PROSPECTS OF A BIODEGRADABLE POLYMER (POLYHYDROXYALKANOATE) PRODUCTION WITH WASTEWATER

By

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
Master of Science in Biotechnology

Department of Mathematics and Natural Sciences
BRAC University
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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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Approval

The thesis/project titled “Prospects of Biodegradable Polymer (Polyhydroxyalkanoate) Production with Wastewater” submitted by

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of Fall, 2019 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Master of Science in Biotechnology on 3rd October 2019.

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Abstract

Plastic production and accumulation have devastating environmental effects, and consequently, the world is in need to focus on environmentally friendly plastic substitutes such as sustainable biofuels, biomaterials, and fine chemicals production. Polyhydroxyalkanoates represent one of the biomaterials of the future due to their physicochemical properties, biodegradability, and biocompatibility. Despite having comparable characteristics to common plastics, extensive PHA use is still hampered by its high production cost. PHAs are by-product of bacterial metabolism, and one of the major costs associated with their production derives from the carbon source used for bacterial fermentation. This study illustrates the individual research articles in the current global researches and development landscape related to polyhydroxyalkanoates (PHA) production with cheap carbon source; mainly wastewater. In this dissertation, summary of the relevant research carried out with bacterial culture as well as the challenges and opportunities regarding polyhydroxyalkanoate production are presented and discussed in both global and national perspective. Basic concepts, regarding the metabolism and microbiology along with technological approaches with feedstock evaluation process engineering and polymer processing towards high-value marketable products are also described.

Keywords: Bacterial culture; Biodegradable Polymers; Feedstock; Metabolism; Polyhydroxyalkanoate; Sustainability; Wastewater

Dedicated to

All those people who are concern about environment

Acknowledgement

Undertaking a project of this stature would not have been possible without the strength and blessings of Allah and the help and support of my family.

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Mehbubun Nayeema

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List of Acronyms

BPA- Bisphenol A

CAGR- Compound Annual Growth Rate

CFSTR- Continuous Flow Stirred Tank Reactor

COD- Chemical Oxygen Deamnd

CPD- Centre for Policy Dialogue

DAHP- Diammonium Hydrogen Phosphate

GAO- Glycogen-Accumulating Organisms

HDPE- High-Density Polyethylene

HRT- Hydraulic Retention Time

IKT- Institut für Kunststofftechnik

MMC- Mixed Microbial Cultures

NADPH- Nicotinamide Adenine Dinucleotide Phosphate

OLR- Organic Loading Rate

PAO- Phosphorus-Accumulating Organisms

PE- Polyethylene

PLA- Polylactic Acid

PhaG -(R)-3-hydroxyacyl-ACP-CoA transferase

PH3B- Polyhydroxybutyrate

PHV- Polyhydroxyvalerate

PHBHV- Poly (HydroxyButyrate-co-HydroxyValerate)

SRT- Solids Retention Time

SASP- South Asian Seas Program

SACEP- South Asia Cooperative Environment Programme

SAS- South Asian Seas

UNEP- The United Nations Environment Programme

VFA- Volatile Fatty Acid

VS- Volatile Solid

WWTP- Wastewater Treatment Plant

1. Introduction

Plastic pollution has turned out to be one of the most prominent natural issues, as rapidly increasing production of disposable plastic products overpowers the world's ability to manage them. Plastic contamination is most noticeable in Asian and African countries, where garbage collection systems are often inefficient or nonexistent. The vast majority of the plastic rubbish in the seas streams from the land. Trash is also carried to sea by major rivers, which act as conveyor belts, getting more plastic waste as they move downstream. Once at sea, much of the plastic trash remains in coastal waters. But once caught up in ocean currents, it can be transported around the world. The ocean is getting polluted by plastic waste every day as around 73,000 tons of plastic waste ends up in the ocean through Padma, Meghna and Jamuna rivers per day. Environmental experts of the country, however, predicted that "If plastic pollution continues at the current pace, Bangladeshis will be living on islands of plastic, not chars. Though the major portion of these wastes is from India, Nepal, and China floating down the Ganges, Yamuna and Brahmaputra, Bangladesh is also producing 3000 tons of plastic waste every day that accumulates in the sea. The United Nations Environment Programme (UNEP), on the occasion of World Environment Day, has published this in their report regarding the production and usage of plastic worldwide. According to UNEP, the world is currently producing 300 million tons of plastic waste each year, of which a significant portion roughly 8 million tons- fall into the seas via 10 river basins. Eight of the 10 rivers originate in China. Among them, Bangladesh is directly affected by Brahmaputra pollution.

The Bay of Bengal is rich in marine resources and produces 6 million tons of fish that correspond to nearly 4 percent of the total global catch. It is an important source of animal protein for nearly 400 million people in this region. But the Bay is heavily littered with plastics and huge amounts of plastic waste are found on the shorelines, on the seabed, and suspended in the water column. The corals of St. Martin Island are almost dead, littered with marine debris, plastic packages, and food wrap discarded by hundreds of tourists daily. The Bay of Bengal and the South China Sea are the new plastic hotspots in Asia. Every year about 2 lakh tons of plastics enter the Bay of Bengal from Bangladesh. According to the Earth Day Network of USA (2018) plastics can have a direct impact on animals, both through entanglement and ingestion that cause starvation or suffocation of wildlife. It also affects biota through leakage of harmful chemicals often contained in plastics or attached to them. Perhaps what is most disturbing is the fact that plastic has been found inside fish and large

mammals in different parts of the world. For instance, a dead whale found in Thailand had 80 plastic bags in its stomach. Fishes of the Pacific coast are estimated to consume over 2,200 tons of plastic in a year. The disintegrated plastic debris can potentially be ingested by the humans when they eat seafood, resulting in a number of fatal diseases including cancer. It is of the utmost importance to minimize plastic pollution. Microplastics, which are tiny pieces of plastic or fibers smaller than 5 mm, are even a more dangerous form of marine debris/litter. About 80 percent of marine litter is microplastics. Face wash, toothpaste, scrubs, and anti-aging creams contain thousands of microplastics per product. A single shower can result in 100,000 plastic particles entering the ocean. Microplastics (also known as microbeads) are too small to be retained by the filters used at sewage treatment plants. Fish and shellfish ingest microplastics. Hence the saying, "what goes in the sea goes in you." The problem lies in the way plastics are consumed and the way consumers use plastics. We have a tendency to use plastic materials which are less durable and less valuable. Our single-use or throw-away culture is deteriorating the problem. Lack of coordination among different ministries, divisions, NGOs and the private sector, the absence of a dedicated agency to harness the potential of Blue Economy, and having no system in place to make consumers and industries responsible for the final deposition of plastics, among others, are major barriers to reducing plastics and fisheries waste from the Bay. The topic of excessive plastic use and its dumping into the marine environment has already been on international, regional and national agendas for several years. Prevention of Marine Pollution by Dumping of Wastes, 1972, and Prevention of Pollution from Ships, 1988, are two notable international conventions. At the national level, Marine Pollution Ordinance 1989 and Bangladesh Environment Protection Act 1995 are dealing with marine pollution. Besides, according to SDG 14.1, by 2025, countries have to prevent and significantly reduce marine pollution of all kinds including marine debris or litter. Sadly, in spite of all the agreements and action plans, plastic is still entering the world's oceans in huge amounts every year, both in the form of microplastics and much larger debris.

Plastic pollution is a growing problem in Bangladesh. Our three major cities (i.e. Dhaka, Chittagong, and Sylhet) are, particularly at great risk. In the last few years, production and consumption of diversified plastic products have been extended from households to industrial purposes. That means the range of plastic waste has also increased. Dr. Khondaker Golam Moazzem, additional research director at the Centre for Policy Dialogue (CPD), says, "Bangladesh is perhaps one of the countries with the fastest growth in consumption of plastic

products during the last decade. Between 2005 and 2014, plastic consumption in urban areas of the country has increased significantly (by 169 % although overall national consumption has increased by 16.2 %) which is even higher compared to other countries and regions such as North America (32 %), Asia (80 %), Europe (37 %) and world (25 %). The growth in consumption of plastic products will continue in the coming decade. This possible rise in plastic consumption may lead to huge plastic waste in municipal areas which need to be properly managed." Though Bangladesh was the first country to ban plastic bags, there are no specific laws, rules or guidelines for plastic waste management. Also, proper management and operation of the supply chain of recycling plastic waste are absent. Approximately 50 % of all plastic waste like lightweight, single-use plastic products and packaging materials are not properly deposited for subsequent removal to particular landfills, recycling centers, or burners. A pervasive culture for littering continues to add to the problem. And, to make matters worse, water channels such as rivers, are used for dumping industrial and domestic wastes that contain a huge amount of plastic and ultimately end up in the sea. Bangladesh, as a part of the South Asian Seas (SAS) region, currently faces the worst-case scenario of sea pollution due to plastic one of the worst pollutants that are harming the seas and oceans around the world. Marine litter or debris, which include plastic wastes, are the persistent, manufactured, processed solid material found in marine and coastal areas predominantly the result of poor waste management is a fundamental problem due to its harmful effect on the environment, wildlife and human health in the Bay of Bengal, says a country report based on the reviews of scientific and policy documents together with a recent preliminary survey on marine litter along four beaches of Bangladesh. The report, titled "National Status including Database, Proposed Recycling Enterprise, and Interventions on Marine Litter," is the outcome of a South Asian Seas Program (SASP) called the Preparation of Regional Action Plan on Marine Litter in the SAS region. The Department of Environment under the Ministry of Environment, Forest and Climate Change prepared the report, is the first of its kind in Bangladesh, with technical support from the South Asia Cooperative Environment Programme (SACEP) and funding from the United Nations Environment Programme (UNEP). According to the report, marine litter reaches the ocean from the land through river runoff, drainage system, wind action and intentional or unintentional discharge of materials in the sea due to human activities. A total of 6,705 pieces of waste products were found on an 18.5km stretch of the four sea beaches Laboni and Inani in Cox's Bazar, and Ananda Bazar and Patenga in Chittagong in Bangladesh during the survey. Among the litter, 63% were found to be plastic, 13% foamed plastic, 2% cloth, 1% glass and ceramic, 1% metal, 9%

paper and cardboard, 3% rubber, 1% wood, and 7% other materials. Plastic bags were found to be the most common type of litter: at least 2,182 pieces of plastic bags were found on the beaches. The survey also found 589 pieces of insulation and packaging foam, 470 pieces of cigarette butts and filters, and 300 bottles. The survey also found bottle caps and lids, drums, jerry cans, buckets, disposable utensils, straws, stirrers, drink packaging, food containers, bags, gloves, cigarette lighters, syringes, baskets, crates and trays, mesh bags, fishing gear, and many other kinds of plastic waste on the four beaches. The report made a number of recommendations to bring down and control the reckless dumping of plastic waste on the beaches.

Table 1. Plastic found at four beaches of Cox's bazar and Chittagong (Amin, 2018)

PLASTIC FOUND AT FOUR BEACHES OF COX'S BAZAR AND CHITTAGONG				
	Length of beach in sample (km)	Total waste found (pieces)	Plastic found (pieces)	Proportion of waste that is plastic (%)
Inani Beach, Cox's Bazar	5.5	3742	2619	70
Laboni Beach, Cox's Bazar	5.5	831	432	52
Ananda Bazar Beach, Chit-tagong	6	1918	997	52
Patenga Beach, Chittagong	1.5	214	126	59

Table 2. Waste collected from four beaches of Cox's bazar and Chittagong (Amin, 2018)

WASTE COLLECTED FROM FOUR SEA BEACHES OF COX'S BAZAR AND CHITTAGONG		
	NUMBER OF PIECES	PROPORTION OF TOTAL (APPROX %)
Plastic	4193	63
Foamed Plastic	860	13
Paper and Card-board	610	9
Rubber	237	3
Cloth	146	2
Glass and Ceramic	90	1
Metal	36	1
Wood	90	1
Others	443	7
Total	6705	100

There is no process of collecting or recycling this. This plastic waste produces harmful dioxin and hydrogen cyanide, deadly for the human body. The harmful chemicals enter plants and fish and then the human body through the food cycle. According to ESDO research, plastic waste causes asthma, lung cancer, stomach problems, and various skin diseases. The research team has found plastic traces in the stomachs of fish and livestock. Shahriar Hossain, the secretary-general of ESDO said the use of environment-friendly products is increasing and the use of plastic and polythene is being reduced worldwide. “Polythene bags in our country were banned in 2002. In 2010 a law on using jute bags was passed. In recent years the use of polythene and plastic bags has increased again and it is affecting the environment as well as clogging the drainage system,” he said. There are more than 100 factories in different areas of Lalbagh, Hazaribagh, Sadarghat, Gazipur in Dhaka and in Chattogram that manufacture polythene bags.

Figure 1. Girani Khal Manda in Dhaka (Molla, 2019)

2. The history of Plastic:

The first synthetic polymer was invented in 1869 by John Wesley Hyatt, who was inspired by a New York firm's offer of $10,000 for anyone who could provide a substitute for ivory. The growing popularity of billiards had put a strain on the supply of natural ivory, obtained through the slaughter of wild elephants. By treating cellulose, derived from cotton fiber, with camphor, Hyatt discovered a plastic that could be crafted into a variety of shapes and made to imitate natural substances like tortoiseshell, horn, linen, and ivory. This discovery was revolutionary. For the first time, human manufacturing was not constrained by the limits of nature. Nature only supplied so much wood, metal, stone, bone, tusk, and horn. But now humans could create new materials. This development helped not only people but also the environment. Advertisements praised celluloid as the savior of the elephant and the tortoise. Plastics could protect the natural world from the destructive forces of human need.

In 1907 Leo Baekeland invented Bakelite, the first fully synthetic plastic, meaning it contained no molecules found in nature. Baekeland had been searching for a synthetic substitute for shellac, a natural electrical insulator, to meet the needs of the rapidly electrifying United States. Bakelite was not only a good insulator; it was also durable, heat resistant, and, unlike celluloid, ideally suited for mechanical mass production. Marketed as "the material of a thousand uses," Bakelite could be shaped or molded into almost anything, providing endless possibilities.

World War II necessitated a great expansion of the plastics industry in the United States, as industrial might prove as important to victory as military success. The need to preserve scarce natural resources made the production of synthetic alternatives a priority. Plastics provided those substitutes. Nylon, invented by Wallace Carothers in 1935 as synthetic silk, was used during the war for parachutes, ropes, body armor, helmet liners, and more. Plexiglas provided an alternative to glass for aircraft windows. A Time magazine article noted that because of the war, "plastics have been turned to new uses and the adaptability of plastics demonstrated all over again." (Nicholso and Leighton, 1942, p.306) During World War II plastic production in the United States increased by 300%.

The surge in plastic production continued after the war ended. After experiencing the Great Depression and then World War II, Americans were ready to spend again, and much of what they bought was made of plastic. According to author Freinkel (2011, p.4), "In product after product, market after market, plastics challenged traditional materials and won, taking the

place of steel in cars, paper, and glass in packaging, and wood in furniture." The possibilities of plastics gave some observers an almost utopian vision of a future with abundant material wealth thanks to an inexpensive, safe, sanitary substance that could be shaped by humans to their every whim.

3. Current state of Plastic:

Plastic's reputation fell further in the 1970s and 1980s as anxiety about waste increased. Plastic became a special target because, while so many plastic products are disposable, plastic lasts forever in the environment. It was the plastics industry that offered to recycle as a solution. In the 1980s the plastics industry led an influential drive encouraging municipalities to collect and process recyclable materials as part of their waste-management systems. However, recycling is far from perfect, and most plastics still end up in landfills or in the environment. Grocery-store plastic bags have become a target for activists looking to ban one-use, disposable plastics, and several American cities have already passed bag bans. The ultimate symbol of the problem of plastic waste is the Great Pacific Garbage Patch, which has often been described as a swirl of plastic garbage the size of Texas floating in the Pacific Ocean. The reputation of plastics has suffered further thanks to a growing concern about the potential threat they pose to human health. These concerns focus on the additives (such as the much-discussed bisphenol A (BPA) and a class of chemicals called phthalates) that go into plastics during the manufacturing process, making them more flexible, durable, and transparent. Some scientists and members of the public are concerned about evidence that these chemicals leach out of plastics and into our food, water, and bodies. In very high doses these chemicals can disrupt the endocrine (or hormonal) system. Researchers worry particularly about the effects of these chemicals on children and what continued accumulation means for future generations.

Despite growing mistrust, plastics are critical to modern life. Plastics made possible the development of computers, cell phones, and most of the lifesaving advances of modern medicine. Lightweight and good for insulation, plastics help save fossil fuels used in heating and in transportation. Perhaps most important, inexpensive plastics raised the standard of living and made material abundance more readily available. Without plastics, many possessions that we take for granted might be out of reach for all but the richest Americans. Replacing natural materials with plastic has made many of our possessions cheaper, lighter, safer, and stronger. Since it's clear that plastics have a valuable place in our lives, some

scientists are attempting to make plastics safer and more sustainable. Some innovators are developing bioplastics, which are made from plant crops instead of fossil fuels, to create substances that are more environmentally friendly than conventional plastics. Others are working to make plastics that are truly biodegradable. Some innovators are searching for ways to make recycling more efficient, and they even hope to perfect a process that converts plastics back into the fossil fuels from which they were derived. All of these innovators recognize that plastics are not perfect but that they are an important and necessary part of our future. So we need an ecofriendly alternative for petrochemical plastic which we can use. PHAs are biodegradable, readily compostable thermoplastics, produced by microbial fermentation of carbon-based feedstock. The properties of PHA polymers are customizable to the application, depending on the specific combinations of different monomers incorporated into the polymer chain.

4. Bioplastic:

Biodegradable polymers represent a growing field (Kaplan *et al*., 1993). A vast number of biodegradable polymers (e.g. cellulose, chitin, starch, polyhydroxyalkanoates, polylactide, polycaprolactone, collagen, and other polypeptides) have been synthesized or are formed in a natural environment during the growth cycles of organisms. Some microorganisms and enzymes capable of degrading such polymers have been identified (Kaplan *et al*., 1993, Chandra and Rustgi, 1998). Here of various biodegradable polymers have been proposed. We propose to classify the biodegradable polymers according to their synthesis process (Fig. 1): (i) polymers from biomass such as agro-polymers from agro-resources (e.g., starch or cellulose), (ii) polymers obtained by microbial production such as the polyhydroxyalkanoates (PHAs), (iii) polymers conventionally and chemically synthesized from monomers obtained from agro-resources, e.g., the polylactic acid (PLA), and (iv) polymers obtained from fossil resources. Only the first three categories (i–iii) are obtained from renewable resources. We can further classify these biodegradable polymers into two main categories: the agro-polymers (category i) and the biodegradable polyesters or biopolyesters (categories ii–iv).

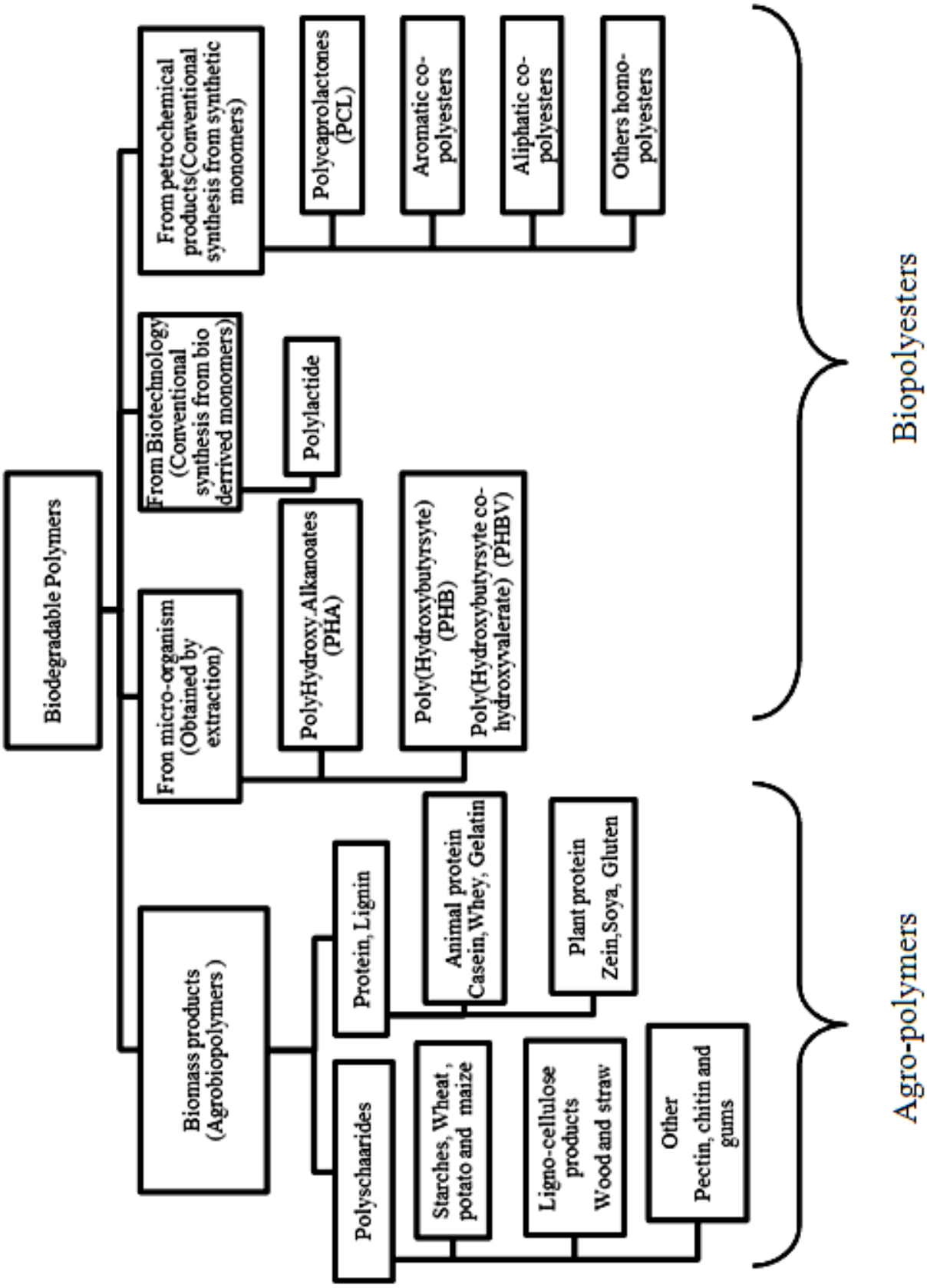


Figure 2. Classification of the main biodegradable polymers.(Avérous and Pollet,2012)

5. Polyhydroxyalkanoates:

Polyhydroxyalkanoates (PHAs) are bio-polyesters, stored within cells as energy storage materials by various microorganisms. Due to their biocompatibility and biodegradability, PHAs have a wide range of applications in various industries such as biomedical sector including tissue engineering, bio-implant patches, drug delivery, surgery, and wound dressing. PHAs are green plastics and they have a positive social and environmental impact when compared with conventional plastics in terms of production and recycling. Moreover, PHAs do not possess acute and chronic health effects when used in vivo. These bioplastics represent a renewable and sustainable resource to reduce landfill requirements without being persistence or causing pollution. A wide range of carbon sources, bacterial strains, fermentation conditions, and recovery methods have been proposed by various researchers for better yield and economical perspectives. Recent advancements in synthetic biology and genetic engineering have led to the production of PHAs from non-PHAs producing strains with no toxins. Progression in recovery techniques has improved the extraction efficacy from biomass with high purity. This review outlines production and characteristics of PHAs, developments in their production, and applications in various industries including nanotechnology.

PHAs are regarded as a renewable resources-based alternative to petrochemical polymers. The advantageous character of PHAs lies in its environmental biodegradability and biocompatibility (Muhammadi *et al*., 2015). PHAs cover a large scale of biological polyesters having properties in the range from thermoplastic to elastomers (Koller, 2018). The first identified and still the most investigated PHA is poly(3-hydroxybutyrate) (PHB). The chemical structure of formed PHA is primarily influenced by the microbial strains, carbon source, and eventually by the addition of precursors (Braunegg *et al*., 2002). A crucial role is played by PHA synthase enzymes (Smolke, 2009). Biochemists, microbiologists, and bioengineers have focused their interest in forced bioproduction of PHAs using different substrates and employing various microorganisms with the aim to produce industrially acceptable concentrations of PHAs in bioreactors and to isolate bio-polyesters and copolyesters with different chemical compositions and physical properties (Koller *et al*., 2017; Kumar and Kim 2018). However, the production costs of PHAs compared to conventional non-biodegradable plastics are still much higher than can compete in most significant industrial sectors (e.g., packaging). Besides, other disadvantages such as limited thermo-mechanical stability, environmental instability, insufficient worldwide production

facilities, and strict requirements for sterile fermentation conditions, hinder the larger industrial application of PHAs (Kovalcik *et al*., 2017). Therefore, intensive research for the production of PHA under non-sterile conditions, for example by using mixed microbial consortia or mutant strains of microorganisms, have been applied and tested as means to simplify operational requirements (Anjum *et al*., 2016; Kourmentza *et al*., 2017).

Taking into account the recalcitrance of conventional plastics in the environment, replacement of synthetic plastics with PHAs would have huge benefits for the society and the environment (Kourmentza, 2017). Wide commercialization and industrialization of PHAs is still struggling due to their high production cost, resulting in higher prices compared to conventional polymers. While the price of polymers such as PP and PE is around US$0.60–0.87/lb, PHA biopolymer cost is estimated to be 3–4 times higher, ranging between US$2.25–2.75/lb (Eno and Hill, 2017). Although several companies have initiated and industrialized the production of PHAs, as presented in Table 3, there are still major issues that need to be addressed in an effort to reduce the overall production cost. The main reasons for their high cost is the high price of high purity substrates, such as glucose, production in discontinuous batch and fed-batch cultivation modes, and a large number of solvents and/or labor regarding their downstream processing. The increasing availability of raw renewable materials and increasing demand and use of biodegradable polymers for bio-medical, packaging, and food applications along with favorable green procurement policies are expected to benefit PHA market growth. According to a recent report, published in 2017, the global PHA market is expected to reach US$93.5 million by 2021, from an estimated US$73.6 million within 2016, characterized by a compound annual growth rate (CAGR) of 4.88%.

Table 3: Pilot and industrial scale PHA manufacturers currently active worldwide.

Name of the Company	Product	Substrate	Biocatalyst	Production Capacity
Biomatera, Canada	PHA resins (Biomatera)	Renewable raw materials	Non-pathogenic bacteria isolated from soil	--
Biomer, Germany	PHB pellets (Biomer®)	Sugar (sucrose)	--	--
Bio-On Srl., Italy	PHB, PHBV spheres(minerv®-PHA)	Sugar beets	*Cupriavidus necator*	10,000 t/a
BluePHA, China	Customized PHBVHHx, PHV, P3HP3HB, P3HP4HB, P3HP, P4HB synthesis	--	Microbial strains via synthetic biology	--
Danimer Scientific, USA	mcl-PHA (Nodax® PHA)	Cold pressed canola oil	--	--
Kaneka Corporation, Japan	PHB-PHHx (AONILEX®)	Plant oils		3500 t/a
Newlight Technologies LLC, USA	PHA resins (AirCarbonTM)	Oxygen and carbon from captured methane emissions	Newlight's 9X Biocatalyst	--
PHB Industrial S.A., Brazil	PHB, PHBV (BIOCYCLE®)	Saccharose	*Alcaligenes sp.*	--
PolyFerm, Canada	mcl-PHA (VersaMerTM PHA)	Sugars, vegetable oils	Naturally selected Microorganisms	3000 t/a
Shenzhen Ecomann Biotechnology Co. Ltd., China	PHA pellets, resins, microbeads (AmBio®)	Sugar or glucose	--	5000 t/a
SIRIM Bioplastics Pilot Plant, Malaysia	Various types of PHA	Palm oil mill effluent, crude Palm Kernel oil	--	2000 t/a
TianAn Biologic Materials Co. Ltd., China	PHB, PHBV(ENMATTM)	Dextrose deriving from corn of cassava grown in China	*Ralstonia eutropha*	10,000 t/a, 50,000 t/a by 2020
Tianjin GreenBio Material Co., China	P (3, 4HB) films, pellets/foam pellets (Sogreen®)	Sugar	--	10,000 t/a

Despite having comparable characteristics to common plastics, extensive PHA use is still hampered by its high production cost. PHAs are bacterial produced, and one of the major costs associated with their production derives from the carbon source used for bacterial fermentation. So this study is for reviewing the cost-effective processes to produce PHAs using Municipal wastewater, where we will go to focus on following topics.

a. Studying most common Bacteria for the Production of polyhydroxyalkanoates
b. Focusing on using Wastewater as a carbon source.
c. Studying the advance processes for Production of polyhydroxyalkanoates
d. Production of polyhydroxyalkanoates on the perspective of Bangladesh

6. Chemical Structure:

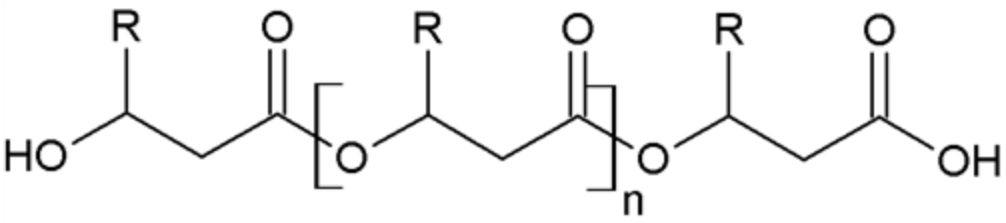
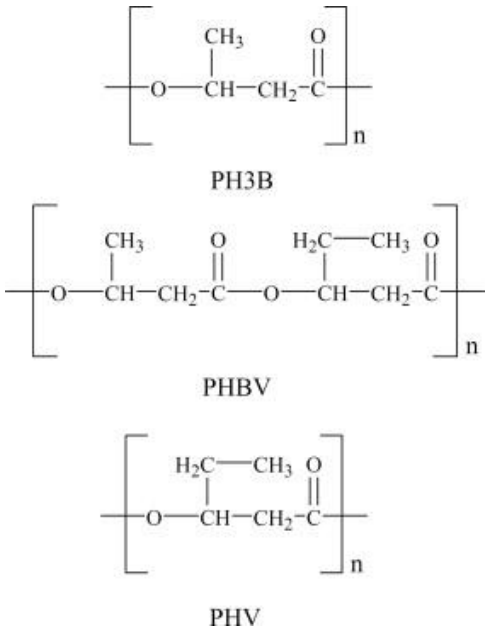


Figure 3. Chemical structure of PHAs

Polyhydroxyalkanoates(PHAs) are naturally produced. Between brackets, one monomer (hydroxyalkanoate) is shown, 'R' denotes residual group. The residual group can consist of different carbon chain lengths. The type of PHA depends on both the structure and length of the main chain, but also on the side chains (Figure 4). Examples of different monomers with different residual chain lengths can be found include poly-3-hydroxybutyrate (PHB or PH3B), PHV, and PHH. A PHA copolymer called poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) is less stiff and tougher, and it may be used as packaging material.

Chemical structures of some of these polymers are shown:



PH3B: Polyhydroxybutyrate

PHV: Polyhydroxyvalerate

PHBV: Copolymer of the above

Figure 4. Chemical structure of PH3B, PHBV, PHV

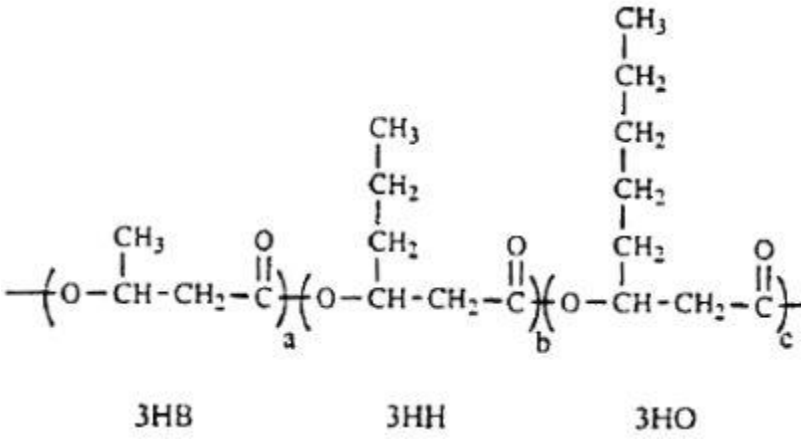


Figure 5: Examples of different monomers with different residual chain lengths (Kato et al., 1996; via Cyberlipid, 2016)

The chain-length of the VFA is of great influence on the composition and properties of PHA (Lee *et al*, 2014). In mixed culture PHA production, the presence of acetic and butyric acids results in the production of 3-hydroxybutyrate (3HB) while propionic and valeric acids lead to the production of 3- hydroxyvalerate (3HV). Poly(3-hydroxybutyrate) (P(3HB)) is brittle and stiff and has limited applications. The incorporation of 3HV into P(3HB) results in P(3HB-co-3HV) which is more flexible and tougher. It is less permeable to oxygen compared to PE and PP, making it suitable for food packaging. Singh and Mallick (2009) have proposed other categories in spite of this, for *Pseudomonas aeruginosa* cultures: Long-Chain-Length (LCL)-PHAs, SCL-MCL-PHA co-polymer, and SCL-LCL-PHA.

7. PHA accumulation in mixed and monocultures

A limited number of pathways for PHA accumulation exist, assuming that other growth parameters are constant: Mixed culture is the culture of many different PHA accumulating microorganisms in the same reactor. Serafim *et al*. (2008) give an overview of the mixed culture processes used for PHA production by reporting the important achievements regarding the use of complex substrates and providing future prospective of this technology. These authors describe a three-step PHA production process consisting of:

1) Feedstock production, where waste-based substrates are converted through anaerobic digestion to substrates for PHA production: organic and fatty acids.

2) Selection of PHA producing microbial cultures under changing feeding conditions.

3) PHA production.

Monoculture is the culture of a single PHA accumulating microorganism. Tamis (2015) notes that the treatment of waste streams with pure cultures is often not economic due to a.o. the requirement for sterile conditions. This is hard to combine with the open (nonsterile) bioconversion processes that are required for the efficient valorization of waste streams. Furthermore, several modes of feeding can be applied, such as:

7.1. Mixed culture with a constant feed

Part of a stock culture is used to produce PHAs in batch, which results in a constant mixture of PHAs (even though some variability cannot be prevented). When applying continuous fermentation, a mixed culture will contain a limited amount of microorganisms due to natural selection. This will result in changes in the mixture of accumulated PHAs until steady-state conditions are reached. After reaching steady-state the composition of the PHA mixture will remain relatively constant.

7.2. Mixed culture with variable feed

When feeding a mixed culture with variable feed, the mixture of accumulated PHAs will vary accordingly. The extent to which this occurs depends on the composition of the mixed culture and the feed.

7.3. Monoculture with a constant feed

A specific microorganism will accumulate a specific PHA or PHA mixture when feed is constant, assuming other growth conditions remain constant as well. The type of PHA or composition of the PHA mixture will depend on feed and microorganism.

7.4. Monoculture with variable feed

When feed is variable, the accumulated type of PHA or composition of the PHA mixture will vary. Generally speaking, using monocultures to produce PHA will result in higher PHA concentrations but fermentation costs are also higher. When mixed cultures are fed with suitable VFA types or when process conditions are optimized, higher PHA content (40-77 %) can be achieved from fermented food waste, waste activated sludge, sugar cane molasses, and paper mill effluent (Lee *et al*., 2014).

8. PHA Production by Pure Bacterial Cultures:

Reddy *et al*. (2003) and Tan *et al*. (2014) stated that PHA are biodegradable polymers obtained from a wide range of gram-negative and gram-positive bacteria that accumulate them in the cytoplasm, to act as storage materials such as carbon, energy, and reducing power, that is manufactured by these organisms under unbalanced culture conditions. Pure culture biotechnology is implemented on an industrial scale since a wide variety of food, pharmaceutical, and cosmetic agents derive as metabolic compounds from certain bacterial strains. Within the last few decades, research has been focused on finding ways to decrease the high production cost of PHAs.

One of the main contributors to their high cost is the use of high purity substrates, which can account for 45% of the total production cost (Kourmentza *et al*., 2015). Instead, renewable feedstocks are being explored and researchers have been developing bioprocesses for the valorization of waste streams and by-products. In addition, current legislation and policies promote biodegradable waste management solutions other than disposal in landfills. Since every type of waste stream or by-product has a different composition, selecting the appropriate biocatalyst is of great importance. In cases where the raw material is rich in carbon and nutrients, a growth-associated PHA producer would be selected, such as *Alcaligenes latus* or *Paracoccus denitrificans*. Conversely, in cases where the feedstock lacks an essential nutrient for growth such as nitrogen, phosphorous, etc., PHA accumulation using non-growth-associated bacteria would be ideal, i.e., *Cupriavidus necator*. Apart from well-known species involved in industrial PHA production such as *Alcaligenes latus*, *Cupriavidus necator*, and *Pseudomonas putida*, bacteria need to combine several features in order to be selected and regarded as promising PHA producers. Such features include their performance utilizing renewable feedstocks and/or environmental pollutants, seawater instead of freshwater, the possibility of PHA production under open, non-sterile conditions, and their potential to develop contamination-free continuous bioprocesses. The use of agricultural byproducts and forest residues as an abundant and renewable source of lignocellulosic material for PHA production is mainly considered after its physicochemical or biological hydrolysis. However, a few microorganisms possess the ability to saccharify cellulose and simultaneously produce PHAs. Moreover, PHA producers isolated from contaminated sites may be regarded for combined PHA production and bioremediation of toxic pollutants and post-consumer plastics. In addition, microorganisms isolated from hypersaline environments are considered the most promising ones, since they combine several benefits with a huge

potential for reducing PHA production cost, namely in the downstream step. Last but not least, within recent years synthetic biology tools are continuously being developed in order to provide solutions to industrial challenges such as maximizing cellular capacity to 'make more space' for PHA accumulation, manipulating PHA composition to design polymers for high value-added applications and enhancing PHA efficiency.

Singh, Kumari, Mittal, Yadav, & Aggarwal in 2013 have described the most convenient and cost-effective way to isolate the PHA producing bacteria from contaminated soil. Following their instruction Aljuraifani, Berekaa and Ghazwani1 also did successful isolation in 2018. They have collected ten grams of the contaminated soil sample in a sterile poly bag, sealed, and transferred to the laboratory or stored at 4°C for later analysis. Approximately 1 g of each sample was dispersed in 99 ml of sterile distilled water and agitated gently for 2 min. Subsequently, the liquid portion of the soil suspension was serially diluted and spread on nutrient agar medium containing peptone (5 g/L), beef (3 g/L), NaCl (5 g/L), and agar (15 g/L). The inoculated plates were incubated at 28–37°C for 24–48 hr. The isolated bacterial colonies were further subcultured to obtain pure cultures of each of the respective bacterial strains. Subsequently, the pure cultures were maintained on nutrient agar slants at 4°C for use in later analyses (Singh, Kumari, Mittal, Yadav, & Aggarwal, 2013). All bacterial candidates were qualitatively analyzed for PHA accumulation using the viable colony screening technique, which involves the application of Sudan Black B dye. For this purpose, nutrient agar plates containing 1% glucose and an ethanolic solution of 0.3% Sudan Black B dye were inoculated with bacterial isolates and incubated for 24 hr at 37°C. All of the Sudan Black B-positive isolates were subjected to quantification of their PHA production (Aswini, Kavitha, Revathy, & Babujanarthanam, 2014; Sunitha & Ujwala, 2013). The positive isolates were further subjected to Nile staining for confirmation of PHA accumulation. During this process, bacterial smears were stained with Nile Red stain for 20 min, cleaned with sterile water and allowed to dry, and then viewed with a fluorescence microscope at a wavelength of 490 nm. PHA-accumulating bacteria exhibited a bright yellowish-orange color (Bhuwal, Singh, Aggarwal, Goyal, & Yadav, 2013)

Polyhydroxyalkanoates (PHA), produced by around 300 different bacterial strains (Raj *et al*., 2007). Some of the bacteria responsible for the production of polyhydroxyalkanoates are listed below.

Table 4. Production of polyhydroxyalkanoates in bacteria (Prados and Maicas, 2016)

Bacteria	**PHA Composition**	**Reference**
Bacillus cereus	PHB	Ali & Jamil, 2014
Bacillus megaterium	PHB	Gouda *et al., 2001.*
Bacillus megaterium R11	PHB	Zhang *et al., 2013*
Bacillus megaterium strain JK4h	PHB	Dhangdhariya *et al., 2015*
Bacillus mycoides RLJ B-017	PHB	Borah *et al., 2002*
Comamonas testosteroni	PHB	Thakor *et al., 2003*
Cupriavidus necator H16	PHB	Batcha *et al., 2014*
Cupriavidus necator H16	PHB	Obruca *et al., 2014*
Haloferax mediterranei	PHB	Huang *et al. ,2006*
Ralstonia eutropha H16	PHB	Kahar *et al., 2004*
Recombinant *Escherichia coli*	PHB	Nikel *et al., 2005*
*Recombinant *Aeromonas hydrophila* 4AK4	P(3HB-co-3HHx)	Tian *et al., 2004*
*Recombinant *Ralstonia eutropha* H16 strain	P(3HB-co-3HHx)	Kahar *et al., 2011*
*Recombinant *E. coli* DH5α	P(3HB-co-3HHx-co-3HO-co-3HD)	Li *et al., 2012*
Bacillus sp.	P(3HB-co-3HV)	Shamala *et al., 2012*
Bacillus sp. 256	P(3HB-co-3HV)	Kumar *et al., 2007*
Brevibacillus invocatus MTCC 9039	P(3HB-co-3HV)	Sankhla *et al., 2010*
Cupriavidus necator	P(3HB-co-3HV)	García *et al., 2013*
Halomonas campisalis	P(3HB-co-3HV)	Kulkarni *et al., 2010*
Methylobacterium sp. GW2	P(3HB-co-3HV)	Yezza *et al., 2006*
*Recombinant *E. coli* XL1	P(3HB-co-3HV)	Yang *et al., 2014*
Serratia ureilytic	P(3HB-co-3HV)	Reddy & Mohan, 2015
***Pseudomonas aeruginosa* MTCC 7925	P(3HB-co-3HV-co-3HHDco-3HOD)	Singh & Mallick, 2009

3HB: 3-hydroxybutyrate, 3HV: 3-hydroxyvalerate, 3HHx: 3-hydroxyhexanoate, 3HO: 3-hydroxyoctanoate, 3HD: 3-hydroxydecanoate, 3HHD: 3-hydroxyhexadecanoic acid, 3HOD: 3-hydroxyoctadecanoic acid, *SCL-MCL-PHA co-polymer producers, **SCL-LCL-PHA co-polymer producers. Modified from Kumar *et al.*

9. Metabolism and precursors associated with PHA synthesis:

Polymer synthesis is related to essential metabolic pathways for the cell (Figure 6), because the necessary precursors may be produced in different metabolic pathways as the tricarboxylic acid cycle (TCA). Aldor and Keasling (2003) described that the β-oxidation of fatty acids and the de novo synthesis of fatty acid, involving central metabolites such as acetyl-CoA and cofactors like NADPH. In the genus Pseudomonas, MCL biosynthesis is closely related to the central metabolic pathways for cell precursors that can provide for polymer synthesis, as the de novo synthesis of fatty acids and β-oxidation fatty acids (Eugenio *et al*. 2007). In the bacterial metabolism, fatty acids are degraded by ß-oxidation by 2 carbon loss in the form of acetyl-CoA. In each cycle, the acyl-CoA molecule generated is oxidized to 3-keto-acyl-CoA using (S)-3-hydroxyacyl-CoA as intermediate. The result of this cycle is a fatty acid molecule with n-2 carbon atoms, which can reenter the degradation cycle or be diverted into other pathways (Tsuge, 2002). The intermediate (S)-3-hydroxyacyl-CoA cannot be used directly for the biosynthesis of PHA, and require the action of the enzyme (R)-enoyl-CoA hydratase (PhaJ), which provides (R)-3-hydroxyacyl-CoA thioester which acts as a substrate for the PHA polymerase (Fiedler, 2002).

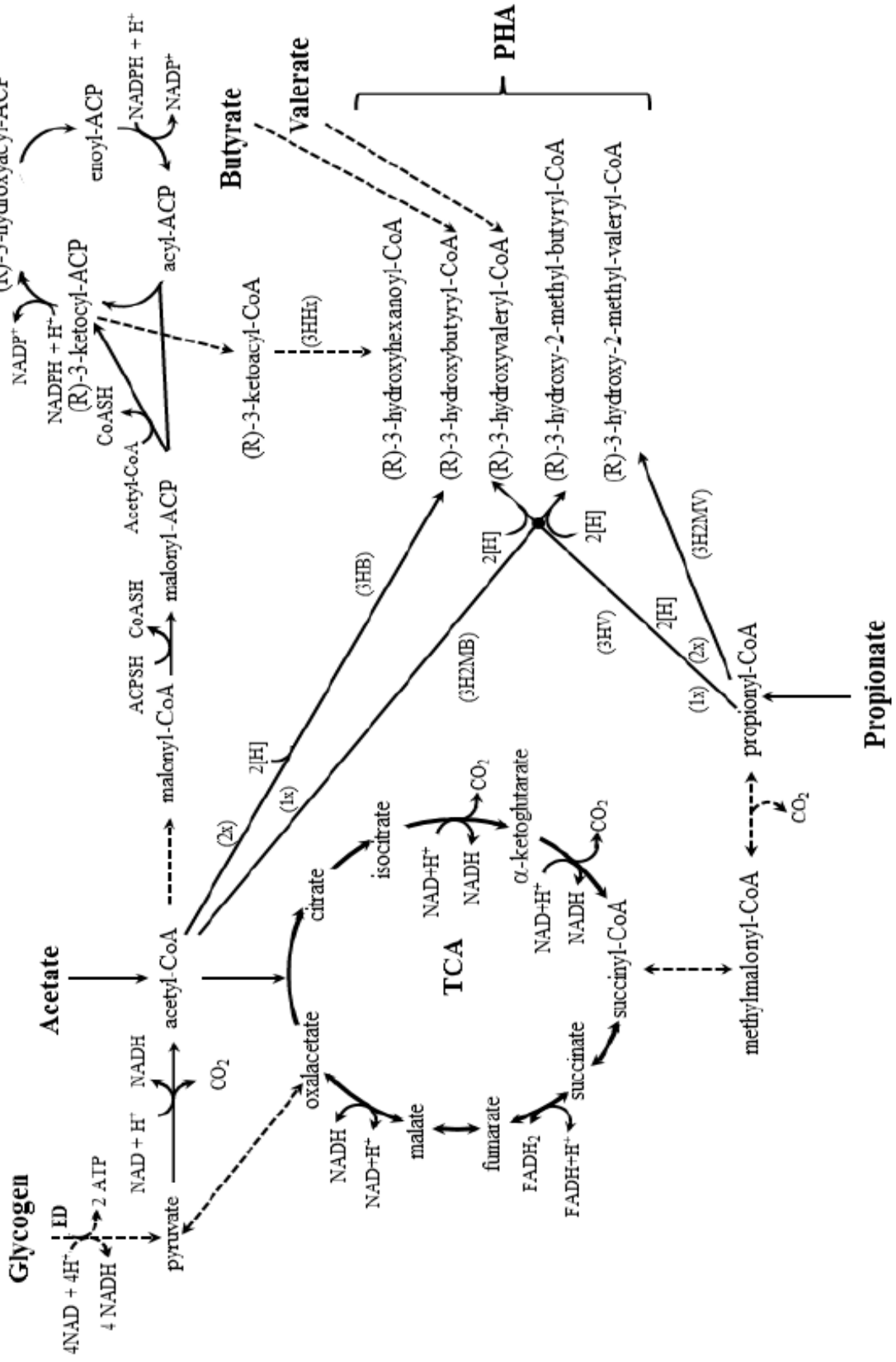


Figure 6: PHA metabolic pathways: ED, Entner-Doudoroff; TCA, tricarboxylic acids.

10. Fatty acids as substrate for PHA accumulating microorganisms

10.1. ß-oxidation of fatty acids

In the bacterial metabolism, fatty acids are degraded by ß-oxidation by 2 carbon loss in the form of acetyl-CoA. In each cycle, the acyl-CoA molecule generated is oxidized to 3-keto-acyl-CoA using (S)-3-hydroxyacyl-CoA as intermediate. The result of this cycle is a fatty acid molecule with n-2 carbon atoms, which can reenter the degradation cycle or be diverted into other pathways (Nomura *et al*., 2004). The intermediate (S)-3-hydroxyacyl-CoA cannot be used directly for the biosynthesis of PHA, and require the action of the enzyme (R)-enoyl-CoA hydratase (PhaJ), which provides (R)-3-hydroxyacyl-CoA thioester which acts as a substrate for the PHA polymerase (Fiedler *et al*., 2002).

10.2. De novo synthesis of fatty acids

Fatty acid synthesis is carried out by a series of enzymatic steps which can involve the activity of enzyme complexes (FASI) or enzymes with a single catalytic domain (FASII). In the first stage, a molecule of acetyl-CoA is carboxylated to form malonyl-CoA, therefore this group is transferred to a small carrier molecule of acyl groups (ACP, acyl carrier protein) to form malonyl-ACP. In a successive process, the required residues are added to rent the final fatty acid (Knoll *et al*., 2009). The enzyme (R)-3-hydroxyacyl-ACP-CoA transferase (PhaG) plays an important role by connecting the de novo synthesis of fatty acid with the MCL synthesis. PhaG catalyzes the conversion of (R)-3-hydroxyacyl-ACP to (R)-3-hydroxyacyl-CoA and contributes to the biosynthesis of MCL from gluconate or other non-carbon sources (Tsuge, 2002 and Fiedler *et al*., 2002)

Generally speaking, waste streams may be acidified to convert persistent (and highly variable) carbon sources into shortchain fatty acids (Table 5), which can then be used by PHA accumulating microorganisms. PHA production takes places by the ‘de novo’ pathway. Formation of PHB and PHV may take place starting from different substrates.

The report “Bioplastic uit slib” (Hart, de *et al*., 2014) describes varying PHA composition during accumulation in mixed cultures (secondary sludge) that is achieved by feeding different shortchain fatty acids. The fatty acids mentioned are mainly acetate and propionate and the PHAs are PHB and PHV (and to some extent PH-2-methyl butyrate and PH-2-methyl valerate).

Table 5: Structure and name of short-chain fatty acids

Carbon	Structure	Name
C1	HCOOH	Formic acid
C2	CH_3-COOH	Acetic acid
C3	CH_3- CH_2-COOH	Propionic acid
C4	$CH_3(CH_2)_2COOH$	Butyric acid
C5	$CH_3(CH_2)_3COOH$	Valeric (pentanoic) acid

However, the mentioned PHA accumulation takes place under aerobic conditions, while the mentioned literature regards anaerobic PHA accumulation (Salehizadeh and van Loosdrecht, 2004). It should be further investigated in the literature to what extent the composition of PHA accumulated under aerobic conditions can be influenced by the short-chain fatty acid composition of the feed. The literature on aerobic PHA accumulation focuses almost entirely on acetate. There is literature on the influence of ethanol, glucose and glutamic acid during aerobic PHA accumulation, but it focuses mainly on removal of these compounds and not on the composition of the produced PHAs (Majone *et al*., 2001; Beccari *et al*., 2002).

Dias *et al*. (2006) explain how PHA can be produced under aerobic conditions from acetate via acetyl- CoA, propionate via propionyl-CoA, butyrate and valerate via hydroxyalkyl-CoA, and longer fatty acids via the β-oxidation route. VFA molecules are assimilated by the cells through active transport (1 mol ATP/mol VFA) following their activation to acyl-CoA molecules. Simpler VFA (acetate and propionate) are activated directly to acetyl- CoA and propionyl-CoA; meanwhile the remaining VFA go through the β-oxidation pathway to be converted to acetyl-CoA and propionyl-CoA by consumption of one more ATP molecules (Pardelha *et al*., 2014). Marang *et al*. (2013) mention that butyrate is an interesting substrate for PHA production. The stoichiometric yield of PHA on butyrate (0.94 mol/mol on a carbon basis) is 40 % higher than the yield on acetate. Also, butyrate is produced in large amounts during acidogenic fermentation of organic waste streams, like fermented sugar cane molasses, olive oil mill effluents, paper mill wastewater, waste activated sludge, or food waste. Their results show that for optimized waste-based PHA production the pre-fermentation process should be directed towards butyrate production. Cerrone *et al*. (2014) describe a two-step biological process for the conversion of ensiled grass biomass to MCL-PHAs through the use of anaerobic and aerobic microbial processes. The main FA produced

was butyric acid and the VFA mixture was concentrated and converted into PHA (mainly 3-hydroxydecanoic acid).

Chakraborty *et al*. (2009) tested the growth of *Ralstonia eutropha* and production of PHAs on acetic, butyric, lactic and propionic acids. They concluded that butyric and propionic acids were the most efficient carbon sources to maximize PHA production when added at a concentration of 5 g/L.

Hassan *et al*. (1996) investigated a two-stage process for the production of PHA from palm oil mill effluent, combining anaerobic treatment to obtain mostly acetic and propionic acid, followed by conversion into PHA by the phototrophic *Rhodobacter sphaeroides*. During the first step formic acid was formed when the pH was maintained below 4. The results clearly indicated that the presence of formic acid substantially decreased PHA yield and content in the cells. Production of PHA by enriched cultures of phosphorus-accumulating (PAO) or glycogen-accumulating organisms (GAO) with acetate or propionate as the sole carbon source has been described often (Jiang and Chen, 2009). To produce PHA with a high 3-hydroxyvalerate (3HV) content they investigated enriched cultures of GAO using different ratios of propionate and acetate (P/A) as carbon sources in this study. With increasing P/A ratio, total PHA decreased and more 3HV was incorporated into PHA. Zhang *et al*. (2008) found that phosphorus removal by PAO increased with P/A ratio. Phosphorus removal also had a linear positive relation with the concentration of (PHV + PH2MV). Jiang *et al*. (2011) modeled SBR and fed-batch experiments with a microbial community dominated by *Plasticicumulans acidivorans* and found that propionate induced mainly PHV production whereas only PHB was produced on acetate. Accumulation experiments with acetate-propionate mixtures yielded PHB/PHV mixtures in ratios directly related to the acetate and propionate uptake rate. The model they developed can be used as a useful tool to predict the PHA composition as a function of the substrate composition for acetate/propionate mixtures. Physical and mechanical properties of PHA can be improved by incorporating 3-hydroxyvalerate (3HV) into the polymer (Jiang and Chen, 2009). Cai *et al*. (2001) found that production of poly (3- hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-co-3HV)) from glucose and propionic (or valeric) acid by *Alcaligenes eutrophus* yielded the same results.

10.3 VFA production

As mentioned earlier volatile fatty acids (VFAs) can be used as a substrate for PHA production by PHA accumulating micro-organisms.

In general, the production of storage compounds with microbial cultures from diluted waste streams improves recovery efficiency since the product is concentrated inside the biomass and can be readily separated from the water using standard sludge settling (or other separation) methods (Tamis, 2015). At present VFAs are mostly produced through chemical routes1, but there is increasing attention to producing them through biological routes (Lee *et al*., 2014). Waste materials are especially interesting for this purpose, such as wastewater sludge, food waste, organic fractions of municipal solid waste, and industrial wastewaters. Koller *et al*. (2009) produced an overview of several organic waste streams of different components that can be converted into PHA through different intermediate compounds and via lactic acid. Medium-chain length (MCL) PHA is mentioned to be formed through fatty acids coming from triglycerides that in turn originate from waste lipids. Lee *et al*. (2014) reviewed various wastes for VFA production, factors influencing VFA production, and various applications of the resulting VFAs. The type of VFA produced can be manipulated by adjusting process parameters. Wastes, in general, should be rich in organic matter with COD concentrations greater than 4 g/L. Also, the ammonium content of the waste should be lower than 5 g/L to prevent inhibition of VFA production. Availability (e.g. due to seasonal harvests) and amounts are important parameters as well.

Before fermented waste that is rich in VFA can be used for PHA production, ammonium and phosphorus concentrations should be low, since these compounds promote microbial growth and decrease the conversion of VFA to PHA. N and P limitations lead to higher PHA contents and yields. They can be removed by struvite precipitation. Sludge particles can lead to failure of PHA production and should, therefore, be filtered out (Lee *et al*., 2014). Waste materials (e.g. primary and secondary sludge from WWTPs, see 4.3) can be used as a source for VFA production under anaerobic fermentative conditions. This can be achieved in reactors with either attached or suspended growth (Lee *et al*., 2014). The VFAs are produced in a two-step process: the first step is the hydrolysis phase and the second step is the acidification phase. During hydrolysis, complex organic matter is converted into soluble amino acids, sugars, and higher (long-chain) fatty acids. In the acidification phase, these components are converted to VFAs and alcohols (acidogenesis or acidogenic/dark fermentation) which are then converted into acetate, hydrogen, and CO_2 (acetogenesis). Both processes involve a complex group of obligate and facultative anaerobic bacteria such as Bacteriocides, Clostridia, Bifidobacteria, Streptococci, and Enterobacteriaceae (Lee *et al*., 2014). Zhang *et al*. (2015) describe several bacterial species that produce different VFAs.

Fusibacter-related organisms, for example, produce acetate and butyrate from carbohydrates, while *Clostridium aminobutyricum* and other *Clostridium*-like organisms use amino acids. *Dethiosulfatibacter aminovorans* produced acetate and propionate from various organic compounds. Zhang *et al*. (2015) suggest investigating, for example by tag-pyrosequencing, types, and functions of microbes which could improve VFA production. When conditions are favorable, methanogenic bacteria will produce methane (Van *et al*., 2011). For PHA accumulation purposes the methane production should, however, be prevented as this means loss of VFAs. Pretreatment of wastes can improve the rate-limiting hydrolysis step (Lee *et al*., 2014), as e.g. cell walls and extracellular polymeric substances (EPS) pose chemical and physical barriers. Also, lignocellulosic material, fats, and proteins lead to lower biodegradation rates. Mixing in other types of waste (e.g. starch-rich industrial wastewater) can enhance VFA production due to better synergistic hydrolysis waste.

Zhang *et al*. (2015) also describe the different pretreatment methods for VFA production (during anaerobic digestion of sludge). Several process parameters are known to influence the concentration, yield and composition of VFAs from waste: pH, T, retention time, organic loading rate, and additives (Lee *et al*., 2014). pH is important for acidogens since these cannot survive at pH values below 3 or above 12. Optimal pH values for VFA production are usually in the range between 5.25 to 11, but this is dependent on the feedstock used. Research suggests that alkaline conditions are beneficial for VFA production from sludge, and neutral and acidic conditions for food waste and wastewater respectively. pH can also influence the type of VFA (particularly acetic, propionic and butyric acid) produced. For example propionic acid formation from dairy wastewater is favored at pH 4-4.5, while acetic and butyric acids are favored at pH 6-6.5. The same was found for gelatine-rich wastewater. However, it was different for whey and other substrates. The optimal pH for the production of a specific VFA is thus dependent on the type of feedstock used (Lee *et al*., 2014). Different temperature ranges have been applied for the production of VFAs: psychrophilic (4-20 °C), mesophilic (20-50 °C), thermophilic (50-60 °C) and extreme/hyper-thermophilic (60-80 °C). The concentration, production rate, and yield of VFAs increased when increasing temperature within the lowest two ranges, due to increased hydrolysis. The effect of still higher temperatures is not clear, as different organisms were probably present in the investigations. Also, higher production temperatures invoke higher costs, which may not be outweighed by the profits of the improved VFA yield. It seems that temperature has a relatively small effect on VFA production (Lee *et al*., 2014). The retention times of the feedstock (hydraulic

retention time = HRT) and the microbial population (solids retention time = SRT) are important process parameters. They are the same during VFA production from sludge since feedstock and microbes are present in the same phase. HRT has an influence on both VFA production and composition, but depends on the feedstock and has an optimum above which VFA production stagnates. A longer HRT also requires a larger reactor volume and as such should be carefully evaluated since this constitutes the main cost factor of the process. In addition, HRT influences the propionic/butyric acid ratio. According to Lee *et al*. (2014) research showed that shorter SRTs lead to higher VFA production since the growth of methanogens is prevented. With primary sludge, it was found that acidogenic conditions prevail at an SRT < 8 days, while methanogenic conditions prevail at an SRT > 10 days. As with other process parameters, the influences of SRT on the VFA composition varies between studies and are often contradictory.

The organic loading rate (OLR) can be expressed as COD, VS, VSS, or DOC fed daily to the reactor. There seems to be a linear increase of VFA concentration with OLR until an optimum is reached. The frequency of feeding also has an influence, with lower frequencies leading to higher VFA concentrations. It seems that higher OLR leads to a decreased proportion of acetic acid in the VFA mixture. However, mainly pH and HRT influence the VFA composition, and these factors should also be taken into account. Additives such as surfactants and enzymes can be used to improve VFA production. They are added during acidogenic fermentation. Surfactants enhance the production of EPS (mainly carbohydrate and protein). Examples are SDS and SDBS. At higher doses, however, the surfactant can be toxic. Combining surfactants with enzymes, e.g. α-amylase and neuter protease, has more effect than the individual substances. Enzymes are costly and recycling them is difficult, therefore immobilization may be a good alternative. A third option is a use of specific (2-bromoethanesulfonate, 2-chloroethanesulfonate, mevastatin, lovastatin) or non-specific (chloroform, fluoroacetate and methyl fluoride) inhibitors of methanogenesis. The drawback of the latter option is their possible toxicity to other bacteria (Lee *et al*., 2014). Zhang *et al*., (2015) describe mixing as a pretreatment method to increase the contact surface area between substrates and microbes. According to the authors, some studies mention agitation speeds ranging from 350-650 rpm. The optimal mixing speed for VFA production from excess sludge is not yet known.

Conversion of proteins, sugars, and fats results in different VFAs during fermentation. Miron *et al*., (2000) investigated the effect of SRT on hydrolysis, acidification, and methanogenesis

of domestic sewage. Carbohydrates, in general, can be easily and rapidly hydrolyzed to simple sugars and subsequently fermented to VFA. Proteins are hydrolyzed to amino acids and degraded to VFA by either anaerobic oxidation linked to hydrogen production, or by fermentation according to the Stickland reaction. The first route depends on the presence of hydrogen scavengers while the second route is independent of the methanogenic activity in the reactor. Triglycerides (lipids) can be hydrolyzed to (medium and long-chain) fatty acids. Accumulation of hydrogen inhibits β-oxidation since it is thermodynamically unfavorable under standard conditions, which means that β-oxidation only occurs in the presence of hydrogen scavengers. It is not clear whether the presence of hydrogen also affects the hydrolysis of lipids, although recent research shows that the presence of methanogenic activity enhances the hydrolysis of lipids. In addition, the lipid concentration influences lipid hydrolysis. LCFA is known to inhibit both their own degradation as well as the methane production from acetate. Miron *et al.*, (2000) found that the hydrolysis of lipids and carbohydrates increased with increasing SRT, whereas protein hydrolysis only occurred under methanogenic conditions. Hydrolysis was found to be the rate-limiting step for the conversion of carbohydrates. Under acidogenic conditions, acidification was the rate-limiting step for conversion of lipids, while both hydrolysis and acidification were limiting for the conversion of proteins. Alibardi and Cossu (2015) evaluated the effects of carbohydrate, protein, and lipid content of organic waste on hydrogen yields, volatile fatty acid production, and the fate of organic carbon from a dark fermentation process. Dark fermentation is the fermentative conversion of organic substrate to hydrogen, with as its main challenge the relatively low energy conversion efficiency from the organic source. Typical H_2 yields range from 1 to 2 mol of H_2 per mol of glucose, which results in 80-90 % of the initial COD remaining in the wastewater in the form of various volatile organic acids (VFAs) and solvents, such as acetic acid, propionic acid, butyric acid, and ethanol. Biogas and hydrogen production were linearly dependent on substrate carbohydrate concentration, but not on protein and lipid concentrations. The end products of dark fermentation were also dependent on the composition of the substrate: the main products were acetic and butyric acid and their ratio were dependent on carbohydrate and protein concentrations.

Overview of methods for improvement of hydrolysis steps in waste streams

Chemical	Acid
	Alkali
	Ozone
	H_2O_2
Biological	Hydrolytic enzyme
	Cellulomonas uda, C. biazotea
	Aspergillus awamori
	Mature compost
	WWTP activated sludge
Physical	Microwave
	Thermal(60-180)
	Ultrasound
Combination	e.g combined alkaline and ultrasonic pretreatment

11. Waste streams to be used for PHA production

Wolf *et al*., (2005) describe four different cheap resources with complex growth and production media for PHA production.

• Carbohydrates: Molasses, starch and whey hydrolysates (maltose), lactose from whey, cellulose hydrolysates (e.g. paper industry waste)

• Alcohols: Wastes from biodiesel production: methanol plus glycerol, methanol

• Fats and oils: lipids from plant and animal wastes

• Organic acids: lactic acid from the dairy industry

Some of these streams can be used directly for PHA formation, while others first need to be converted into fatty acids (Nikodinovic-Runic *et al*., 2013). Khosravi-Darani *et al*. (2013) give an overview of cheap waste streams that were used in the past for PHA production: they include whey, wheat bran and rice bran, corn steep liquor as well as starch, molasses, waste water from olive mills and starch, waste glycerol, waste of vegetable, sweet sorghum, enzyme hydrolysate of potato starch, sesame oil cake, groundnut oil cake, cassava powder, jackfruit seed powder and cornflour, waste of potato starch, canola oil and waste oil.

Nikodinovic-Runic *et al*. (2013) made a comprehensive review of the use of wastes for PHA production.

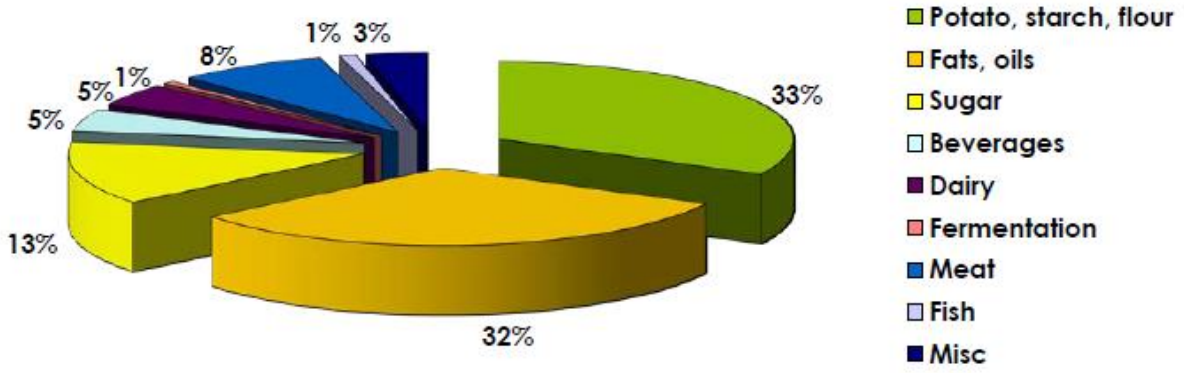


Figure 7 : Overview of waste streams from food and beverage industries in the Netherlands (based on de Boer and van Doorn, 1997; Boersma and Hemmes, 2001; Elbersen et al., 2002; Vis, 2002; Bouwmeester et al., 2006; Bondt and Meeusen, 2008; Boekhoff et al., 2008; OPNV, 2009). Data are indicative only as they are averages of several reports and originate from different years.

12. Activated sludge for PHA production:

The total wastewater treatment capacity in the Netherlands is estimated at 24 million population equivalents (p.e.), with 10 million p.e. represented by wastewater treatment plants (WWTPs) equipped with digesters (Hart *et al*., 2014). One population equivalent is defined as an amount of waste with a Biological Oxygen Demand (BOD) of 54 g O2 per 24 hours. BOD is a unit commonly used in wastewater treatment, as is COD (Chemical Oxygen Demand), mostly for mass balance reasons and process control. (Note: for people not accustomed to BOD and COD: it should be noted that expressing concentrations as BOD and COD does not carry information about the concentration of specific components. As an example, a COD of 60 mg/L means that 1 L contains an amount of material that would demand 60 mg of O_2 to oxidize). Lee *et al*. (2014) mentioned that compared with industrial wastewater, domestic wastewater (influent) has low organic content, with typical COD values in the range of 175–600 mg/L, thus it is not attractive for VFA production. Sludge produced during treatment of this wastewater contains higher COD values. In a case study, de Hart *et al*. (2014) mention a PHA production potential from Dutch wastewater sludge of 2.9 kilo tonne/year using the rich culture route, and 5.2 kilo tonne/year if produced in mixed culture. Their estimates for an indicative cost-effective price (break-even) of PHA produced from wastewater sludge are shown in Table 6.

Table 6: Estimates of indicative cost-effective PHA price (€/kg) for PHA produced from sewage sludge in the Netherlands (adapted from Hart, de et al., 2014).

Factors		Existing sludge facilities	New sludge facilities
Excluding purification	Rich culture	9.1 (4.6-13.7)	6.0 (3.0-9.0)
	Mixed culture	5.3 (2.7-8.0)	2.5 (1.3-3.8)
Including purification	Rich culture	10.9 (5.5-16.4)	7.8 (3.9-11.7)
	Mixed culture	8.5 (4.3-12.8)	5.7 (2.9-8.6)

Van Nieuwenhuijzen *et al*. (2011) reported that primary sludge (PS) is collected during primary settling of suspended solids which originate from municipal wastewater. Secondary sludge, also known as activated sludge (WAS), is produced in the aerobic zone of a municipal WWTP. The bacteria that make up this sludge mainly remove leftover organic residues after the primary settling step. Secondary sludge consists of flocs composed of a diversity of microorganisms and other organic/inorganic matter.

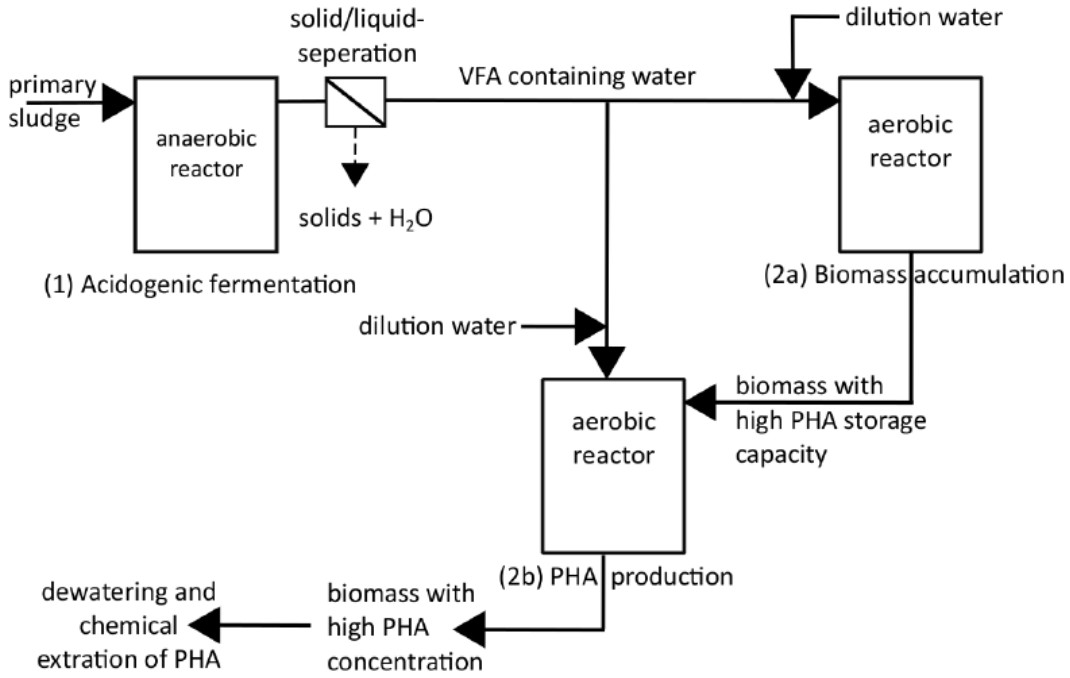


Figure 8: PHAs production scheme

Zhang *et al*. (2015) review methods that improve the production of volatile fatty acids (VFA) from excess sludge during anaerobic digestion. Research by Ucisik and Henze (2008) compared the VFA production of PS and WAS from different WWTPs and a sludge mix

from one WWTP. In semi-continuous reactors, PS showed the greatest VFA potential. They found a soluble COD (SCOD) production of 168-270 mg/g volatile suspended solids (VSS) and VFA yield (VFA/SCOD) that varied from 77 % to 100 %. Fermentation of the sludge mix and WAS showed a SCOD production of 114 and 62 mg/g VSS respectively with a VFA yield of 77 % and 82 % respectively. The solids retention time (SRT) in this research was 5 days in order to prevent methane production. The difference in SCOD production between the different sludge types is explained by the inaccessibility of organic matter in WAS, which mainly consists of living organisms. SCOD in sludge is often low in comparison to total COD, which slows down VFA production since hydrolysis is the rate-limiting step (Lee *et al*., 2014). The composition of the produced mixture of VFAs in semi-continuous reactors from PS showed little variation between the different WWTPs: 25 % to 30 % acetate, 40 % to 50 % propionate, 15 % to 20 % butyrate, and +/- 10 % other. When comparing PS, WAS and mixed sludge from a WWTP it was observed that both WAS and mixed sludge produced a VFA mixture relatively lower in acetate than PS. The percentages propionate and butyrate were mainly higher in the mixed sludge and the percentage of (mainly C5 and C6 VFAs) increased in the fermentation of WAS and mixed sludge compared to PS.

The fermentation of PS at different pH results in different VFA yields. It was concluded that optimal fermentation pH was PS pH found in the collected research material, which ranged from pH 6.72 to pH 6.84. Both higher (7.5) and lower (5.5) controlled pH had a negative effect on the VFA yield. The lowest VFA-COD/SCOD yields of 58 % and 56 % were observed at controlled pH 6 and 7.5 respectively. The highest yield of 90 % VFA-COD/SCOD was observed at a non-controlled initial pH of 6.72 and during fermentation, pH dropped to 5.84. The hydrolysis process seems to be enhanced at higher pH levels. A controlled pH of 7.5 resulted in a 26 % higher SCOD concentration compared to the uncontrolled pH of 6.82 for the same PS composition. VFA composition differed with fermentation pH as the relative concentration of acetate was approximately 37 mass % at pH 6.5, while this was 50 % at pH 7.0, and 70 % at pH 5.5. All results were generated in experiments performed at 20 ^{0}C. Changing fermentation temperature affected VFA production composition. Sludge containing ~15 g/L VSS produced 610 mg/L VFA at 10 ^{0}C while a fermentation temperature of 24 ^{0}C produced 3 g/L VFA in experiments lasting around 120 hours. Relative acetate content dropped from over 80 % at 10 ^{0}C to around 50 % at 20 ^{0}C and practically stabilized between 20 ^{0}C and 24 ^{0}C (Cokgor *et al*., 2008). Khardenavis *et al*. (2005) studied P(3HB) production from sludge by using different carbon and nitrogen

sources. Maximum accumulation of polymer was observed with carbon source acetic acid and diammonium hydrogen phosphate (DAHP) as the nitrogen source. Further studies were carried out to optimize the carbon/nitrogen ratio using acetic acid and DAHP. A maximum of 65.8 % (w/w) P(3HB) production was obtained at a C/N ratio of 50 after 96 hours of incubation. To improve VFA yield from WAS, Chen *et al*. (2007) investigated its hydrolysis and acidification at different pH settings. pH values ranged from 4 to 11 including control in which pH was not adjusted or controlled. The research showed that under alkaline conditions (pH 10 and 11) fermentation of WAS yielded a much higher SCOD/TCOD (total COD) ratio and soluble protein and carbohydrate concentrations compared to the control. At room temperature the extent of sludge hydrolysis was in the following order: alkaline (pH 9-11) >acidic (pH 4-5) >(neutral and blank test). It was found that methane production had its optimal rate at pH 6-7. VFA production was highest at pH 10 and the final concentration of VFA was 2770 mg/L VFA-COD after 12 days of fermentation. High pH values enhance the hydrolysis of WAS and decrease or prevent the methanogenic conversion of VFAs to methane. Acetic, propionic and iso-valeric acid were the three main products.

Khiewwijit *et al*. (2015a, b) investigated a combined process of bio-flocculation (to concentrate sewage organic matter) and anaerobic fermentation (to produce volatile fatty acids (VFA)). The bio-flocculation step was performed in a high-loaded aerobic membrane bioreactor (HL-MBR), after which the concentrate was anaerobically fermented. More than 75 % of sewage COD was diverted to the concentrate, but only 15% sewage COD was recovered as VFA, due to incomplete VSS degradation at the short treatment time applied. The VFA mixture produced consisted of 50 % acetate, 40 % propionate, and 10 % butyrate. In their next paper, they discussed the optimization of VFA production with respect to SRT and alkaline pH (pH 8–10). Application of pH shock to a value of 9 at the start of a sequencing batch cycle, followed by a pH uncontrolled phase for 7 days, gave a 50 % higher yield of 440 mg VFA-COD per g of VSS. The authors explained this by

(1) a reduction of methanogenic activity, or

(2) a high degree of solids degradation or

(3) enhanced protein hydrolysis and fermentation.

They suggest that VFA production can be further optimized by fine-tuning pH level and longer operation, possibly allowing enrichment of alkalophilic and alkali-tolerant fermenting microorganisms. Zoetemeyer *et al*. (1982) studied the anaerobic formation of fatty acids from

glucose in a continuous flow stirred tank reactor (CFSTR) inoculated with activated sludge over the temperature range 20-60 °C. A mesophilic and a thermophilic region could be distinguished, with optimum temperatures of 37 °C (Dmax = 0.51/h) and 52 °C (Dmax = 0.71/h) respectively. Maximum sludge loading rates at these temperatures were 77 and 112 kg COD per kg per day, respectively. In the liquid phase, mainly acetate, propionate, butyrate, lactate, and ethanol were found. They concluded that separate acidification should be designed at a mesophilic temperature below optimal temperature and at high, almost maximal, sludge loading rates. Suboptimal temperatures lead to more stable conditions (broader optima and stable product distribution, e.g. prevention of lactate formation) and require less energy. High sludge loadings lead mainly to the formation of butyrate instead of acetate/propionate.

13. Recent Advancement in PHAs production:

Kourmentza and colleagues provide a comprehensive review on current challenges and opportunities in PHA production in 2017. This article covers all hot spots during the multi-facetted PHA production lines. From the microbiological point of view, the application of both pure (monoseptic) cultures and MMCs are addressed. In the case of MMCs, the coupling of PHA to WWTPs is strongly encouraged by the authors. Special emphasis is also dedicated to raw material selection, process design, and the downstream processing for PHA recovery from microbial biomass. Regarding raw materials, the authors suggest abundant lignocelluloses as the future materials of choice to run a sustainable PHA production facility and discuss recent advances in using toxic substrates like aromatic compounds, which would provide for bioremediation coupled to PHA biosynthesis. Moreover, halophile microbes are presented as stable production strains; their application should contribute to running PHA production processes at reduced sterility requirements. Finally, the outlook of this review refers to synthetic biology as a tool to achieve competitive PHA production by facilitating downstream processing, and to boost PHA productivity.

Takahashi *et al*. in 2017 screened marine bacteria in order to assess their potential to thrive and accumulate PHA on the inexpensive substrate combination crude glycerol from the biodiesel industry and seawater. Out of 150 isolates, the authors report the identification of two auspicious new marine strains with high potential of PHA production on this substrate combination, which, in the future, should be subjected to detailed investigation and optimization in order to assess their applicability for industrial-scale PHA production.

In addition, Bhattacharya and associates used crude glycerol in 2016, in this case stemming from Jatropha curcas-based biodiesel production, as a carbon substrate for PHA production. Here, a Gram-positive production strain of marine origin, *Bacillus licheniformis* PL26, was used. Valappil *et al*. (2007) stated that such Gram-positive strains display the advantage of generating endotoxin-free PHA especially suitable for in vivo application in the biomedical field. In addition to the intracellular storage product PHA, the authors also investigated the excretion of the extracellular product poly(ε-lysine), a material of significance inter alia for food preservation, by this organism. Regarding PHA production, the authors revealed that this organism accumulates the copolyester poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBHV) from waste glycerol as the sole carbon source without the need to add 3-hydroxyvalerate (3HV)-related precursor substrates.

Table 7: Results of the parameters used to analyze the PHA production of the isolates grown in the mineral medium (MM) supplemented with starch, carboxymethylcellulose, glycerol, glucose and Tween 80.

Isolate	Carbon Source	Total Biomass ($g \cdot L^{-1}$)	P(3HB) Concentration ($g \cdot L^{-1}$)	P(3HB) Content in Total Biomass (%)	P(3HB) Productivity ($g \cdot L^{-1} \cdot h^{-1}$)
LAMA 748	Starch	16.80 ± 0.97	0	0	0
LAMA 737	Starch	25.31 ± 1.09	0	0	0
LAMA 748	Carboxymethylcellulose	1.19 ± 0.19	0	0	0
LAMA 674	Carboxymethylcellulose	1.24 ± 0.20	0	0	0
LAMA 677	Glycerol	1.41 ± 0.18	0.4	28.28	0.0058
LAMA 685	Glycerol	2.03 ± 0.27	0.67	32.79	0.0098
LAMA 685	Glucose	1.73 ± 0.22	0.17	9.62	0.0025
LAMA 737	Glucose	0.68 ± 0.10	0.05	7.85	0.0008
LAMA 726	Tween 80	0.91 ± 0.08	0	0	0
M 199 A	Tween 80	0.56 ± 0.05	0	0	0

Table 8: Results of the parameters used to analyze the P(3HB) production of the isolates LAMA 677 and LAMA 685, in seawater and residual biodiesel glycerol, with different formulations.

Isolate	Culture Medium Composition	Total Biomass ($g \cdot L^{-1}$)	P(3HB) Concentration ($g \cdot L^{-1}$)	P(3HB) Content in Total Biomass (%)	P(3HB) Productivity ($g \cdot L^{-1} \cdot h^{-1}$)
LAMA 677	90% mineral medium + 5% glycerol + 5% distilled water	1.11 ± 0.04	0.71	64.28	0.0103
	90% mineral medium + 10% glycerol	0.85 ± 0.05	0.55	64.04	0.0079
	90% seawater + 5% glycerol + 5% distilled water	0.10 ± 0.03	0.03	35.04	0.0005
	90% seawater + 5% residual glycerol + 5% distilled water	0.06 ± 0.01	0.02	31.70	0.0003
	90% seawater + 10% residual glycerol	*	*	*	*
	90% mineral medium + 5% residual glycerol + 5% distilled water	1.39 ± 0.05	0.74	52.94	0.0107
LAMA 685	90% mineral medium + 5% glycerol + 5% distilled water	0.95 ± 0.06	0.42	43.64	0.0060
	90% mineral medium + 10% glycerol	0.36 ± 0.07	0.10	28.08	0.0015
	90% seawater + 5% glycerol + 5% distilled water	0.27 ± 0.03	0.13	48.26	0.0019
	90% seawater + 5% residual glycerol + 5% distilled water	0.32 ± 0.01	0.17	53.60	0.0024
	90% seawater + 10% residual glycerol	0.10 ± 0.02	0.01	10.97	0.0002
	90% mineral medium + 5% residual glycerol + 5% distilled water	2.71 ± 0.96	1.22	44.95	0.0177

* Unobserved growth.

In the experiment used to characterize the growth kinetics, LAMA 677 presented a higher maximum specific growth rate (μmax = 0.087 h−1) than LAMA 685 (μmax = 0.049 h−1). LAMA 677 also reached a D-3-hydroxybutyrate (P(3HB)) content of 78.63% (dry biomass), which was 3.5 times higher than that of LAMA 685. In the assay of the production of P(3HB) from low-cost substrates (seawater and biodiesel waste glycerol), LAMA 677 reached a polymer content of 31.7%, while LAMA 685 reached 53.6%. Therefore, it is possible to conclude that the selected marine strains have the potential to produce PHA, and seawater and waste glycerol may be alternative substrates for the production of this polymer.

Salgaonkar and Bragança in 2017 investigated *Halogeometricum borinquense*, a new haloarchael PHA producer, as a PHA production strain on hydrolyzed sugarcane bagasse. Using this abundant lignocellulose substrate, *Hgm. borinquense* exhibited a superior performance in terms of PHA productivity and intracellular PHA fraction when compared to other scientifically rather undescribed haloarchaea, which were studied in parallel to this work. Furthermore, this strain was shown to produce a PHBHV copolyester from hydrolyzed bagasse without the need for a precursor compound. Generally, this work contributes to the current quest for extremophile PHA producers, which are frequently described as the "rising stars" in the consortium of industrially production strains (Yin *et al*., 2015)

A similar substrate was used to cultivated two Burkholderia strains (*B. cepacia* and *B. sacchari*) on the hydrolysate of spruce sawdust, a lignocellulosic wood waste (Kucera *et al*., 2017). Sawdust hydrolysis was carried out both by applying strong acids to hydrolyze the hemicellulose fraction, and enzymatically to hydrolyze the cellulose fraction. This approach generates considerable amounts of fermentable sugars, which are converted by the two applied organisms towards biomass and PHA. Because this hydrolysate also contains growth-limiting components like polyphenols, furfural, acetic acid, or levulinic acid, the authors present a new, convenient upstream processing strategy to remove growth-inhibiting compounds from the hydrolysate by using inexpensive lignite (brown coal) instead of overlining or the commonly used, more expensive charcoal. As a further benefit, the authors suggest the value-added use of lignite after detoxification as an energy carrier.

Hokamura *et al*., in 2017 used soybean wastewater from a Japanese miso production process for the accumulation of an intracellular PHA blend by a recombinant Pseudomonas sp. 61-3 harboring two different PHA synthase enzymes. For substrate preparation, soybean wastewater was spray-dried and used as a feedstock with and without subsequent hydrolysis. The intracellular blend consisted of poly(3-hydroxybutyrate) (PHB) homo-polyester, and a randomly distributed copolyester consisting of 3-hydroxybutyrate (3HB) and longer 3-hydroxyalkanoates with four to 12 carbon atoms. Using hydrolyzed spray-dried soybean wastewater as the sole carbon source, the highest achieved the concentration of this PHA blend amounted to 1 g/L, which corresponds to a PHA fraction of 35% of the cell dry mass. In this case, the blend contained about 80% 3HB and 20% longer building blocks and displayed a flexible material with considerable toughness comparable to the characteristics displayed by poly(ethylene) (PE).

Pittmann and Steinmetz (2017) studied the production potential for PHA as a by-product of municipal WWTPs. Here, differently composed WWTP sludge lots were investigated as substrates under different operational conditions regarding pH-value, retention time, and withdrawal/refilling for optimized VFA production; short retention time and low withdrawal/refilling rate turned out to be the most beneficial for high VFA formation. In a second stage, generated VFA were used for high and stable production of PHA of constant monomeric composition in a feast/famine regime under fluctuating conditions. The authors suggest that this process when using the entire quantity of sludge for all municipal WWTP in the European Union, could theoretically provide the feedstock for the production of about 20% of the current global PHA production.

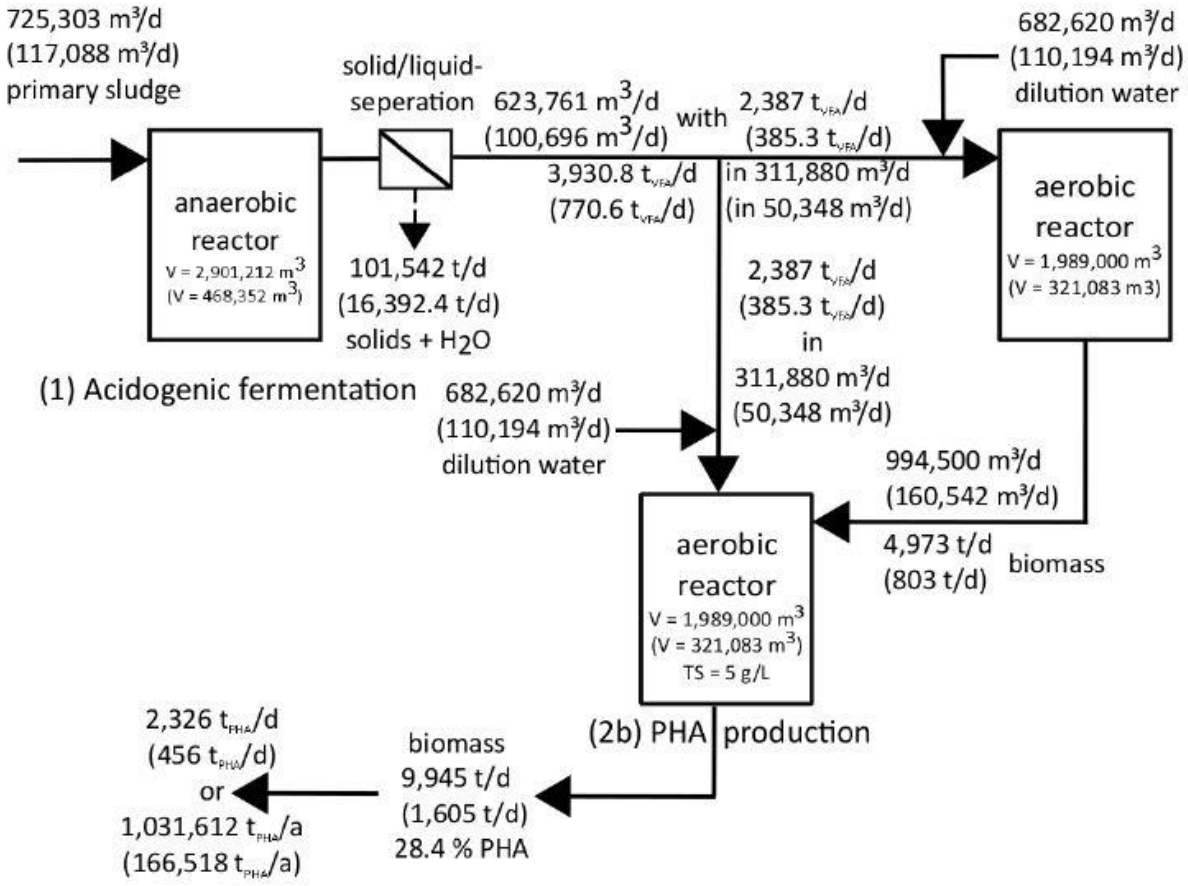


Figure 9: Biopolymer potential scheme for European and German (figures in brackets) WWTPs.

Another concept for a PHA-based biorefinery was presented by Troschl *et al.*, in 2017, who studied the solar-driven autotrophic pilot-scale cultivation of cyanobacteria for PHA production over extended periods in a 200-L horizontal tubular photobioreactor. As a carbon source, CO_2 from the gaseous effluents of an Austrian coal power plant was used. The authors describe the challenges they were confronted with during process development and highlighted several issues considered as especially crucial for developing a stable cyanobacterial PHA production process, namely strain selection, CO_2-availability, and process design and automation. Regarding strain selection, the authors suggest the use of rather small, unicellular cyanobacterial species like *Synechocystis sp.*, which should be more resistant against shear stress when compared to the well-known filamentous cyanobacterial PHA-producer *Arthrospira sp.*

14: Factors of PHAs Production

14.1. Compostability of PHAs

Degradation There are several routes for metabolization of degradable polymers, basically: a) thermal degradation: by the effect of temperature, b) hydrolytic degradation: due to contact with water, c) photodegradation: by sunlight, and d) biodegradation: made by microorganisms. Degradation is faster when low molecular weight polymers are involved. Higher molecular weight polymers require the combination of photosensitive and hydrolyzable functional groups to achieve effective environmental degradation (González, 2013). PHAs are degraded by two major pathways, one intracellular and other extracellular by PHA-hydrolases and PHA-depolymerases (Knoll, 2009 and Voinova, Gladyshev and Volova, 2008). However, the degradation time of a piece of PHA ranges from a few months to years depending on the plastic composition and environmental conditions (Voinova *et al*., 2008).

PHA's are materials manufactured by companies from natural occurring biodegradation of products. PHA's are manufactured by bacteria which can be used for polymers to be molded, extruded into useful products. PHA's are vast and the difference between each strain and polymer of PHA have different abilities in our environment. In some cases, PHA's can be home composted without industrial compost facilities like PLA. PHA material has a barrier to entry due to the costs of the material.

PHA has special properties In Cologne, some companies highlighted the special properties of their PHA. Metabolix specializes in PHA copolymers. Together with PLA, it produces a plastic that can compete with HDPE. And in a mix with biopolymers, PHAs improve biodegradability. Bioplastics, a spin-off of the University of Dublin, produce PHA from plastic waste through bacteria. KANEKA, a major Japanese plastic producer, develops varieties with good heat-resistant properties (e.g. for plastic cups) and varieties that can be used as biodegradable agricultural plastic. Spanish AIMPLAS develops fire-resistant PHA panels for the automotive industry. And levulinic acid improves PHA properties, according to GF Biochemicals, that produces and markets biobased levulinic acid. All said the PHA sector should not leave out of sight the advantages of biodegradability, according to Ravenstijn. The sector should always highlight that plastics have and end-of-life phase. What will happen if the product breaks down, or is discarded because it is no longer useful? The problem of plastic soup in the oceans is staggering. In nature, ordinary plastics break down to little

unnoticeable fragments that can easily enter into the food chain, according to Christian Bonten of the Institut für Kunststofftechnik (IKT). We should not allow them to escape, they should be controlled and recycled. But at present over 85% of all packaging materials are discarded to the environment, which equals 1 to 2 truckloads of plastic each minute (right now; without any subsequent action, this might be 3 to 4 times as much in 2050). Biodegradable plastics like PHA will be at least part of the solution to that problem.

Figure 10: Biodegradable PHA bottles disintegrate in the soil within 2 months (but remain intact as long as they are not discarded) (Hoeven, 2016)

14.2 Cost of production:

To date, the price of PHAs is in general much higher than that of starch polymers and other bio-based polyesters due to high raw material costs, high processing costs (particularly purification of the fermentation broth), and small production volumes (Wolf *et al.*, 2005). The PHA market price in 2014 was €4-5 per kg of biopolymer, for which applications vary from high-value medical products to low-value consumables for agri and horticultural purposes (Hart *et al.*, 2014). Currently, raw material costs account for as much as 40 % to 50 % of the total production costs for PHA. Use of cheap carbon sources, recombinant *E.coli* or genetically engineered plants should lead to cost reduction (Wolf *et al.*, 2005). However, the production of PHA generates a large amount of biomass waste: about 5 kg of raw material is required to obtain 1 kg product. As such, both low conversion rates as well as waste management are issues.

15. PHAs Production with wastewater in Prospect of Bangladesh.

In the last few years, we are getting concern about the water pollution problem in the rivers of Bangladesh. Bangladesh is still lagging behind in the drainage and water treatment system. A World Bank study said four major rivers near Dhaka; the Buriganga, Shitalakhya, Turag, and Balu — receive 1.5 million cubic meters of wastewater every day from 7,000 industrial units in surrounding areas and another 0.5 million cubic meters from other sources. Food processing waste, clothing industry, leather industry, almost every industry is responsible for the pollution of the rivers of Bangladesh. on April 11, 2019, the Daily Star reported that the Khondokia Khal In Chittagong has turned into pitch-black which is stream to the Halda river. Even Prime Minister Sheikh Hasina today urged all, particularly mills and factories; to stop discharging wastes into rivers as water pollution has appeared to be a severe problem for the country. "I would like to request you all to stop discharging wastes into rivers...every industrial unit will have to have waste management system so that it doesn't pollute rivers," she said adding that sea and river pollution has turned to be a global problem. The prime minister made this call while addressing a function organized by Water Resources Ministry at Bangabandhu International Conference Centre in the city, marking the World Water Day 2019.

Figure 11: The water of Khondokia Khal in Chittagong has turned pitch-black due to unabated dumping of waste from nearby industrial units. The canal flows into the Halda river. The photos were taken recently. (The Daily star, 2019)

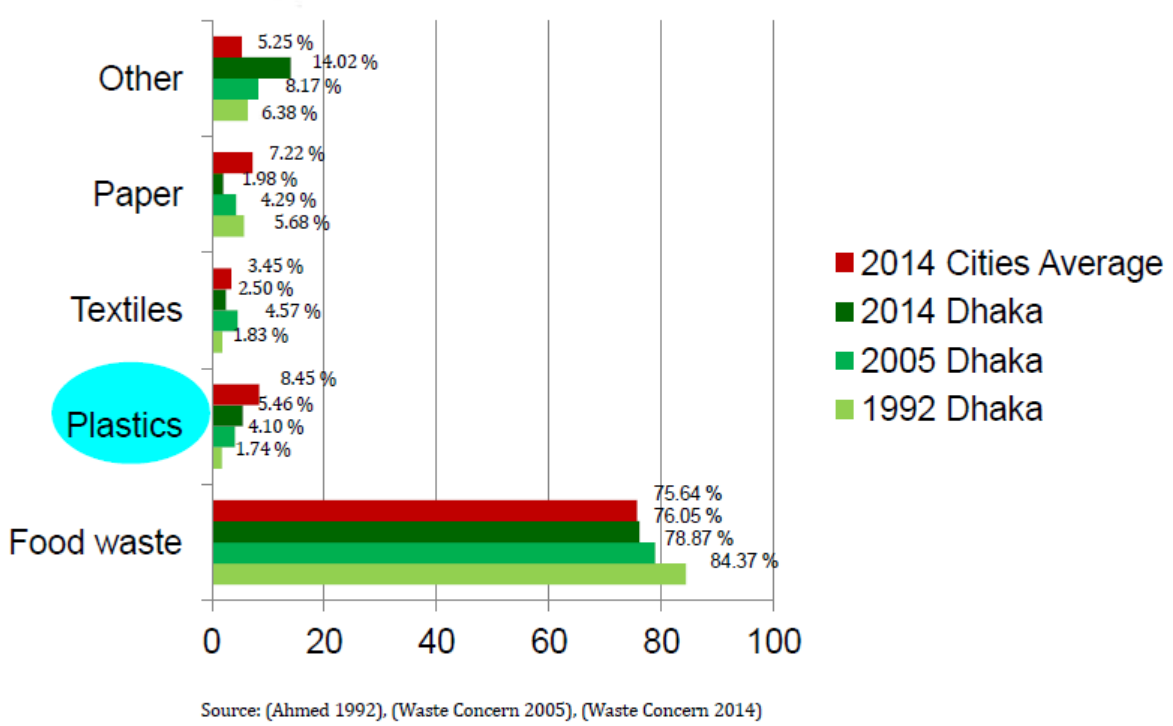


Figure 12: Comparative Analysis of Composition of Waste at Landfill Sites (Huq, 2015)

Buriganga is now one of the most polluted rivers in Bangladesh because of the rampant dumping of industrial and human waste. "Much of the Buriganga is now gone, having fallen to ever insatiable land grabbers and industries dumping untreated effluents into the river," said Ainun Nishat (2009), a leading environmental expert. "The water of the Buriganga is now so polluted that all fish have died, and increasing filth and human waste have turned it like a black gel. Even rowing across the river is now difficult for it smells so badly," he told reporters. Bangladesh has about 230 small and large rivers, and a large chunk of the country's 140 million people depend on them for a living and for transportation. The plight of the Buriganga symbolizes the general state of many rivers in Bangladesh, a large flat land crisscrossed by hundreds of rivers which faces an uphill battle to keep them navigable and their waters safe for human and aquatic lives.

The previous study on the production of PHAs with wastewater can actually help us to get through the pollution of the rivers as well as plastic pollution. Khardenavis *et al*. in 2009 describe the PHA production in wastewater from food industries in India. The process showed an excellent result which can be applicable in our country. Munir and Jamil (2015) have successfully proved that with the industrial wastewater 45% PHA obtained in 48 hours of incubation by *Psudomonus aeruginosa*. Aljuraifani, Berekaa, and Ghazwani in 2018 succeeded in producing PHA with low-cost carbon source in Saudi Arabia.

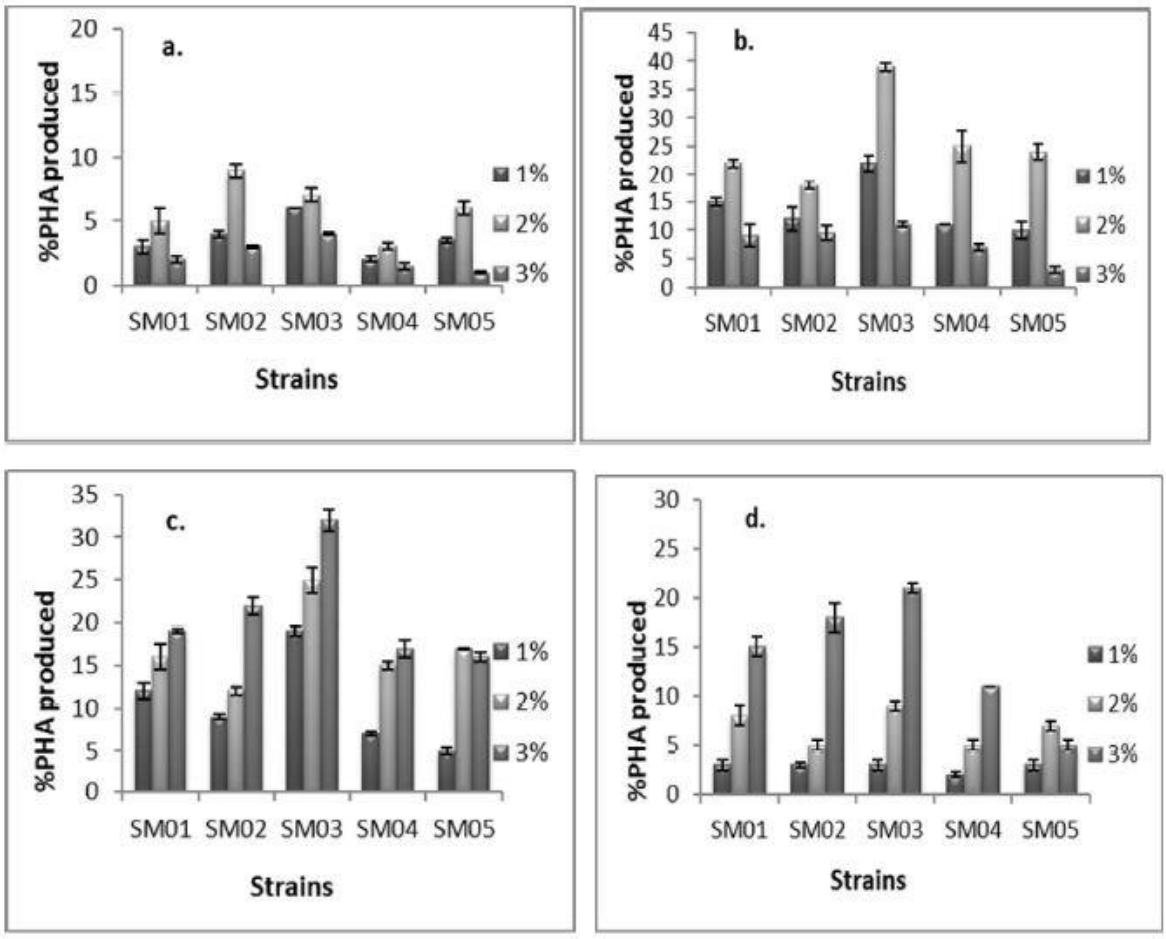


Figure 13: PHA production by bacteria at various concentrations of glucose; (a). after 24 h, (b). after 48 h, (c). after 72 h, and (d) after 96 h (Munir and Jamil, 2015)

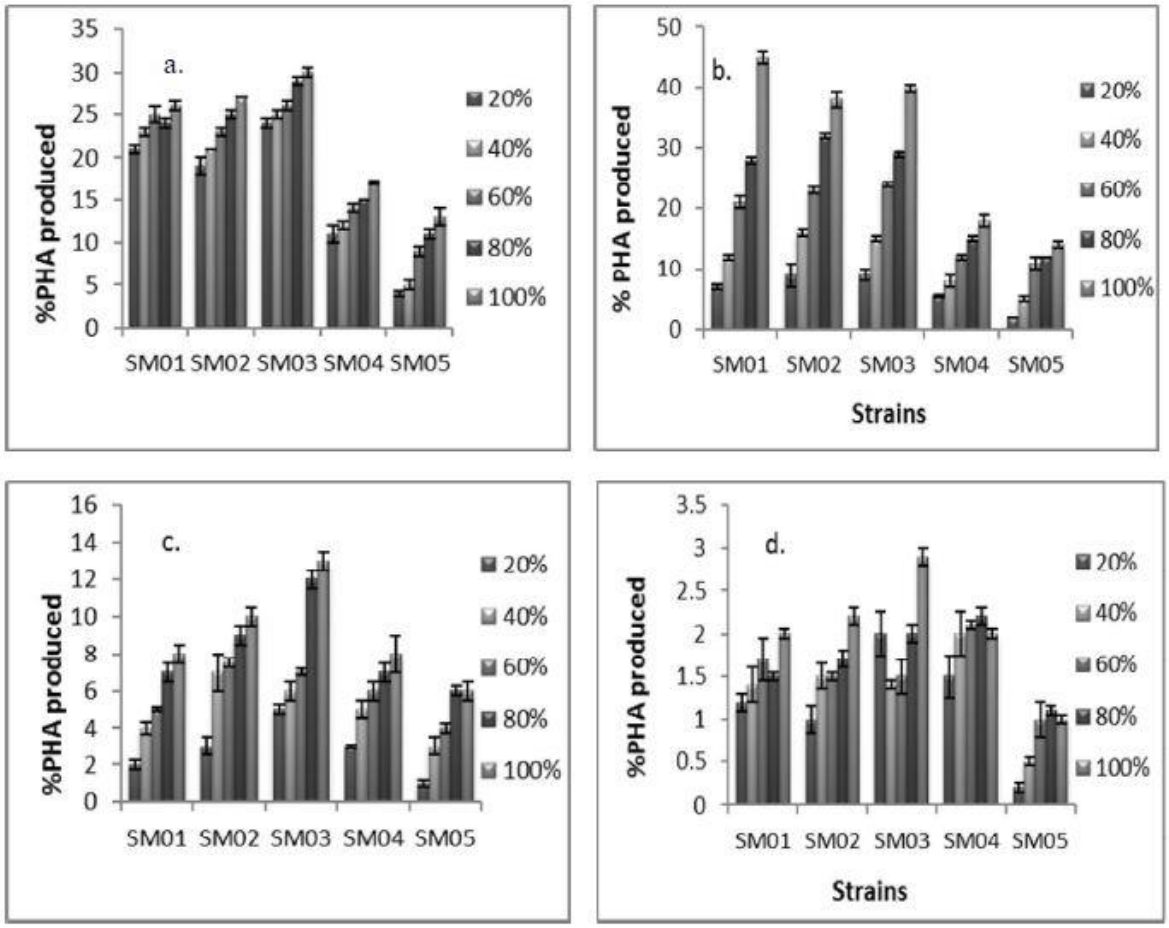


Figure 14: PHA production by bacteria at different concentrations of wastewater; (a). after 24 h, (b). after 48 h, (c). after 72 h, and (d) after 96 h (Munir and Jamil, 2015)

Bangladesh is a densely populated country and in the last ten years, our rivers and water are one of the major concern for being polluted. The PHA production in wastewater can help us get through one of the major problems.

16. Discussion

PHA can create a cleaner and better future for Bangladesh. The current pollution rate will increase in the near future with the growing number of population. If we don't start to make people concern and find alternatives, our normal life will be hampered, our health will face major risk. We need to find a solution that helps is in multiple concern, PHA production with wastewater serves the purpose. Wide production, commercialization, and thus the application of PHAs as a biodegradable alternative to conventional plastics is still limited due to high production cost. Bioprocess technologies are still being developed while bacterial resources are still being explored. Pure cultures are constantly investigated for their potential to valorize

waste byproducts as a low-cost feedstock. The ability of certain bacteria to directly utilize lignocellulosic biomass as the carbon source for PHA production is a huge bioprocess advantage. In this case, the need for chemicals, energy, and labor is minimized. PHA production can be also used as a tool for the bioremediation of oil-contaminated sites, as bacterial strains can degrade environmental pollutants, minimizing their toxicity and environmental impact. Moreover, halophilic PHA producers combine a series of advantages, such as growth on seawater and the possibility of continuous processes under non-sterile conditions. Those features will significantly contribute to the PHA cost reduction and minimize the requirements for freshwater. Synthetic biology tools are expected to aid in the enhancement of PHA production efficiency, simplify the downstream process, and regulate PHA composition providing customized materials for specific applications. However, robust strains are yet to be developed. Efforts have been also made towards PHA production using mixed microbial cultures (MMC). MMC-based processes, apart from offering a reduction in the operational costs and the possibility to adapt to a wider range of waste substrates, they could be integrated in current wastewater treatment plants. Recent developments regarding different enrichment strategies and the PHA production step offer new opportunities to make the PHA production more feasible. Cell densities and derived productivities attained with MMC are the main current bottleneck. The development of economic and simple downstream processes is crucial for the recovery of PHAs. Methods based on the utilization of environmentally friendlier techniques are constantly being investigated, with enzymatic methods advancing the bio-based profile of the process. Australia has a very wide vision of PHA production by 2025. We need a well-managed and proper vision to eradicate the pollution that is harming both human and animal health along with our environment.

Figure 15: Future products made of PHA

17. Conclusion:

Global research efforts are currently devoted to the individual aspects needed to be addressed in order to facilitate the quick success of PHA-based materials on the polymer market. I hope the current issue regarding the plastic pollution at hand will motivate and inspire researchers all over the world (and undergraduates interested in getting their feet on the ground of biopolymers!) to dedicate intensified efforts to further improve PHA production in terms of economics, product quality, and sustainability.

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