: 99%; Yield: 89% | [112] |
| | Palmitoyl carnitine | *C. necator*, *Alcaligenes latus* | Degree of lysis: 56–78% | [113] |
| | SDS-Sodium hypochlorite | *Azotobacter chroococcum G-3* | Purity: 98%; Yield: 87% | [114] |
| | Triton X-100-sodium hypochlorite | *C. necator* DSM 545 | Purity: 98% | [115] |
| | Sodium hypochlorite | *C. necator* Recombinant *Escherichia coli* | Purity: 86%; Purity: 93% | [116] |
| | Sodium hypochlorite | *Staphylococcus epidermidis* | | [117] |
| | Triton X-100-EDTA | *Sinorhizobium meliloti* | Purity: 68% | [118] |
| | Betaine-EDTA disodium salt | *C. necator* DSM 545 | Purity: >96%; Yield: 90% | [119] |
| | Chloroform-sodium hypochlorite | *B. cereus* SPV | Purity: 95%; Yield: 30% | [107] |
| | Chloroform-sodium hypochlorite | *C. necator* Recombinant *Escherichia coli* | Purity: >98% | [116] |
| | Selective dissolution by protons | Sulfuric acid | *C. necator* | Purity: >97%; Yield: >95% | [120] |
| | Enzymatic digestion | Enzyme combined with SDS-EDTA | *P. putida* | Purity: 93% | [121] |
| | | Alcalase combined with SDS and (EDTA), *Cupriavidus necator* DSM 428 | | Purity: >90%; Yield: >90% | [71] |
| | | Bead mill | *A. latus* | | [122] |
| | | High pressure homogenization | *A. latus* | | [122] |
| | | SDS-High pressure homogenization | *Methylobacterium sp V49* | Purity: 95%; Yield: 98% | [123] |
| | | Spontaneous liberation | *E. coli* | Autolysis of 80% | [68] |
8. Conclusions

Poly-β-hydroxybutyrate (PHB) is one of the best biodegradable polymers among those proposed as alternatives to conventional plastics. The photoautotrophic system of cyanobacteria is considered the system of choice for a low-cost production of PHB. One of the challenges is to render the PHB production by cyanobacteria, a competitive process, using various strategies described in this paper: (i) medium optimization; (ii) genetic engineering approach; (iii) tuning of mathematical model for cyanobacterial growth and coupled PHB production; (iv) PHB production using continuous operations. The synergic effect of the improvements achieved with these approaches will give the best chances to maximize PHB production by cyanobacteria.

So far, the main drawback of the commercial production and application of PHB in the industry is its high production cost compared with conventional plastics. The recovery of PHB granules from cytoplasm significantly increases total processing costs. The other big challenge of the PHB production process from cyanobacteria is to find a low-cost and environmentally friendly strategy to recover it.

Author Contributions: R.C.: conceptualization, writing—original draft, writing—review & editing. G.O.: conceptualization, writing—original draft, writing—review & editing. K.J.H.: writing—review & editing. A.P.: writing—review & editing. A.M.: writing—review & editing.

Funding: Part of the work of R.C. was supported by a grant from the Stichting tot Bevordering van het Wetenschappelijk Onderzoek in de Biochemie, Amsterdam, The Netherlands.

Conflicts of Interest: The authors report no conflict of interest.

References

1. Sharma, L.; Kumar Singh, A.; Panda, B.; Mallick, N. Process optimization for poly-beta-hydroxybutyrate production in a nitrogen fixing cyanobacterium, Nostoc muscorum using response surface methodology. Bioresour. Technol. 2007, 98, 987–993. [PubMed]
2. Hempel, F.; Bozarth, A.S.; Lindenkamp, N.; Klingl, A.; Zauner, S.; Linne, U.; Steinbüchel, A.; Maier, U.G. Microalgae as bioreactors for bioplastic production. Microb. Cell Fact. 2011, 10, 81. [PubMed]
3. Costa, S.S.; Miranda, A.L.; de Morais, M.G.; Costa, J.A.V.; Druzan, J.I. Microalgae as source of polyhydroxyalkanoates (PHAs)—A review. Int. J. Biol. Macromol. 2019, 131, 536–547.
4. Singh, A.K.; Sharma, L.; Mallick, N.; Mala, J. Progress and challenges in producing polyhydroxyalkanoate biopolymers from cyanobacteria. J. Appl. Phycol. 2017, 29, 1213–1232.
5. Brandl, H.; Gross, R.A.; Lenz, R.W.; Fuller, R.C. Pseudomonas oleovorans as a source of poly(beta-hydroxyalkanoates) for potential applications as biodegradable polyesters. Appl. Environ. Microbiol. 1988, 54, 1977–1982. [PubMed]
6. Nomura, C.; Taguchi, K.; Gan, Z.; Kuwabara, K.; Tanaka, T.; Takase, K.; Koyama, N.; Doi, Y. Expression of 3-ketoacyl-acyl carrier protein reductase (fabG) genes enhances production of polyhydroxyalkanoate copolymer from glucose in recombinant Escherichia coli JM109. Appl. Environ. Microbiol. 2005, 71, 4297–4306. [PubMed]
7. Steinbüchel, A.; Wiese, S.A. Pseudomonas strain accumulating polyesters of 3-hydroxybutyric acid and medium-chain-length 3-hydroxyalkanoic acids. Appl. Microbiol. Biotechnol. 1992, 37, 691–697.
8. Braunegg, G.; Lefebvre, G.; Genser, K.F. Polyhydroxyalkanoates, biopolymers from renewable resources: Physiological and engineering aspects. J. Biotechnol. 1998, 65, 127–161.
9. Balaji, S.; Gopi, K.; Muthuvelan, B. A review on production of poly β hydroxybutyrates from cyanobacteria for the production of bio plastics. Algal Res. 2013, 2, 278–285.
10. Amaro, T.M.M.; Rosa, D.; Comi, G.; Iacumin, L. Prospects for the Use of Whey for Polyhydroxyalkanoate (PHA) Production. Front Microbiol. 2019, 10, 992.
11. Roland-Holst, D.; Heft-Neal, S. Bioplastics in California. Economic Assessment of Market. Conditions for PHA/PHB Bioplastics Produced from Waste Methane; Department of Resources Recycling and Recovery: Sacramento, CA, USA, 2013.
12. Chandani, N.; Mazumder, P.B.; Bhattacharjee, A. Production of polyhydroxybutyrate (biopolymer) by Bacillus tequilensis NCS-3 Isolated from municipal waste areas of silchar, assam. Int. J. Sci. Res. 2014, 3, 198–203.
13. Jiang, Y.; Marang, L.; Kleerebezem, R.; Muyzer, G.; van Loosdrecht, M.C.M. Polyhydroxybutyrate production from lactate using a mixed microbial culture. Bioresource Technology. Biotech. 2011, 108, 2022–2035.

14. Budde, C.F.; Riedel, S.L.; Hübner, F.; Risch, S.; Popovi, M.K.; Rha, C.; Sinskey, A.J. Growth and polyhydroxybutyrate production by Ralstonia eutropha in emulsified plant oil medium. Appl. Microbiol. Biotech. 2011, 89, 1611–1619.

15. Han, J.; Qiu, Y.Z.; Liu, D.C.; Chen, G.Q. Engineered Aeromonas hydrophila for enhanced production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) with alterable monomers composition. FEMS Microbiol. Lett. 2004, 239, 195–201.

16. Wong, P.A.L.; Cheung, M.K.; Lo, W.; Chua, H.; Hoi, P.; Yu, F. Investigation of the effects of the types of food waste utilized as carbon source on the molecular weight distributions and thermal properties of polyhydroxybutyrate produced by two strains of microorganisms. e-Polymers 2004, 31, 1–11.

17. Pozo, C.; Martín-Toledo, M.V.; Rodelas, B.; González-López, J. Effects of culture conditions on the production of polyhydroxyalkanoates by azotobacter chroococcum H23 in media containing a high concentration of alpechin (wastewater from olive oil mills) as primary carbon source. J. Biotechnol. 2002, 97, 125–131.

18. ŁAbuzek, S.; Radecka, I. Biosynthesis of PHB tercopolymer by Bacillus cereus UW85. J. Appl. Microbiol. 2001, 90, 353–357.

19. Chaijamrus, S.; Udpuay, N. Poly(3-Hydroxybutyrate) production with high productivity and high polymer content by a fed-batch culture of alcaligenes latus under nitrogen limitation. Agric. Eng. Int. CIGRE J. 2008, 27, 10.

20. Thirumala, M.; Reddy, S.V.; Mahmood, S.K.; Sultanpuram, M.T.; Reddy, V.; Mahmood, S.K. Production and characterization of PHB from two novel strains of Bacillus spp. isolated from soil and activated sludge. J. Ind. Microbiol. Biotech. 2010, 37, 271–278.

21. Brämer, C.O.; Vandamme, P.; Da Silva, L.F.; Gomez, J.G.C.; Steinbüchel, A. Burkholderia sacchari sp. nov., a polyhydroxyalkanoate-accumulating bacterium isolated from soil of a sugar-cane plantation in Brazil. Int. J. Syst. Evol. Microbiol. 2001, 51, 1709–1713.

22. Silva, L.F.; Taciro, M.K.; Michelin Ramos, M.E.; Carter, J.M.; Pradella, J.G.C.; Gomez, J.G.C. Poly-3-hydroxybutyrate (P3HB) production by bacteria from xylose, glucose and sugarcane bagasse hydrolysate. J. Ind. Microbiol. Biotech. 2004, 31, 245–254.

23. Qi, Q.; Rehm, B.H.A. Polyhydroxybutyrate biosynthesis in caulobacter crescentus: Molecular characterization of the polyhydroxybutyrate synthase. Microbiology 2001, 147, 3353–3358.

24. Daneshi, A.; Younesi, H.; Chasempouri, S.M.; Sharifzadeh, M. Production of poly-3-hydroxybutyrate by Cupriavidus necator from corn syrup: Statistical modeling and optimization of biomass yield and volumetric productivity. J. Chem. Technol. Biotech. 2010, 85, 1528–1539.

25. Cavalheiro, J.M.B.T.; Catarina, M.; de Almeida, M.D.; Grandfils, C.; da Fonseca, M.M.R. Poly(3-hydroxybutyrate) production by Cupriavidus necator using waste glycerol. Process. Biochem. 2009, 44, 509–515.

26. Ceyhan, N.; Ozdemir, G. Poly-β-hydroxybutyrate (PHB) production from domestic wastewater using enterobacter aerogenes. Afr. J. Microbiol. Res. 2011, 5, 690–702.

27. Nikel, P.I.; Pettinari, M.J.; Galvagno, M.A.; Méndez, B.S. Poly(3-hydroxybutyrate) synthesis by recombinant Escherichia coli arcA mutants in microaerobiosis. Appl. Environ. Microbiol. 2006, 72, 2614–2620.

28. Quillaguamán, J.; Hashim, S.; Bento, F.; Mattiasson, B.; Hatti-Kaul, R. Poly(beta-hydroxybutyrate) production by a moderate halophile, Halomonas boliviensis LC1 using starch hydrolysate as substrate. J. Appl. Microbiol. 2005, 99, 151–157.

29. James, B.W.; Mauchline, W.S.; Dennis, P.J.; Keevil, C.W.; Wait, R. Poly-3-hydroxybutyrate in Legionella pneumophila, an energy source for survival in low-nutrient environments. Appl. Environ. Microbiol. 1999, 65, 822–827.

30. Wendlandt, K.D.; Geyer, W.; Mirschel, G.; Al-Haj Hemidi, F. Possibilities for controlling PHB accumulation using various analytical methods. J. Biotechnol. 2005, 117, 119.

31. Akar, A.; Akkaya, E.U.; Yesiladali, S.K.; Çelikyilmaz, G.; Çokgor, E.U.; Tamerler, C.; Orhon, D.; Çakar, Z.P. Accumulation of polyhydroxyalkanoates by Microtunatus phosphovorus under various growth conditions. J. Ind. Microbiol. Biotech. 2006, 33, 215–220.

32. De Paula, F.C.; Kakazu, S.; de Paula, C.B.C.; Gomez, J.G.C.; Contiero, J. Polyhydroxyalkanoate production from crude glycerol by newly isolated Pandoraea sp. J. King Saud Univ. 2017, 29, 166–173.
33. Fernández, D.; Rodríguez, E.; Bassas, M.; Viñas, M.; Solanas, A.M.; Llorens, J.; Marqués, A.M.; Manresa, A. Agro-industrial oily wastes as substrates for PHA production by the new strain Pseudomonas aeruginosa NCIB 40045: Effect of culture conditions. Biochem. Eng. J. 2005, 26, 159–167.

34. Koller, M.; Bona, R.; Chiellini, E.; Fernandes, E.G.; Horvat, P.; Kutschera, C.; Hesse, P.; Braunegg, G. Polyhydroxyalkanoate production from whey by Pseudomonas hydrogenovora. Bioresour. Technol. 2008, 99, 4854–4863. [PubMed]

35. Coelho, V.C.; Klasener, C.; Terra, A.L.; Alberto, J.; Costa, V.; Morais, M.G. De Polyhydroxybutyrate production by Spirulina sp. LEB 18 grown under different nutrient concentrations. Afr. J. Microbiol. Res. 2015, 9, 1586–1594.
56. Panda, B.; Mallick, N. Enhanced poly-beta-hydroxybutyrate accumulation in a unicellular cyanobacterium, *Synechocystis* sp. PCC 6803. *Lett. Appl. Microbiol.* 2007, 44, 194–198.

57. Carpine, R.; Raganati, F.; Oliweri, G.; Hellingswerf, K.J.; Pollio, A.; Salatino, P.; Marzocchella, A. Poly-β-hydroxybutyrate (PHB) production by *Synechocystis* PCC 6803 from CO₂: Model development. *Algal Res.* 2018, 29, 49–60.

58. Haase, S.M.; Huchzermeyer, B.; Rath, T. PHB accumulation in *Nostoc muscorum* under different carbon stress situations. *J. Appl. Phycol.* 2012, 24, 157–162.

59. Smith, A.J. Modes of cyanobacterial carbon metabolism. In *The Biology of Cyanobacteria*; Carr, N.G., Whitton, B.A., Eds.; Blackwell: London, UK, 1982; pp. 47–85.

60. Di Pippo, F.; Ellwood, N.T.W.; Gismondi, A.; Bruno, L.; Rossi, F.; Magni, P.; de Philippis, R. Characterization of exopolysaccharides produced by seven biofilm-forming cyanobacterial strains for biotechnological applications. *J. Appl. Phycol.* 2013, 25, 1697–1708.

61. Carpine, R.; Raganati, F.; Oliweri, G.; Hellingswerf, K.J.; Pollio, A.; Salatino, P.; Marzocchella, A. Poly-β-hydroxybutyrate (PHB) polymer from *Aulosira fertilissima*. *J. Appl. Phycol.* 2012, 24, 803–814.

62. Toh, Y.; Jau, M.-H.; Yew, S.-P.; Abed, R.M.M.; Sudesh, K. Comparison of polyhydroxyalkanoates biosynthesis, mobilization and the effects on cellular morphology in *Spirulina platensis* and *Synechocystis* sp. uniwg. *Biomolecules* 2019, 9, 198.

63. Singh, M.K.; Rai, P.K.; Rai, A.; Singh, S.; Singh, J.S. Poly-β-hydroxybutyrate production by the cyanobacterium *Synechocystis* sp. under varying environmental conditions. *Biomolecules* 2019, 9, 198.

64. Miyake, M.; Takase, K.; Narato, M.; Khatipov, E.; Schnackenberg, J.; Shirai, M.; Kurane, R.; Asada, Y. Polyhydroxybutyrate production from carbon dioxide by *Calothrix scytonemicola* TISTR 8095. *Bioresour. Technol.* 2016, 212, 342–347.

65. Khetkorn, W.; Incharoensakdi, A.; Lindblad, P.; Jantaro, S. Bioresource technology enhancement of *Synechocystis* sp. PCC 6803 for poly-β-hydroxybutyrate overproduction. *Algal Res.* 2017, 25, 117–127.

66. Haase, S.M.; Huchzermeyer, B.; Rath, T. PHB accumulation in *Nostoc muscorum* under different carbon stress situations. *J. Appl. Phycol.* 2012, 24, 157–162.

67. Smith, A.J. Modes of cyanobacterial carbon metabolism. In *The Biology of Cyanobacteria*; Carr, N.G., Whitton, B.A., Eds.; Blackwell: London, UK, 1982; pp. 47–85.

68. Smith, A.J. Modes of cyanobacterial carbon metabolism. In *The Biology of Cyanobacteria*; Carr, N.G., Whitton, B.A., Eds.; Blackwell: London, UK, 1982; pp. 47–85.

69. Ducat, D.C.; Way, J.C.; Silver, P.A. Engineering cyanobacteria to generate high-value products. *Trends Biotechnol.* 2011, 29, 95–103.

70. Ruffing, A.M. Engineered cyanobacteria: Teaching an old bug new tricks. *Bioeng. Bugs* 2011, 2, 136–149.

71. Koksharova, O.A.; Wolk, C.P. Genetic tools for cyanobacteria. *Appl. Microbiol. Biotechnol.* 2002, 58, 123–137.

72. Angermayr, S.A.; Gorchs Rovira, A.; Hellingwerf, K.J. Metabolic engineering of cyanobacteria for the synthesis of commodity products. *Trends Biotechnol.* 2015, 33, 352–361.

73. Miyake, M.; Takase, K.; Narato, M.; Khatipov, E.; Schnackenberg, J.; Shirai, M.; Kurane, R.; Asada, Y. Polyhydroxybutyrate production from carbon dioxide by cyanobacteria. *Appl. Biochem. Biotechnol.* 2000, 84, 991–1002.

74. Carpine, R.; Du, W.; Olivieri, G.; Pollio, A.; Hellingswerf, K.J.; Marzocchella, A.; Branco dos Santos, F. Genetic engineering of *Synechocystis* sp. PCC 6803 for poly-β-hydroxybutyrate overproduction. *Algal Res.* 2017, 25, 117–127.

75. Khetkorn, W.; Incharoensakdi, A.; Lindblad, P.; Jantaro, S. Bioresource technology enhancement of poly-3-hydroxybutyrate production in *Synechocystis* sp. PCC 6803. *Int. J. Biol. Macromol.* 2002, 30, 97–104. [PubMed]

76. Sudesh, K.; Taguchi, K.; Doi, Y. Effect of increased PHA synthase activity on polyhydroxyalkanoates biosynthesis in *Synechocystis* sp. PCC 6803. *Int. J. Biol. Macromol.* 2002, 30, 97–104. [PubMed]

77. Wu, G.F.; Shen, Z.Y.; Wu, Q.Y. Modification of carbon partitioning to enhance PHB production in *Synechocystis* sp. PCC 6803. *Enzym. Microb. Technol.* 2002, 30, 710–715.
78. Hondo, S.; Takahashi, M.; Osanai, T.; Matsuda, M.; Hasunuma, T.; Tazuke, A.; Nakahira, Y.; Chohnan, S.; Hasegawa, M.; Asayama, M. Genetic engineering and metabolite profiling for overproduction of polyhydroxybutyrate in cyanobacteria. J. Bioenerg. Biomembr. 2015, 47, 120, 510–517.

79. Osanai, T.; Numata, K.; Oikawa, A.; Kuwahara, A.; Iijima, H.; Doi, Y.; Tanaka, K.; Saito, K.; Hirai, M.Y. Increased bioplastic production with an RNA polymerase sigma factor SigE during nitrogen starvation in Synechocystis sp. PCC 6803. DNA Res. 2013, 20, 525–535.

80. Rippka, R.; Deruelles, J.; Waterbury, J.B.; Herdman, M.; Stanier, R.Y. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. 1979, 111, 1–61.

81. Koller, M.; Braunegg, G. Potential and prospects of continuous polyhydroxyalkanoate (PHA) production. Bioengineering 2015, 2, 94–121.

82. Braunegg, G.; Lefebvre, G.; Renner, G.; Zeiser, A.; Haage, G.; Loidl-Lanthaler, K. Kinetics as a tool for polyhydroxyalkanoate production optimization. Can. J. Microbiol. 1995, 41, 239–248.

83. Koyama, N.; Doi, Y. Continuous production of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) by Alcaligenes eutrophus. Biotechnol. Lett. 1995, 17, 281–284.

84. Luengo, J.M.; García-Gonzalez, L.; De Wever, H.; Volcke, E.I.P. Poly (3-hydroxybutyrate) (PHB) production by Bacillus megaterium poly[(R)-3-hydroxyalkanoates] by Pseudomonas putida GPo1: Batch versus chemostat production. Biotechnol. Bioeng. 2006, 93, 737–746. [PubMed]

85. Kollner, M.; Salerno, A.; Dasi, M.; Reiterer, A.; Braunegg, G. Modern biotechnological polymer synthesis: A review. Food Technol. Biotechnol. 2010, 48, 255–269.

86. Islam Mozumder, M.S.; Garcia-Gonzalez, L.; De Wever, H.; Volcke, E.I.P. Poly (3-hydroxybutyrate) (PHB) production from CO₂ to PHB biopolymer with non-halogenated solvents. J. Microbiol. Biotechnol. 2015, 25, 1936–1943.

87. López-Abelairas, M.; García-Torreiro, M.; Lu-Chau, T.; Lema, J.M.; Steinbüchel, A. Comparison of several methods for the separation of poly(3-hydroxybutyrate) from Cupriavidus necator H16 cultures. Biochem. Eng. J. 2015, 93, 250–259.

88. Jendrossek, D.; Fleiffer, D. New insights in the formation of polyhydroxyalkanoate granules (carbonosomes) and novel functions of poly (3-hydroxybutyrate). Environ. Microbiol. 2014, 16, 2357–2373.

89. Gerngross, T.U.; Reilly, P.; Stubbe, J.; Sinskey, A.J.; Peoples, O.P. Immunocytotoxic analysis of the metabolism and potential for CO₂ recycling. Int. J. Biol. Macromol. 1999, 25, 37–42.

90. Zhang, D.; Dechativongp, S.; Hellgardt, K. Modelling light transmission, cyanobacterial growth kinetics and fluid dynamics in a laboratory scale multiphase photo-bioreactor for biological hydrogen production. Algal Res. 2015, 8, 99–107.

91. Kunasundari, B.; Sudesh, K. Isolation and recovery of microbial polyhydroxyalkanoates. Express Polym. Lett. 2011, 5, 620–634.
100. Jacquel, N.; Lo, C.; Wei, Y.; Wu, H.; Wang, S.S. Isolation and purification of bacterial poly(3-hydroxyalkanoates). Biochem. Eng. J. 2008, 39, 15–27.

101. Ramsay, J.A.; Berger, E.; Voyer, R.; Chavarie, C.; Ramsay, B.A. Extraction of poly-3-hydroxybutyrate using chlorinated solvents. Biotechnology 1994, 8, 589–594.

102. Kobayashi, D.; Fujita, K.; Nakamura, N.; Ohno, H. A simple recovery process for biodegradable plastics accumulated in cyanobacteria treated with ionic liquids. Appl. Microbiol. Biotechnol. 2014, 99, 1647–1653.

103. Jung, I.L.; Phyo, K.H.; Kim, K.C.; Park, H.K.; Kim, I.G. Spontaneous liberation of intracellular polyhydroxybutyrate granules in escherichia coli. Res. Microbiol. 2005, 156, 865–873.

104. Rosengart, A.; Cesário, M.T.; de Almeida, M.C.M.D.; Raposo, R.S.; Espert, A.; de Apodaca, E.D.; da Fonseca, M.M.R. Efficient P(3HB) extraction from Burkholderia sacchari cells using non-chlorinated solvents. Biochem. Eng. J. 2015, 103, 39–46.

105. Wang, G.; Gislum, M.; Filipppov, G.; Montgomery, S. An environment-friendly and efficient method for extraction of PHB biopolymer by non-halogenated solvents. J. Microbiol. Biotechnol. 2015, 31, 785–794.

106. Martino, L.; Cruz, M.V.; Scoma, A.; Freitas, F.; Bertin, L.; Scandola, M.; Reis, M.A.M. Recovery of amorphous polyhydroxybutyrate granules from Cupriavidus necator cells grown on used cooking oil. Int. J. Biol. Macromol. 2014, 71, 117–123. [PubMed]

107. Valappil, S.P.; Misra, S.K.; Bocaccini, A.R.; Keshavarz, T.; Bucke, C.; Roy, I. Large-scale production and efficient recovery of PHB with desirable material properties, from the newly characterised Bacillus cereus SPV. J. Biotechnol. 2007, 132, 251–258. [PubMed]

108. Fioresse, M.L.; Freitas, F.; Pais, J.; Ramos, A.M.; De Aragão, G.M.F.; Reis, M.A.M. Recovery of polyhydroxybutyrate (PHB) from Cupriavidus necator biomass by solvent extraction with 1,2-propylene carbonate. Engr. Life Sci. 2009, 9, 454–461.

109. Wampfler, B.; Ramsauer, T.; Rezzonico, S.; Hischier, R.; Köhling, R.; Thöny-Meyer, L.; Zinn, M. Isolation and purification of medium chain length poly (3-hydroxyalkanoates) (mcl-PHA) for medical applications using nonchlorinated solvents. Biomacromolecules 2010, 11, 2716–2723. [PubMed]

110. Fei, T.; Caseneuve, S.; Wen, Z.; Wu, L.; Wang, T. Effective recovery of poly-b-hydroxybutyrate (PHB) biopolymer from Cupriavidus necator using a novel and environmentally friendly solvent system. Am. Inst. Chem. Eng. 2016, 32, 678–685.

111. Elbahloul, Y.; Steinbüchel, A. Large-scale production of poly (3-hydroxyoctanoic acid) by Pseudomonas putida GP01 and a simplified downstream process. Appl. Environ. Microbiol. 2009, 75, 643–651.

112. Choi, J.; Lee, S.Y. Efficient and economical recovery of poly (3-Hydroxybutyrate) from recombinant escherichia coli by simple. Biotechnol. Bioeng. 1999, 62, 546–553.

113. Lee, K.M.; Chang, H.N.; Chang, Y.K.; Kim, B.S.; Hahn, S.K. The lysis of Gram-negative Alcaligenes eutrophus and recombinant Escherichia coli by simple. Process. Biochem. 1993, 7, 295–300.

114. Dong, Z.; Sun, X.; Zhaolin, D.; Xuenan, S.U.N. A new method of recovering polyhydroxyalkanoate from Azotobacter chroococcum. Chin. Sci. Bull. 2000, 45, 252–256.

115. Ramsay, J.A.; Berger, E.; Ramsay, B.A.; Chavarie, C. Recovery of poly-3-hydroxyalkanoic acid granules by a surfactant-hypochlorite treatment. Biotechnol. Bioeng. 1990, 4, 221–226.

116. Hahn, S.K.; Chang, Y.K.; Lee, S.Y. Recovery and characterization of poly (3-hydroxybutyric acid) synthesized in Alcaligenes eutrophus and recombinant Escherichia coli. Recovery and characterization of poly-(3-Hydroxybutyric Acid) synthesized in Alcaligenes eutrophus and recombinant Es. Appl. Environ. Microbiol. 1995, 61, 34–39. [PubMed]

117. Marjadi, D.; Dharaiya, N. Recovery and characterization of poly (3-Hydroxybutyric acid) synthesized in Staphylococcus epidermidis. Afr. J. Environ. Sci. Technol. 2015, 8, 319–329.

118. Lakshman, K.; Shamala, T.R. Extraction of polyhydroxyalkanoate from Sinorhizobium meliloti cells using Microbispora sp. culture and its enzymes. Enzym. Microb. Technol. 2006, 39, 1471–1475.

119. Chen, Y.; Yang, H.; Zhou, Q.; Chen, J.; Gu, G. Cleaner recovery of poly (3-hydroxybutyric acid) synthesized in Alcaligenes eutrophus. Process. Biochem. 2001, 36, 501–506.

120. Yu, J.; Chen, L.X.L. Cost-effective recovery and purification of polyhydroxyalkanoates by selective dissolution of cell mass. Biotechnol. Prog. 2006, 22, 547–553.

121. Kathiraser, Y.; Aroura, M.K.; Ramachandran, K.B.; Kit, I.; Tan, P. Chemical characterization of polyhydroxyalkanoates (PHAs) recovered by enzymatic treatment and ultrafiltration. J. Chem. Technol. Biotechnol. 2007, 82, 847–855.
122. Tamer, I.M.; Moo-Young, M.; Christi, Y. Disruption of alcaligenes latus for recovery of poly (-hydroxybutyric acid): Comparison of high-pressure homogenization, bead milling, and chemically induced lysis. *Ind. Eng. Chem. Res.* **1998**, *37*, 1807–1814.

123. Ghatnekar, M.S.; Pai, J.S.; Ganesh, M. Production and recovery of poly-3-hydroxy-butyrate from *Methyllobacterium* sp. V49. *J. Chem. Technol. Biotechnol.* **2002**, *77*, 444–448.

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).