Novel bioprocessing strategy for polyhydroxybutyrate production from agro-industrial effluents: A waste biorefinery approach

A Dissertation

Submitted in the partial fulfillment for the degree of

Doctor of Philosophy

Jayakrishnan U

(Roll no. 156152005)

Thesis supervisor: Prof. Gopal Das

Thesis Co-supervisor: Dr. Deepmoni Deka

Centre for the Environment

Indian Institute of Technology Guwahati

Assam, India-781039

Dedicated to my family, peers, and all those scholars trying to make a difference

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

Centre for the Environment

CERTIFICATE

This is to certify that Mr. Jayakrishnan U has been working under our supervision since July 2015 as a regular registered Ph.D. student. His thesis entitled **"Novel bioprocessing strategy for polyhydroxybutyrate production from agro-industrial effluents: A waste biorefinery approach"** is an authentic record of the results obtained from the research work carried out under our supervision in the Centre for the Environment, Indian Institute of Technology Guwahati, Assam, India. We are forwarding his thesis to submit for the award of the degree of Doctor of Philosophy from this institute. We hereby certify that he has fulfilled all the requirements according to the rules of this institute regarding the investigations embodied in his thesis and this work has not been submitted elsewhere for a degree.

Prof. Gopal Das
(Thesis Supervisor)
Professor
Department of Chemistry
IIT Guwahati
Assam - 781039, India

Dr. Deepmoni Deka
(Thesis Co-Supervisor)
Technical Officer Gr-I
Centre for the Environment
IIT Guwahati
Assam - 781039, India

Acknowledgment

My research odyssey would be incomplete without acknowledging the inspiration and support that kept me going even in adverse situations. Foremost, I convey my gratitude to my family for bringing me up to the person I am now, my mom, and the sacrifices she endured helped me reach the position that I am and my better half for backing me during difficult times. I express my heartfelt gratitude to the 'God-The Almighty' for the invisible hand that helped me tide over adverse situations and complete my work.

I would like to express my gratitude to thesis supervisors Prof. Gopal Das and Dr. Deepmoni Deka for their unwavering support, constant guidance, encouragement, knowledgeful insights, and fostering my research interest during all the ups and down research work. Their calm and composed nature while guiding me brought confidence in me to face the challenges of the Ph.D. I am indebted to my supervisors for bringing out the honest researcher in me and equipping me to face the life ahead. I am always overwhelmed by the support, apt suggestion, and expert comments provided by the doctoral committee member comprising of Prof. K. Pakshirajan, Prof. Mihir Purkait, Dr. S. Senthil Kumar and Ph.D viva-voce committee members comprising of Prof. L.M Kundu, Prof. A. Ramesh, Prof. K. Muthukumar (external). I would like to express my sincere gratitude to Prof. Utpal Bora (HOC, Centre for the Environment), Mr. Kaustubh Rakshit, Mr. Rajeev Gogoi, Mr. Mridul Das for their continuous assistance throughout the Ph.D work. I am thankful to the staff, Mr. Partha P. Bakal for his ceaseless, unbiased, and unwavering help and suggestion during the course of the thesis work.

The account of colleagues to thank for their endless and impartial support and motivation is long. Firstly, it would be many of my batch mates, especially Anupama, Himali, Arnab, Tanushree, Ankur, Poulami, and Sayanti. Then, I thank my lab mates Debolina, Biswajith, Asesh, Sagnik, Debojit, Deepa for their cooperation and support. I sincerely thank for the selfless assistance and encouragement imparted by my colleagues Smruti, Narendra, Rajneesh, Arul, Saran, Papu, Anirudha, Rupam, Sounak, Dibyjyoti, Prithwi, Naveen, Deepti, Veluchamy, Divya, Mouna, Srimoyee, Srishti, Rashmi, Shravan, Srineesh, Nayan, Manjay, Rahul, and many other countless peers in IIT Guwahati. I am delighted to express the assistance provided by Dr. S. Venkata Mohan, Omprakash, and Naresh during many hurdles during the thesis work. I take this opportunity to show my appreciation to my friend Shashi for the spark that kept me going in the path of science and research. I am highly indebted to Mrs. Bandana Dutta and Ms. Namrata Rynjah for counseling me through many troublesome moments.

Also, I am pleased to mention the pivotal role of my teachers Mr. Santhosh Kumar, Prof. Pinaki Bhattacharya, Dr. Shaon Ray Chaudhary, and Dr. Shalini Nair, in enlightening the path of science I followed. I thank my peers during graduations and schooling for their companionship that kept me on the clear track. Last but not least, I would like to thank the Centre for the Environment, IIT Guwahati, and the ministry of education, Govt of India, for providing me with the opportunity and fellowship support to continue my Ph.D. work

Jayakrishnan U

The thesis titled “**Novel bioprocessing strategy for polyhydroxybutyrate production from agro-industrial effluents: A waste biorefinery approach**” is divided into six chapters with extensive deliberation of the research work performed and results derived.

Chapter 1: Introduction

Waste management had been a global boiling issue since the 20^{th} century. Presently, waste disposal is unable to match that of waste generation. Hence, the concept of waste biorefinery came up as a feasible solution, adding value to waste disposal and management. Several biological and physical/chemical processes established could reduce or dispose of solid and fluid wastes. Anaerobic digestion of waste under acidogenic conditions is one such process to converts organic contents into H_2, CO_2, volatile fatty acids (VFA) that have high energy and industrial value. Various carbon sources, like industrial effluents and solid wastes like municipal sewage waste, waste sludge, agro residues, etc., have shown VFA generation potential. At the same time, the output of acidogensis gets greatly influenced by the nature of the substrate. Hence, various approaches employed aided in improving the VFA output also depends on the substrate. The pretreatment of seed anaerobic sludge is yet another approach that inactivates the methanogens in the inoculum, thereby increasing the acidogenic activity and VFA accumulation. These manipulations of acidogenic fermentation can render increased VFA production and the desired VFA profile to effect better downstream applications polymer production.

Polyhydroxyalkanoates (PHAs) is another highly investigated waste biorefinery strategy. PHAs are one of the ideal substitutions for conventional plastics like polypropylene. PHAs can reduce the plastic, and microplastic pollution load as they are completely biodegradable Microbial cells accumulate PHA as lipophilic inclusion under appropriate conditions. Activated sludge is the cheapest source of inoculum for microbial production of PHAs. The biological waste treatment in the industrial effluent treatment plant wastes activated sludge as excess sludge. This sludge sequentially enriched with PHA accumulators using aerobic dynamic feeding strategy produces PHA at high density. Modulation of many factors and parameters influences PHA production from activated sludge.

Activated sludge is the ideal inoculum for PHA production from waste feedstocks. Activated sludge is non-axenic and highly stable against environmental perturbations. This makes waste feedstocks an ideal carbon source for polymer production under appropriate conditions. Acidogenic fermentation to improve VFA content increases the efficiency of the bioconversion process further. The VFA profile also plays a vital role in deciding the physiochemical characteristics of the final PHA. This amalgamation of both processes as three-step process converts PHA production as a feasible option for integrating into the existing effluent treatment process, thereby achieving waste treatment or reduction with value generation (Fig 1).

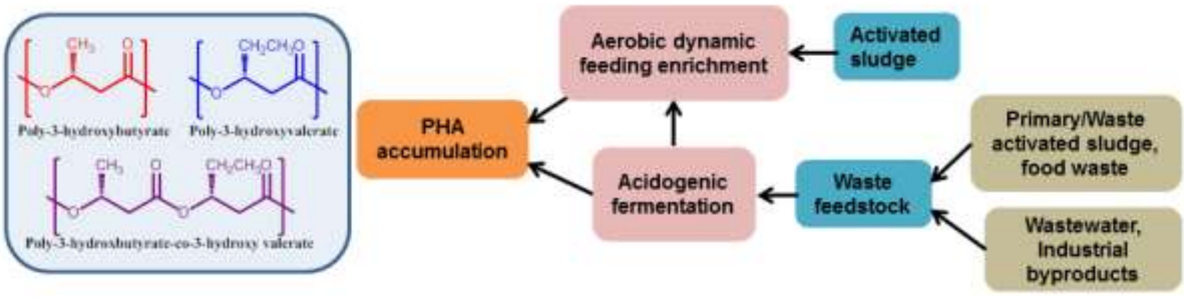


Figure 1: The schematic representation of PHB production from waste feedstocks through acidogenic fermentation -aerobic dynamic feeding strategy (*J.Environ.Chem.Engg. xxx 2021)*

Chapter 2: Experimental methods and materials

This chapter details the materials used for the study like inoculum, effluent, media, reagents etc., the experimental procedures employed for the study, and the protocols, equations, and stoichiometric parameters used for the analysis of experimental outcomes.

Chapter 3: Acidogenic fermentation of agro-industrial effluents to enhance volatile fatty acid production through inoculum pretreatment (*Environ. Sci. Water Res. Technol.* 5, 334–345)

In this chapter, the VFA content of rice mill effluent (RME) and brewery effluent (BE) was influenced by pretreatment of gobar gas plant sludge under cyclic heat and acid shock regime to convert the effluent streams into media suitable for PHA production (Fig. 2). Initial analysis of RME's acidogenic potential was through optimization of the food to microbe ratio (0.5, 1.0, and 2.0). The optimized value was used to study the effect of the pretreated system over the untreated system.

F/M ratio of 1.0 performed better bioconversion resulting in total VFA content of 1897±0.11 mg/L, degree of acidification of 75±0.16 % and VFA profile of HAc:HPr:HBu:HVa::38:31:30.5:0.5. This indicates the capacity of F/M: 1.0 to perform acidification suitable for downstream applications. Further studies used F/M ratio at the optimized value. Pretreatment of the gobar gas plant sludge refined the VFA content of RME with total VFA content (TVFA) and degree of acidification (DA) incrementing from 1897±0.12 to 2437±0.03 mg/L and 75±0.16 to 86±0.13 % at their respective peaks from the untreated system, transforming the profile from 38:31:30.5:0.5 to 58:6.2:29:6.8. Similarly, for BE, the increment was from 1697±0.13 to 2175±0.05 mg/L in TVFA content and 71±0.14 to 85±0.09% in DA. Acetate was in a higher amount among VFAs after the pretreatment for both effluents. Chemical oxygen demand was retained in the pretreated system. pH remained stable in the acidic range for the pretreated system while the pH shifted to an alkaline condition in the untreated system. This projects the possibility of an uncontrolled pH system. Overall, the sludge acidified RME better than BE, probably due to the difference in the effluent matrix. Therefore, pretreatment enhanced the sludge capacity for stable acidification process, transforming the effluents to suit the downstream polymer production.

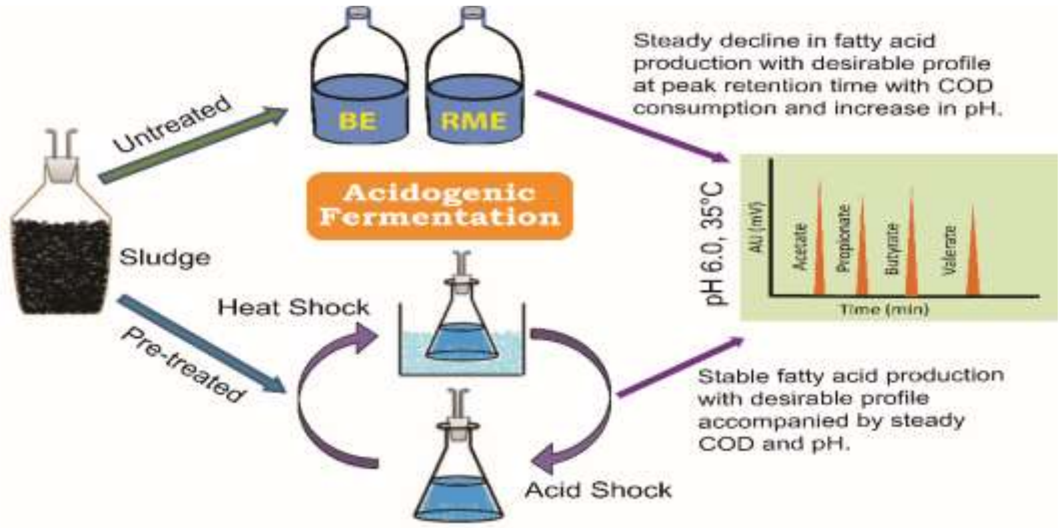


Figure 2: Graphical representation of the pretreated and untreated system influencing gobar gas plant sludge to enhance the VFA content and refine VFA profile of RME and BE

Chapter 4: Regulation of volatile fatty acid accumulation from agro-industrial effluents through heat–acid shock pretreatment of anaerobic inoculum (*Water Environ. Res.*, 00, 1-13)

In contrast to chapter 3, chapter 4 explores the implications of waste feedstock, inoculum origin, and pretreatment on volatile fatty acid accumulation, especially even-numbered fatty acid. The study assesses the effect of cyclic heat and acid shock pretreatment of brewery anaerobic sludge to enhance the even to odd ratio of RME and BE (Fig. 3). The results obtained were encouraging as the pretreatment was successful in refining and stabilizing VFA production from the feedstocks, even though a negative effect on total VFA content was observed.

The fermentation of RME with pretreated sludge had an enhanced acetate yield of 0.37±0.02 mgCOD/mgCOD, even to odd ratio of 20.97±0.08 mg/mg, and the highest butyrate yield of 0.39±0.01 mgCOD/mgCOD compared to untreated system. Meanwhile, the acidogensis of BE remained more or less similar for the pretreated and untreated system, with the former being slower. The pretreated system had stability in COD and pH profile, while VFA content depends on the inoculum's origin. Pretreatment inhibited the carbon sinks and augmented acetate-butyrate type metabolism with stable performance. The fermentation of RME by pretreated sludge produced a higher even-numbered VFAs and enhanced even to odd ratio compared to fermentation of BE. The improvement can positively affect polymer composition and property.

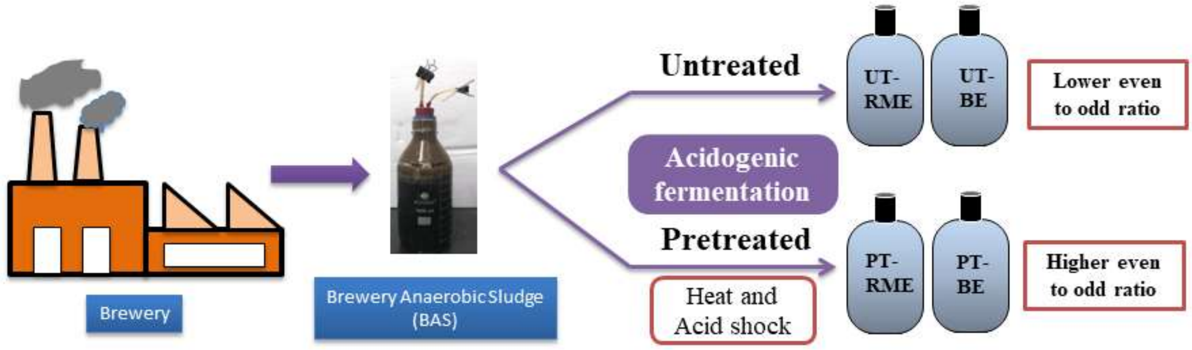


Figure 3: The experimental outlay to influence VFA content through even to odd ratio of RME and BE by brewery anaerobic sludge

Chapter 5: Effect of activated sludge origin and nitrogen availability on the bioconversion of volatile fatty acid into polyhydroxybutyrate by aerobic dynamic feeding strategy (*Int. J. Biol. Macromol.* 163, 2032–2047)

The cumulative effect of the interaction between the origin of the two different activated sludge, petroleum refinery sludge (PRS) and brewery sludge (BS), and nitrogen availability on the polyhydroxybutyrate (PHB) accumulation potential of enrichment culture and the subsequent impact on PHB accumulation and its characteristics are evaluated in the study (Fig 3). Nitrogen excess and limitation enrichment of both sludge produced mix microbial culture with adequate PHB storage of 7.8±0.05%, 14.4±0.04%, 14.4±0.04%, 13.4±0.02%, respectively. The beginning of sludge retention time caused the washout of significant PHB accumulators. The nitrogen limiting culture quickly balanced the loss from the start of proper limitation at C:N:P::100:1:1 due to growth restriction inducing storage. Nitrogen excess condition took a longer time to make up for the lost PHB genotypes due to recurrent feast/famine culture resulting in stronger selective pressure.

In contrast, batch accumulation revealed higher PHB accumulation of 76.1±0.03% and 71.7±0.05% under nitrogen limitation for PRS and BS enriched under nitrogen excess condition compared to any other combination. Long-term exposure to growth limiting conditions might have drained cell machinery resulting in reduced accumulating potential. The PHB extracted had a higher decomposition temperature of 289 ˚C, 293 ˚C and a lower melting point of 168 ˚C, 165 ˚C, but with a higher molecular weight of 4.3×10^5 g/mol and semi-crystalline arrangement for PRS and BS respectively. The enrichment under nitrogen excess is favorable during long term enrichment, while nitrogen limitation is desirable during short term accumulation. The similar physiochemical properties of the PHB extracted reveals its suitability to a broad spectrum of applications. The overlapping microbial community distribution also asserts PRS and BS to have similar PHB production capabilities. Therefore sludge origin does not play any vital role in PHB production under a long term feast-famine enrichment regime.

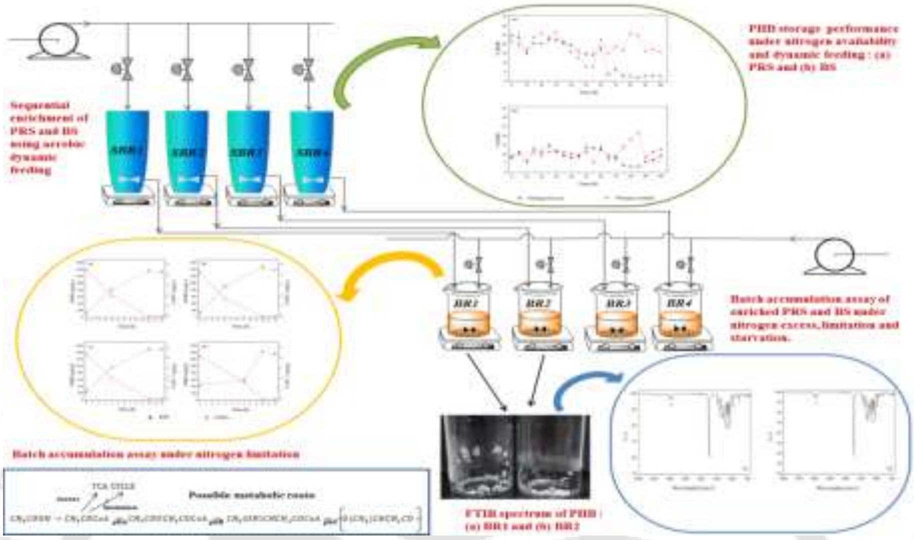


Figure 4: Pictorial representation of the sequential feast-famine enrichment of PRS and BS and subsequent bulking of enriched sludge with PHA accumulated

Chapter 6: Polyhydroxybutyrate production from rice mill effluent by influencing sludge retention time during short-term feast/famine enrichment of activated sludge

Impact of sludge retention time (SRT) on polyhydroxybutyrate (PHB) production from brewery sludge was investigated through feast/famine enrichment. Different accumulation strategies were explored to maximize PHB production from rice mill effluent (RME) fermented by acid-heat shock pretreated gobar gas plant sludge through acidogenic fermentation (Fig 5). Brewery sludge maintained under an SRT of 10 d had a higher storage response of 10 ±0.003 % than an SRT of 5 d (3.9±0.05 %). The higher SRT gave a wide spectrum of microbial communities enough time to adapt to the feast/famine condition. Lower SRT demand a faster growth rate to replace the waste amount with a shorter period that might have resulted in washout of a large portion of PHB accumulators. PHB is generally a non-growth-associated macromolecule. Hence, lower SRT could give more time for the microbial community to adapt the stress condition to divert more carbon towards the storage form. The study indicates that optimum SRT for activated sludge could vary depending on origin and period of enrichment.

PHB accumulation was maximized using batch and fed-batch accumulation. Fed-batch accumulation with two consecutive pulses with a settling phase in between resulted in the highest storage response. PHA accumulation of 60 ±0.004 % resulted from enriched BS and acetate as carbon sources. The accumulation was lower for raw RME at 12.3 ±0.06 %, while double was the storage response with fermented RME at

29.5 ±0.01%. Similar was the case PHA yield. Therefore, PHA production improved through acidogenic fermentation. Thus, a fed-batch accumulation can be an effective tool to improve the storage response for effluent streams with lower organic content like RME. Hence, BS enriched under higher SRT can produce higher PHA storage with fermented RME through fed-batch accumulation. The study indicates possible waste management of medium-strength effluent through the waste biorefinery approach.

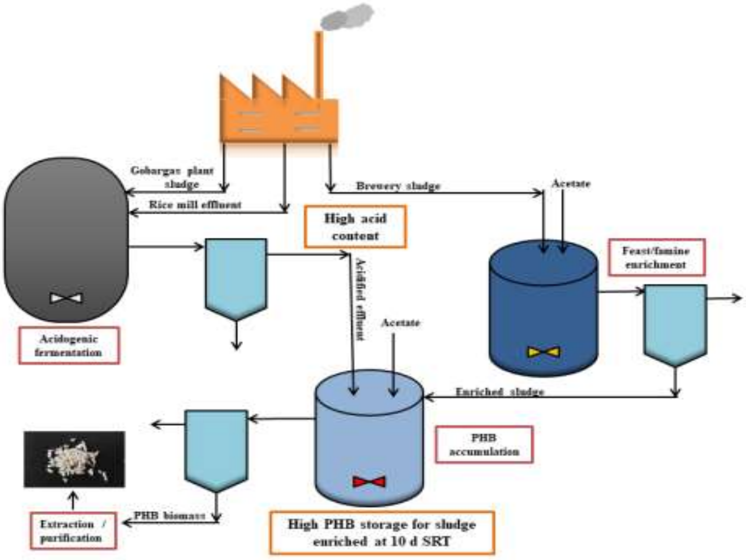


Figure 5: Graphical representation of PHB production by feast/famine enriched activated sludge from acidified rice mill effluent

Conclusion

The three-step process is an effective process to mitigate ever-increasing plastic pollution and waste disposal issues. The research work formulates a feasible route for the production of PHB mix culture and agro-industrial waste streams. The pretreatment of anaerobic seed inoculum through cyclic heat-acid shock enriches the sludge with spore-forming hydrogen producers capable of converting organic content to VFA. The pretreatment redirects the metabolic route resulting in acetate-butyrate-rich fermentate. Among the two anaerobic inoculums examined, gobar gas plant sludge was capable of withstanding the pretreatment with better VFA content than brewery anaerobic sludge. Meanwhile, rice mill effluent with higher readily biodegradable content produced acidogenic effluent with higher VFA content and degree of acidification than brewery effluent. The pretreatment strongly inhibited the acetoclastic communities, which resulted in lower consumption and higher VFA accumulation. The medium-strength of the effluent restricts pH in the range of 5.0-6.0, which points towards the feasibility of a pH-controlled system. Moreover, choosing the correct inoculum and substrate can regulate the even-numbered to odd-numbered VFA ratio. Rice mill effluent fermented with pretreated brewery anaerobic sludge had resulted in one of the highest even to odd ratio reported. Therefore, a combination of heat-acid shock pretreated gobar gas plant sludge and rice mill effluent produces acidified fermentate that promotes higher storage and PHB content during downstream polymer production.

Activated sludge is an ideal source of microorganisms capable of storing carbon in the form of PHB. Aerobic dynamic feeding pushes mix culture in the sludge towards the short feast and long famine phase. The culture with internal storage survives the famine phase that in the extended run gets dominant. PHB production is highly influenced by nutrient availability, especially nitrogen. Nitrogen excess condition develops proper feast/famine phase that asserts stronger ecological selective pressure. The proper metabolism and cell machinery enrich an actively PHB-storing microbial community. Meanwhile, nitrogen limitation causes increased storage response due to imbalance in growth and the resulting metabolic selective pressure. An extended period of enrichment under nitrogen limitation can lead to a loss in the storage response itself due to sludge bulking that diverts more carbon for filamentous production than PHB. Nonetheless, nitrogen limitation is an effective strategy for PHA accumulation for a shorter period, leading to high storage response. Therefore, sludge enriched under nitrogen excess condition maximally stores carbon as PHB when accumulated under nitrogen limitation.

Acetate as a carbon source produces pure PHB from enriched activated sludge. However, raw rice mill effluent led to lower storage and yield. This indicates the diversion of carbon for growth and maintenance. The effluent fermented with pretreated gobar gas plant sludge produced was able to produce a higher PHB content and yield with a concurrent reduction in chemical oxygen demand. Hence, the research potentially demonstrates the treatment of rice mill effluent through PHB production.

Content

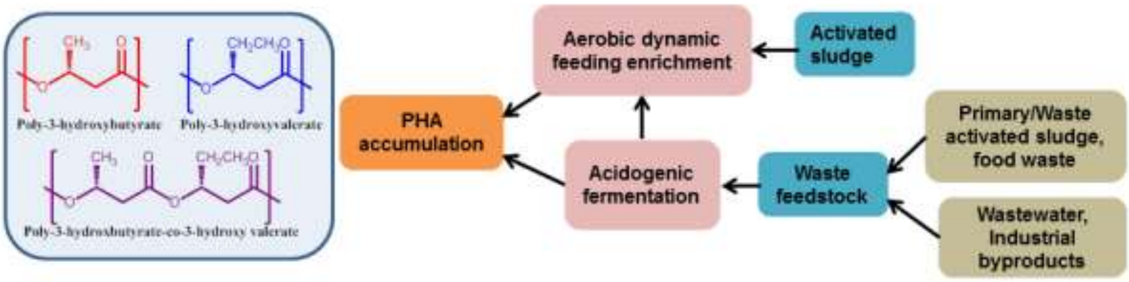
Abbreviations and Symbols

PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate (HB)
PHV	Polyhydroxyvalerate (HV)
ADF	Aerobic dynamic feeding
AF	Acidogenic fermentation
VFA	Volatile Fatty Acid
TVFA	Total volatile fatty acid
nonVFA	organic content contributed by other than VFA
HAc	Acetic acid
HPr	Propionic acid
HBu	Butyric acid
HVa	Valeric acid
CoA	Coenzyme A
RME	Rice mill effluent
BE	Brewery effluent
BAS	Brewery anaerobic sludge
GPS	Gobar gas plant sludge
PRS	Petroleum refinery sludge
BS	Brewery sludge
PT-RME	Acidogenic fermentation of RME with pretreated sludge
PT-BE	Acidogenic fermentation of BE with pretreated sludge
UT-RME	Acidogenic fermentation of RME with untreated sludge
UT-BE	Acidogenic fermentation of BE with untreated sludge
COD	Soluble chemical oxygen demand
HRT	Hydraulic retention time
SRT	Sludge retention time
DO	Dissolved Oxygen
$N\text{-}NH_4$	Ammonical nitrogen
$P\text{-}PO_4$	Phosphate-phosphorus
SBR	Sequential Batch reactor
BR	Batch reactor
FBR	Fed-batch reactor
UASB	Up-flow anaerobic sludge blanket reactor

DA	Degree of acidification, %
VFA_{max}	VFA conc. at the peak,mg/L
$VFA_{initial}$	VFA conc. initially present, mg/L
ΔCOD	COD stability
ΔpH	pH stability
$COD_{nonVFAmax}$	nonVFA concentration, mgCOD/L
$Y_{VFA/nonVFA}$	VFA conversion yield, gCOD/gCOD
PR_{VFA}	VFA production rate, mg/Lh
A_{max} is	Cumulative maximum of acid accumulation, mg/L
R_{max}	Maximum rate of acid accumulation, mg/Lh
λ	Lag time to exponential acidification, h
X	Active biomass, mg/L
f/f	Feast to Famine ratio
%PHB	PHB storage, %
$Y_{P/S}$	PHB yield, gCOD/gCOD
$Y_{X/S}$	Active biomass yield, gCOD/gCOD
q_p	Specific PHB production rate, $mgCOD_{PHB}$ /Lh
$-q_s$	Specific substrate consumption rate, $mgCOD_S$ /Lh
M_w	Weight-average molecular weight (g/mol)
M_n	Number-average molecular weight (g/mol)
PDI	Poly dispersity index
DP_w	Degree of polymerization
CI	Crystallinity index, %

Chapter 1

Introduction



1.1. Emergence of PHA production from waste feedstocks

Attention to environmental pollutants, studying the environment's quality, and waste management are among the main topics discussed in today's world, especially in developed countries (Karimi-Maleh et al., 2020). Plastic pollution is one such issue that is becoming harder to manage. However, the first synthetic plastic, 'Bakelite," was developed by Leo Baekaeland in New York in 1907. This was a small step towards a greater leap in plastics production and use. It was supposed to be a massive stride in reducing the dependence on environment-intensive commodities like paper, clothing, etc. These commodities draw a larger share of natural resources in harvesting the raw materials to the final product. Meanwhile, synthetic polymers were manufactured from petroleum refining byproducts and had a lesser environmental impact (BBC, 2019),(Feldman, 2008). Plastics made from synthetic polymers could be molded according to use, i.e., shape, size, color, thickness, toughness, etc. Hence, over the years, plastic became one of the primordial components of everyday life. Synthetic or conventional plastics went on to be used in every niche of our existence, from packaging, biomedical, construction, etc. This lead to unchecked and inappropriate use of synthetic plastic over the century leading to accumulation in every part of the biosphere. The non-biodegradability of the plastics increased the gravity of the situation. The majority of the conventional plastics we use and depose accumulate in soil without degradation for centuries, depending on the type and thickness of the polymer, or find their way into water bodies (Shah et al., 2008). Over the decades, these plastics in the water bodies got deposited in the oceans. These plastics carried through the ocean current finally merged at a site in the Pacific Ocean called the "great pacific garbage patch" (National Geographical society, 2019). These floating plastics ingested by marine fauna misjudging as a food led to choking, blockage, suffocation, and death of adults and juveniles equally. The plastic pollution of "the midway atoll" and Laysan albatrosses with plastic in their digestive system is a grim view of anthropogenic effects on marine life (CNN, 2016).

Conventional plastics are being dumped more than salvaged and disposed of, choking the biosphere. These plastics disintegrate into "microplastics" upon the action of environmental agents and concentrates on the soil and water. Marine ecosystems harbor the worst case of microplastic pollution. These bioaccumulate even in the lower tier of the marine food chain, which inevitably finds its way to the bigger marine species like fish, whales, etc. Microplastics have become so rampant that plastics are bioaccumulating in the human body through salts, seafood, water, etc. Microplastics elevate the propensity of contracting cancer and other chronic ailments in human beings (Sharma and Chatterjee, 2017). Conventional plastics in soil pose a severe threat to wildlife and agriculture. Plastics were found to have moderate to severe effects on earthworms, affecting soil hydraulics, bioturbation, etc. Microplastics in hen excreta indicate the possibility of bioaccumulation in humans through the soil. A recent study approximates 840 plastics per person as the annual plastic consumption through soil (Chae and An, 2018). Moreover, unscientific disposal also resulted in the clogging of waterways and subsequent inundation.

Fossil fuels are a highly exploited energy source in the world that steered human civilization. As for anything of high utility, extended exploitation of fossil fuel threatens exhaustion in the near future. Hence, alternate energy sources are being investigated, and some have been put to work. The petrochemical industry estimates a 20 % rise in oil demand solely to supply feedstock for plastic in the near future due to declining gasoline demand trends as never before (McKay, 2019). An alternative to the current source is necessary to mitigate any drastic implications of a sudden drop in plastic production on the world economy. Similarly, the impact of plastic on greenhouse gases emission is a matter of urgency. If the production, disposal, and incineration of plastic follow the current trajectory, the emission could reach 1.34 Gt CO_2 emission by 2030, equal to 295 500 MW coal plants. This would be a dent for the climate change initiative to reduce the global temperature rise to 1.5 °C (Center for International Environmental Law, 2019).

Conventional plastics have been unavoidable, ubiquitously utilized, an omnipresent commodity in the world. An all-out ban on plastic usage could ignite a catastrophic effect on the global economy. Some synthetic plastic's applications are so crucial that its removal from the scenario cannot initiate at all. Hence the more sustainable practice of 4 R's of waste management, reduce-reuse-recycle-remediation, is the need of the hour. Recycling of other than thinner plastics has been carried out for a long time and was the reason why the adverse effects of plastic pollution were restricted till a recent decade. The inflow of plastics is more than what could be collected and recycled. Remediation is yet to catch up with the expectations of plastic waste management. Presently, incineration is the widely used method for plastic disposal that adds a considerable amount of CO_2 to the atmosphere. Meanwhile, the available landfill areas are declining along with increasing reports of leaching from landfill sites into groundwater and other water bodies. Many bacteria and fungi degrade conventional plastic depending on plastic characteristics and pretreatment, microorganism, etc. However, bioremediation is hardly discussed or applied for plastic waste management (Shah et al., 2008). Many investigations have been undertaken to reduce plastic dependence by innovating biodegradable polymers that could replace the existing plastics. According to ASTM, biodegradable polymers are those which can be easily broken down into harmless components with or without simultaneous uptake by microbes (BPI, 2015). According to European standards, biodegradable polymers are compostable if the packaging breaks down (primarily into CO_2 and H_2O) under the industrial composting condition of 12 weeks and <10 % of the original material remaining in pieces of $\leq$2mm without any harmful effect on soil (Oakes, 2019). Most of these compostable polymers can be mixed with normal materials without much negative impact on the final compost property. The common of these biodegradable polymers majorly falls under two categories: petrochemicals like polybutylene adipate-co-terephthalate, and polycaprolactone, polybutyrate adipate terephthalate, polyvinyl alcohol, and bio-based like polylactic acid, polyhydroxyalkanoates (PHA), starch blends, cellulose acetate, polyisoprene (Lambert and Wagner, 2017)(Kinhal, 2019).

PHA is one such widely investigated bio-based biodegradable polymer that can reduce the dependence on conventional plastics. PHAs are polyesters of fatty acids formed by the condensation of hydroxylated fatty acids. These synthesized as lipophilic inclusion inside the cell cytoplasm are the storage form of carbon like glycogen, triacylglycerol, wax, etc. A broad spectrum of microbial communities synthesizes PHA granules that are widely spread across ecosystems like esturian sediment bed, rhizosphere, groundwater sediments in addition to activated sludge from waste treatment plants with fluctuations in carbon content or nutrient variability (Koller et al., 2011). More than 300 different microorganisms produce PHA with 100 different types of monomer units. Dictated by the carbon source, PHAs majorly belong to two categories: short-chain length (scl-PHA) or medium chain length (mcl-PHA). scl-PHAs like poly(3-hydroxybutyrate) [PHB], poly(3-hydroxyvalerate) [PHV], and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [PHBV] have been extensively characterized and possess industrial significance (Kumar et al., 2019). BIOCYCLE® (PHB), ENMAT™ and BIOMER® (PHB/PHBV), etc., are some of the trade names of biopolymers currently available in the market. mcl-PHAs are rarely synthesized, especially in the presence of hydrocarbons, higher fatty acids, oils, and lipids as carbon sources. PHAs are thermoplastic polyesters with comparable properties to polypropylene and polyethylene (Kourmentza et al., 2017). Naturally occurring degrading microbes swiftly remove PHA from the environment. Subjected to environmental stimuli like moisture, temperature, etc., PHA gets hydrolyzed into the component 3-hydroxy fatty acids. β–oxidation and TCA cycle metabolize these byproducts into CO_2 and H_2O. In general, PHA degradation does not have any harmful intermediates. Moreover, 3-hydroxybutyrate, a hydrolysis product, is even found in blood plasma. For these reasons, PHAs have found their way into biomedical applications like sutures, bone, and blood vessel replacement, drug delivery, etc. (Shah et al., 2008). PHA homo-copolymers and blends have shown potential applications in food packaging, followed by textile, consumer electronics, automobile parts, catering products, etc. Crude PHA has also found use in building and construction, as mulch for maintaining soil properties, etc., (Albuquerque and Malafaia, 2018),(Ivanov et al., 2015),(Oakes, 2019).

Due to its applicability, bioplastic production had increased to 2.11 million tonnes (Mts) by 2019, < 1 % of the total market share. The biodegradable polymer production is set to increase from 0.9 to 1.3 Mts from 2018-2023. Presently, PHA contributes only 5 % of the biodegradable polymer production that is dominated by starch blends and 1.2 % of the bioplastics produced(Oakes, 2019)(Gameiro, 2019)(European Bioplastic, 2019). However, bioplastic manufactured from PHAs is yet to make their presence feel. The bottleneck is that the cost of PHA is still to be normalized to that of conventional plastics. Over the decade, the price of conventional plastic ranged between 1.3- 1.9 $/Kg while that of bioplastics derived from PHA was higher between 4.5-6.1 $ /Kg (Kourmentza et al., 2017). Cost of the substrate, bioprocess control (aeration, sterilization, temperature, pH), polymer properties, etc., sum up to a higher operational cost that inevitably influences the per kg rate of PHA (Chen et al., 2020). The cost addition to the total operating cost for the downstream processing, extraction, purification, modification, etc., are decided by application (purity) and complexity of the process (Koller et al., 2011). The pharma application could route for higher production cost and price than other applications that demand less purity. These arguments have been dealt from different angles. Many studies tried to balance the cost by improving the physiochemical characteristics of PHA. PHA blends had improved the biopolymer's property and economy compared to conventional plastics (Pietrini et al., 2007). The past two decades' major investigations concentrated on PHA accumulating culture and the impact of renewable carbon sources on these storage-specific microorganisms (mentioned in detail in the following sections).

1.2. Activated sludge as a source of inoculum for PHA production

Between 1923-1927, Dr. Maurice Lemoigne conducted a series of studies to find the cause of acidification of medium containing *Bacillus megaterium* that led to the isolation of a solid material that he characterized as a polymer of 3-hydroxybutyrate (Lenz and Marchessault, 2005). Since then, over 300 bacterial species have been found to produce PHA primarily belonging to generas *Alcaligens*, *Bacillus*, *Pseudomonas*, *Azotobacter*, *Burkholderia*, etc., and recombinant *Escherichia (E.) coli* (Choi and Lee, 1999). A high cell density and PHA storage (100–200 g/L and 75–80 % cell dry weight (CDW)) were obtained from wild type *Cupriavidus necato* (Pagliano et al., 2017).

These developments provided acceptable productivity and yield for PHA. Yet, higher operational costs persisted that denied PHA-derived bioplastics' capacity as a potential substitute for conventional plastics. This bottleneck led to the search for a better culture that could dilute one or the other restriction. The discovery of microbes from certain ecological niches opened up PHA's prospects further. Some hydrogenotrophic strains like *Cupriavidus necator* (wild type) can exist in an oxic-anoxic interface fixing CO_2, taking H_2 as an electron donor and O_2 as an electron acceptor with simultaneous PHA accumulation. Certain extremophiles like *Halomonas* spp., *Saccharophagus degradans*, *Caldimonas taiwanensis*, etc., found to produce PHA under growth-limiting conditions showed the possibility of easing the downstream processing and bioprocess control (non-axenic condition) (Kourmentza et al., 2017). From the 1960s, cyanobacteria like *Spirulina* spp., *Aphanothece* spp., *Gloeothece* spp., and *Synechococcus* spp., have been demonstrated for PHB accumulation under a photoautotrophic condition in the presence of simple inorganic micro/macronutrients (Balaji et al., 2013). Similarly, some methanotrophic bacteria like *Methylococcus* spp., *Methylocystis* spp., *Methylomonas* spp., can produce PHA by utilizing methane and methanol as a carbon source (Strong et al., 2016). These strains point out the applicability of cheaper and readily available carbon sources and uncomplicated fermentation conditions towards affordable PHA production.

By the end of the 90s, the search for a better culture moved on to various ecosystems that lead to an unexploited source, activated sludge. The presence of PHB in activated sludge treating municipal sewage wastewater was known for a while. This might be due to fluctuations in carbon and nutrients availability (Metcalf and Eddy, 2003). Many characteristics of activated sludge propound its applicability as an inoculum like non-axenic, resilient to environmental perturbation, easily adapted to specific molecules or metabolic routes (anaerobic, anoxic, biofilm, granular), uncomplex bioprocess control, the suitability of renewable feedstocks like effluent, etc. Dircks et al. (Dircks et al., 2001) directly used activated sludge procured from an effluent treatment plant for PHB accumulation without any limitation condition and observed a feast phase with a good yield of PHB by consuming acetate (decline) while in the famine phase PHB got consumed for growth, maintenance, and glycogen conversion. Suresh Kumar et al. (Suresh Kumar et al., 2004) went a step ahead to enhance PHA accumulation by activated sludge under the exposure of nutrient (nitrogen) limitation condition. Later, Sakai et al. (Sakai et al., 2015) tried to produce PHB under nutrient limitation with activated sludge collected from municipal wastewater treatment plants working under different operational modes for secondary biological treatment. Both studies at a gap of 10 years found PHB storage of only 0.2 g PHB/g CDW. The value nowhere near pure strain made the activated sludge a weaker candidate for the purpose.

Therefore, various enrichment strategies were employed to improve the PHA accumulating potential (PAP) of activated sludge. The most suitable contenders exposed sludge to an extended period of alternating conditions imposing an ecological selective pressure on the mix microbial culture (MMC), enabling PHA accumulators to survive and dominate. In general, two specific MMCs are involved in anaerobic/aerobic cycle enrichment, i.e., glycogen accumulating organism and phosphorus accumulating organisms. During the anaerobic phase, glycogen accumulating organism gains energy and reducing power by metabolizing glycogen into acetyl-CoA and propionyl-CoA, produced from external carbon sources. Subsequently, these condense into PHAs that get consumed during the aerobic phase for growth and glycogen production. Similarly, phosphorus accumulating organisms consume the external carbon source for PHA production by the energy supplied by the degradation of internal polyphosphate granules, thereby increasing the medium's soluble phosphorus content. The accumulated PHA is then used up in the aerobic phase for growth and energy production via phosphate assimilation from the medium. Thus phosphorus accumulating organisms have shown promise for PHA accumulation, phosphorus recovery, and/or removal. The wasted biomass acts as an excellent biofertilizer as well. But there is always the complexity of bioprocessing when handling anaerobic and aerobic phases alternatively. Simultaneous investigation of such sequencing cycles employing only the aerobic phase also got kicked off. The aerobic dynamic feeding (ADF) strategy employs exposing activated sludge to alternating short carbon excess-feast phase and extended carbon starved-famine phase in a sequential batch operation. The feast/famine ratio puts strong selective pressure and restricts the microbial diversity to PHA accumulators that can survive the long carbon starvation period (Kourmentza et al., 2017). This type of long term ecological selective pressure has resulted in a high PHA accumulation of 0.9 gPHA/gVSS (Tamis et al., 2014). Realizing the PHA accumulating potential of activated sludge had opened up the field to use the vast untapped renewable feedstocks from waste solids to wastewater, etc., thereby extending the reach of the waste biorefinery approach (Valentino et al., 2016).

1.3. Potential waste feedstock for PHA production from mix culture

According to the present calculations, 5 Mts biomass feedstock was used for bio-based plastic production in 2019 with a land-use estimation of 0.79 million hectares (Mha) (European Bioplastic, 2019)(Chinthapalli et al., 2019). At the same time, the cost of carbon feedstock added a substantial portion to the operating cost. Thus the experimentation with cheaper, ubiquitously available, renewable/ waste kind of carbon source was taken up by the scientific community. Such carbon sources must have less interference with the normal consumption and nutrition of humans or animals, i.e. a negative value to comply with such schemes (Venkata Mohan et al., 2016). One possible route was to use pure strain or recombinant strains to produce PHAs from such renewable carbon sources. These carbon feedstocks utilized for PHA production fall in the following categories: starch-based materials, molasses, and sucrose, lignocellulosic material, whey-based, fatty acid and glycerol, agro-industrial byproducts, and waste streams, etc. (Pagliano et al., 2017). Even though commendable advancements were brought about with these attempts, the balance was still tilted towards conventional plastics. In 2009, Metabolix, in partnership with ADM, set up a plant where the starch and sugar-rich effluent from wet corn milling plant was used as a carbon source for PHA production (Dietrich et al., 2017).

Yet, the complexities of bioprocess for PHA production with pure cultures persisted. Added to that, the suitability of pure strain with unsterilized carbon sources was a question. Therefore, simultaneous investigations were undertaken to economize PHA production by utilizing potential activated sludge as a culture source. Thus, polymer production using renewable and cheaper carbon sources using open mix culture came to be considered a possible replacement for the current production strategy to minimize and balance production costs (Kourmentza et al., 2017). Waste feedstocks are such renewable sources that were explored for PHA production with activated sludge. Exploiting these feedstocks serves a dual purpose, i.e., waste management and minimization through resource recovery. The regional availability and seasonal variability of potent waste feedstocks are critical in realizing waste-derived PHA synthesis.

Some initial studies with single-step PHA production from activated sludge and molasses spent wash had revealed reduced production (20 % CDW) compared to that of pure strain (Khardenavis et al., 2009). Later, the inclusion of the sludge feast/famine acclimatization with a cycle length of 4 days increased the PHB storage from biodiesel wastewater (42-62 % CDW) (Dobroth et al., 2011). Consequently, ADF enrichment of activated sludge in wastewater followed by batch PHA accumulation using pure substrate yielded a stable and high storage response $\geq$ 60 % (De Grazia et al., 2017). Thus it became apparent that sequential ADF enrichment succeeded by batch accumulation was the apt route for PHA production from activated sludge and the waste feedstocks. Over the decade, ADF has proven to be a reliable methodology for waste-derived PHA production (Valentino et al., 2016).

However, non-specific substrates possess an issue for the efficiency of ADF. Diversion of carbohydrates as a carbon source towards glycogen always loomed over the enrichment processes. Also, some waste feedstocks required pretreatment to remove recalcitrant or toxic components that could impair the process (Pereira et al., 2020),(Campanari et al., 2014). Meanwhile, there were speculations that fatty acids are better competent for polymer accumulation. VFAs were found to be the preferred carbons over other carbohydrates. VFAs act as a precursor that would improve storage response (Cui et al., 2016). In addition to this, non-fatty acid (>30 % TOC) comprising mainly of proteins and carbohydrates assisted cell growth, which had a negative implication on storage (Jia et al., 2013). The storage response of accumulators is unaffected due to the presence of non-VFA components but reduces the overall PHA production (Marang et al., 2014). This specificity was due to an overlap in the metabolic route between fatty acid and PHA synthesis. One of the intermediate 3-hydroxyacyl-CoA is a common point in particular stages of the corresponding synthesis (Magdouli et al., 2015). Also, an increase in acetate spiked acetyl-

CoA concentration in the cytoplasm through β-oxidation, leading to a reduction in the free Coenzyme-A (CoASH) inhibiting PHA synthesis pathway ameliorating the polymer production (Vallari et al., 1987),(Tan et al., 2014). Long-chain fatty acids tend to produce mcl-PHA. Meanwhile, short-chain fatty acids or volatile fatty acids in the range C_2-C_5 get metabolized into scl-PHAs such that even-numbered VFA corresponds to PHA with higher hydroxybutyrate (HB) content and odd-numbered VFA to that of higher hydroxyvalerate (HV) content (Shen et al., 2014). Because of this entwined relation between VFAs and PHA, acetate had been used as a carbon source to kick start the carbon flux (Kumar et al., 2019). Realizing this potential, the effects of individual VFA on PHA storage, property, composition, etc., were also mapped (Arcos-Hernández et al., 2013). Moreover, acidogenic fermentation (AF) efficiently performed bioconversion of the organic content in the feedstocks into VFAs to overcome the non-specificity and improve PHA storage and accumulation in activated sludge. Extensive investigation of the acidogenic potential and its enhancement using a variety of carbon feeds have aided in implementing an effective three-step methodology with AF-ADF as a core functional unit for efficient biosynthesis of PHA from cheaper feedstocks (Lee et al., 2014),(Valentino et al., 2016). Therefore, the acidogenic potential is an important criterion for selecting suitable waste feedstocks for PHA production.

The waste feedstocks employed in the three-step methodology are waste sludge and solids, wastewater, agro-industrial refuse, and industrial byproducts having some economic value. Two types of sludge are sourced from the activated sludge process in an effluent treatment plant. Primary sludge (PS) is generated in the initial stage of the treatment process during the removal of solids, biomass from wastewater before entering into the biological treatment unit. A higher amount of easily biodegradable protein and carbohydrate content makes up PS. The wastewater acts as the carbon source that aids in the active metabolism of microbiota concomitantly removes organic content leading to augmentation of biomass, which in the course settles down in secondary settler. A substantial part of the biomass gets wasted as waste activated sludge (WAS) or excess sludge (ES). The remaining portion gets recirculated as inoculum for the next batch. WAS is generally bacterial, and other biomass and rate-limiting cell lysis could release more nitrogen and phosphorus into the liquid phase (Ucisik and Henze, 2008). WAS is the most investigated substrate to enhance PHA production due to its disposal requirement and high organic content (Jia et al., 2014). Meanwhile, improved acidification is also reported with a combination of PS-WAS (Zhang et al., 2019). Various treatment conditions employed hydrolyzes and release organic content resulting in a spike in VFA content and PHA production (Lee et al., 2014). Thereby a holistic approach towards waste management can be materialized where sludge minimization and waste disposal get accomplished using sludge as a substrate for acidogensis and as a source of

MMC for PHA production (Morgan-Sagastume et al., 2015). Food waste is another ubiquitously available waste from households, cafeterias, etc., containing a comparatively higher Soluble chemical oxygen demand (SCOD) as sludge, even though enhancing the hydrolysis is necessary to improve soluble components. PHA production from acidified food waste could rise as a sustainable solution for such high carbon loaded waste generated throughout the year without any kind of layovers (Amulya et al., 2015). A combination of food waste and dewatered sludge could produce a high degree of acidification and improve PHA production (Chen et al., 2013). Organic fraction of municipal solid waste (OFMSW) is one of the major solid waste generated that forms leachate in landfill sites that can contaminate groundwater and other water bodies. Recent investigations have shown the possibility of remediating the high organic content of OFMSW (Colombo et al., 2017)(Papa et al., 2020) through resource recovery. Agro-industrial residues and other lignocellulosic biomass generated from biomass processing, harvesting, etc., are also potential and cheap substrates to be explored with activated sludge (Kumar et al., 2019),(Pagliano et al., 2017). Waste grass or cultivated grass is one such lignocellulosic biomass that can produce 403.65 t PHA from 30,000 t fresh grass (Patterson et al., 2020).

Soluble COD contributes to the VFA production from acidogenic fermentation that specifically bulks cells with PHA. Wastewater is the ideal source of SCOD that can be directly acidified without much pretreatment and more straightforward process control. Agro-industrial waste feedstocks are the most investigated among the effluents as they contain readily available and fermentable organic carbon with a mostly non-inhibitory matrix (Valentino et al., 2016). Olive oil mill effluent (OME) and palm oil mill effluent (PME) share a significant part of the AF-ADF PHA synthesis. The effluents originate from the cold/wet extraction of respective fruits from which water gets separated from the oil by gravity settling or cold centrifugation. The investigations for polymer production from acidified OME also involved in dephenolization and phenol recovery (Campanari et al., 2014). The ever-growing demand for palm oil worldwide generates a copious amount of PME with a high COD that requires treatment before disposal (Salmiati et al., 2007). Agro-industrial wastes also contain a high amount of organic carbon that was investigated for resource recovery through PHA using activated sludge like paper mill effluent (Bengtsson et al., 2008), wood mill effluent (Ben et al., 2011), hardwood spent sulfite liquor (Pereira et al., 2020), paperboard mill wastewater (Farghaly et al., 2017). Distillery effluent is known to have one of the highest SCOD ranging from 10 to 100g/L, which can be effectively diverted to storage form through acidification (Khardenavis et al., 2009). Among the industrial byproducts, the most investigated and optimized source on an industrial scale is sugar cane molasses. Sugar cane molasses contain a high amount of fermentable sugars, which can be acidified entirely and recovered as PHA (Oehmen et al., 2014). Similar is whey, cheese whey (Colombo et al., 2016), or whey permeate (Janarthanan et al., 2014), a byproduct produced during cheese manufacturing. Crude glycerol is the major component of biodiesel wastewater, which is the side stream of biodiesel production. A recent study has shown the acidogenic potential with subsequent PHA production from crude glycerol (Burniol-Figols et al., 2018). The point of concern for these kinds of carbon sources is their economic value. Animal husbandry-based waste feedstocks are among the least explored carbon sources for PHA production, primarily for their lipid content. ANIMOL is a project initiated to evaluate such a possibility in animal husbandry-based waste feedstocks (Titz et al., 2012). Dairy effluent, slaughterhouse wastewater (Kumar et al., 2019), Bio-oil (Fidalgo et al., 2014), dairy manure (Coats et al., 2016), meat processing effluent (Hamawand, 2015), etc., are some of the principal animal husbandry-based waste feedstocks worth exploring further. A huge gap exists in utilizing marine-origin waste for PHA production, like the saline and protein-rich mussels cooking wastewater (Roibás-rozas et al., 2021).

It is evident from Table 1.1 that high-strength effluent can deliver maximum VFA, which is a prerequisite component for high accumulation. Even then, low strength waste feedstocks are equally compatible that can effectively prevent the down regulatory effects of a high organic load. A reasonable degree of acidification can ensure a higher polymer storage yield from these low-strength feedstocks (Morgan-Sagastume et al., 2015). Brewery effluent (Tamang et al., 2019), Tomato Cannery wastewater (Liu et al., 2008), cafeteria waste (Din et al., 2012) are some of the promising media for incrementing storage response. Besides, PHA can capture and store some of the greenhouse gases released into the atmosphere. Some *Methylotrophs* can fix greenhouse gasses like CO_2, CH_4, CO, and methanol into intracellular carbon reserves (Strong et al., 2016). Petroleum refinery/petrochemical effluents are yet to get investigated for their PHA production capacity from activated sludge. Recent research with pure strains has shown the possibility of diverting hydrocarbons specifically into PHA (Ghosh and Chakraborty, 2020). Current literature also pointed out that the resource recovery approach reduces waste generation and enables waste conversion to possible utilities (Venkata Mohan et al., 2016). Hence, the possibility of integrating PHA production from renewable carbon source with existing treatment facility are being investigated actively as a waste biorefinery approach to balance the ever-increasing waste management issue.

Deciphering the route for acidogenic conversion can pave the way for such recalcitrant sources to be bioplastics building blocks. In this way, the organic contents in various waste feedstocks can be captured, stored, or utilized as a value-added product through a three step PHA production methodology. Thus PHA production can be accounted for as a dynamic approach for maintaining environmental stability and sustenance. But the necessity is still on the horizon for deliberate analysis of polymer productivity with other potential region-specific carbon sources.

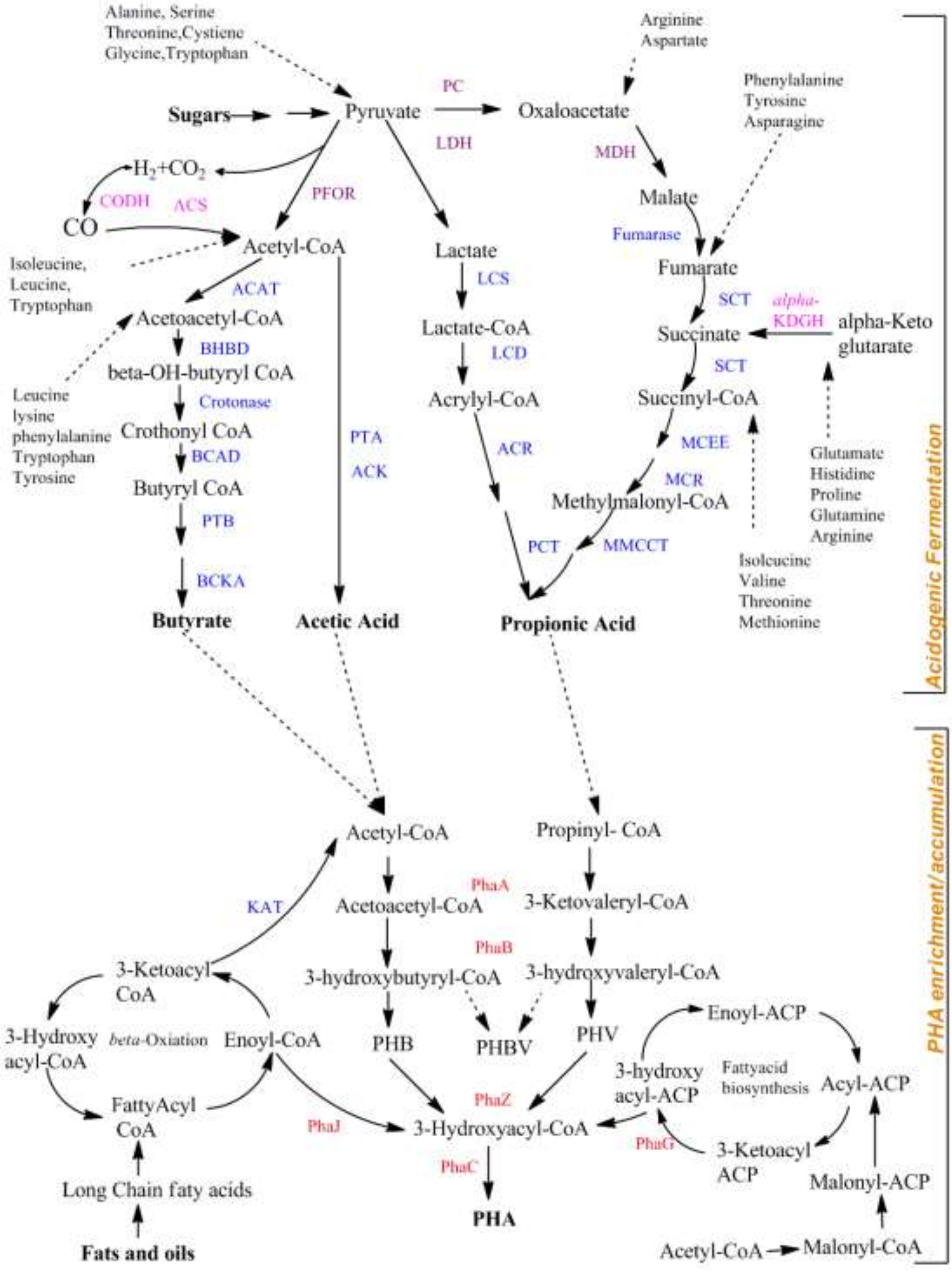


Figure 1.1: Multilevel metabolic network that leads to PHA production through acidogenic fermentation. PhaA: b-ketothiolase; PhaB: NADPH-dependent acetoacetyl-CoA reductase; PhaC: PHA polymerase; PhaZ: PHA depolymerase; PhaG: 3-hydroxyacyl-ACP-CoA transferase; PhaJ: enoyl-CoA hydratase; PFOR : Pyruvate ferredoxin oxidoreductas; LCS: Lactate CoA Synthetase; ACR; Acryly CoA reductase; LCR: Lactoyl CoA reductase; PCT: Propionyl CoA transferase; LDH: Lactic acid dehydrogenase; ACAT: Acetyl CoA acetyltransferase; BHBD: 3-hydroxybutyryl-CoA dehydrogenase; BCAD: Butyryl CoA dehydrogenase; PTB: Phosphotransbutyrylase; BCKA: Butyryl CoA Kinase; ACK: (acety CoA-carboxylase) Kinase; PTA: Phosphotransacetylase; MDH: Malonyl CoA dehydrogenase; SDH: Succinate dehydrogenase; SCT: Succinyl CoA transferase; PC: Pyruvate decaroboxylase; MMCCT: MethylMalonyl CoA carboxytransferase; MCEE: Methylmalonyl CoA epimarase; MCM: Methylmalonyl CoA mutase; CODH: Carbonmonoxide dehydrogenase; ACS: Acetyl CoA synthetase; KAT: 3-Ketoacyl CoA thiolase; α-KGDH: α-ketoglutarate dehydrogenase.

Process optimization needs deliberation for understanding the full potential of PHA production with mixed culture through an increment in VFA content. The multilevel network of PHA synthesis from various substrates through volatile fatty acid is described in Fig. 1.1 (Liu et al., 2016),(Rodriguez et al., 2014),(Sikora et al., 2013),(Lehninger et al., 2008),(Pryde et al., 2002),(Prabhu et al., 2012),(Ojumu et al., 2004),(Ragsdale, 2008).

1.4. Carbon recovery from waste through three-step methodology

Waste feedstocks have been bioconverted to PHA through the three-step process. The PHA inducing potential of VFA was explored for a variety of organic loaded waste feedstocks. An enrichment stage and accumulation explore the full capacity of activated sludge as a catalyst for bioconversion. The general scheme of the methodology explained in the review is shown in Fig. 1.2. The methodology works on three levels :(1) Breaking down complex organic molecules in the waste feedstocks into simpler and readily assimilated carboxylic acids VFA by tuning the process elements (Table1.1), (2) Selection of PHA competent microbial communities from the mix culture by aerobic dynamic feeding strategy using VFAs or fermented feedstock (Table 1.2), and (3) Exploring the full capacity of the mix culture for maximum PHA accumulation from VFA or fermented feedstock (Table 1.3). The subsequent section comprehensively discusses these three steps.

1.4.1. Anaerobic digestion of waste feedstocks towards VFA production

Acidogenic fermentation transforms soluble organic content into volatile fatty acids. Hydrolysis and acidification of substrate in an anaerobic environment lead to the synthesis of C_2-C_5 fatty acids. In the process, some part of the soluble COD is used for cell maintenance and growth, while a significant portion gets diverted to fermentation products (Bengtsson et al., 2008). Acidogenesis is initiated either by the in situ microbial population or by supplying mix culture from the digestate of an anaerobic process like biogas production. Anaerobic sludge from biogas production plants is an ideal seed culture that can be diverted more towards VFA production and accumulation by aligning different parameters discussed in the subsequent sections.

Many operational parameters like pH, temperature, organic loading rate (OLR), sludge retention time (SRT), hydraulic retention time (SRT), etc., influences VFA production from waste feedstocks. Each parameter has a different impact on the VFA content and profile depending on the interaction with substrate and microbial community. The type of substrate, waste solids or waste liquids greatly alter the effect of each parameter. For example, alkaline pH and thermophilic temperature facilitate better hydrolysis and VFA production. Meanwhile, acidic pH and mesophilic temperature facilitates optimal action of the acidogenic microbial community. Similarly, the optimal contact time (OLR, SRT, HRT) for required bioconversion also varies with the type of substrate. The readily available organic content in the form of SCOD in waste liquids promotes shorter contact time and higher OLR capacity. However, the necessity of hydrolysis in waste solids to increase the SCOD demands longer contact, reducing the maximum OLR the system could handle. In addition, the origin of anaerobic inoculum, food to microbe (F/M) ratio, substrate pretreatment methods, etc., also plays a vital role in deciding the acidogenic outcomes. The detailed outline of these parameters is deliberated elsewhere (Lee et al., 2014).

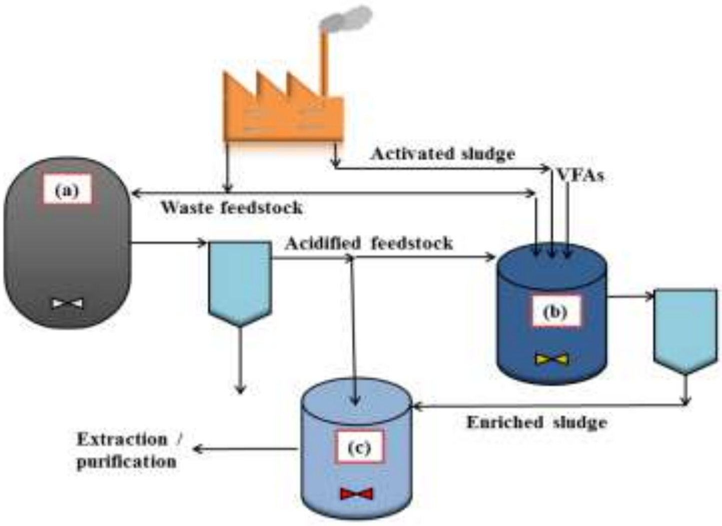


Figure 1.2: The schematic representation of three step method. (a) Acidogenic Fermentation, (b) Aerobic dynamic feeding enrichment, (c) accumulation PHA

1.4.1.1. Anaerobic inoculum sludge pretreatment for enhancing acidogenesis

Different substrate pretreatment has been investigated to improve the interaction between the inoculum and substrate. But in most of these pretreatments, the substrates (solids) get broken, and the SCOD gets released into the medium. Such pretreatments are least applicable when SCOD is readily available, like in the case of wastewater. The conversion yield might increase, but the VFA composition will depend upon the nature of the substrate used (Lee et al., 2014). A primary production strategy enhances VFA production by influencing H_2 production. Glycolysis and oxidative decarboxylation of pyruvate to ATP require NAD+/ferredoxin (ox). Hydrogenase enzyme catalysis this electron transfer generating H_2 and VFA as byproducts (1). Acetate and butyrate are the major VFAs produced in this reaction, with a lower butyrate/acetate ratio tending to maximum theoretical hydrogen production (Hawkes et al., 2007). A widely accepted approach for maximizing H_2 yield is largely a physiological restriction of the microbial community. It primarily depends on selectively inhibiting or killing H_2 and VFA consumers like hydrogenoclastic, acetoclastic, methanogens, sulfate-reducing bacteria, etc. Heat shock treatment (65-100 ˚C), aeration, ultrasound, microwave, electric current, chemical pretreatment like acidic (pH 2.0-3.0), alkali, 2-bromoethane sulphonic acid, ethylenediaminetetraacetic acid, chloroform, etc., have been employed for improving hydrogen production. The absence of any carbon sink leads to higher production and accumulation of VFA along with concomitant hydrogen evolution (Chaitanya et al., 2016),(Pachapur et al., 2019).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (1)$$

Heat-shock pretreatment is one of the most widely investigated inoculum pretreatment for upgrading H_2 production. The heat pretreatment inhibits or removes methanogenic microbes enriching the anaerobic seed sludge with spore-forming hydrogen producers (Venkata Mohan et al., 2008). A study of heat pretreatment of forest soil (65-125 °C) found the acidogenic fermentation to shift from ethanol type to acetate-formate type with an increase in hydrogen production. *Clostridia* spp. dominated the heat pretreated inoculum as high temperature commences the germination of *Clostridia* spp. spore (Ravindran et al., 2010). The variation in the impact of heat treatment is also found between different types of inoculum. Heat pretreatment at 95 °C suppressed hydrogen and acid production for activated sludge, while there was a shift in dominant VFA from acetate to butyrate for anaerobic sludge. Anaerobic sludge had better acid and hydrogen production at 65 °C (Baghchehsaraee et al., 2008). Moreover, presence of some hydrogenotrophic microbes, homoacetogens, irrespective of pretreatment or inoculum origin, could prove to be an added advantage towards VFA production through the consuming H_2 and CO_2 produced (Saady, 2013). Granular anaerobic sludge produced the highest VFA production at heat pretreatment of 120 °C/20 min compared to the highest H_2 production at 95 °C /10 min (Magrini et al., 2020).

A similar shift in acidogenic metabolism is observed irrespective of the substrate. The fermentation of cheese whey without inoculum led to lactate dominated fermentate, while acetate dominated from heat pretreated (100 °C for 1 h) culture (Colombo et al., 2016). Fermentation of food waste and cow slurry by heat pretreated inoculum (94–100 °C for 0.5 h) also produced acetate as the major fatty acid (Tampio et al., 2019). Similarly, acidification of food waste using heat-shock pretreated inoculum (90 °C for 1 h) observed the possibility of high VFA production along with selective adjustment of the process using various buffering agents like Na_2CO_3, $CaCO_3$, and NaOH (Dahiya and Mohan, 2019). High VFA production was observed from brewery spent grains at an initial pH of 9.0 by using heat pretreated inoculum (90 °C for 2 h) with high acetate and butyrate content (Sarkar et al., 2021). However, the even-numbered VFA dominance was severely reduced for thermal-hydrolyzed/raw primary-activated sludge as substrate. The substrate consisted of high protein content leading to higher valerate production by the pretreated inoculum (110 °C-1 h and BES) (Zhang et al., 2019). For these kinds of solid feedstocks, substrate pretreatment could enhance the acidification process by increased solubilization and preference of acid condition. The mechanical pretreatment of rice straw produced higher VFA content using anaerobic inoculum (100 °C for 0.5 h) under mesophilic condition and acidic pH of 6.8-5.7(Sattar et al., 2016).

Another inoculum pretreatment method that provides an upper hand for the survival of acid producing or acid tolerant microbes is acid shock pretreatment. Various literature has used the pH 3.0 with exposure ranging from 10 mins to 24 h for selecting microbial population that can survive and grows under the acidic condition as pH 6.8-7.2 is the preferred condition for methanogens (Pachapur et al., 2019). An earlier investigation by Lin and Chou (Lin and Chou, 2004) had shown the capacity of acid shock treatment for 24 h to produce a higher percent of H_2 and VFAs at different HRTs using sucrose as a substrate. Meanwhile, acid-shock treatment for 24 h (pH 3.0) had maximum specific hydrogen production during the anaerobic digestion of waste activated sludge at initial pH of 7.0 among all the assessed pretreatment methods (Chang et al., 2011). A marine intertidal sludge pretreated under acid shock (pH 3.0 for 10 min) led to the dominance of *Clostridium* spp. and *Bacillus* spp. and higher H_2 production (Liu et al., 2009). Sarkar et al. (Sarkar et al., 2016) were able to enhance VFA production with higher acetate-butyrate content at pH 10.0 from food waste using acid-shock pretreated inoculum (pH 3.0 for 24 h).

Some comparative studies indicate a combination of inoculum pretreatment effective than individual methods. A heat-alkali (100 °C for 15 min-pH 12.0 for 5 min) performed maximum bioconversion of glucose, sucrose, and starch to VFAs among all the pretreatment assessed (Chaitanya et al., 2016). However, the VFA production from dairy wastewater improved with heat-acid shock pretreatment (100 °C for 1 h-pH 3.0 for 24 h) of inoculum compared to other combination or single methods. Meanwhile, acid-BES treatment was the best combination of H_2 production (Venkata Mohan et al., 2008). The similar inoculum pretreatment with 2 h of heat shock produced high VFA content from vegetable market waste extract with pulp than without pulp (Venkata Mohan et al., 2009). Much investigation is necessary to fully understand and exploit the potential of heat-acid shock pretreatment of anaerobic inoculum sludge.

Nevertheless, regardless of acidogenic fermentation conditions, the major VFA produced are acetate and butyrate (Sarkar et al., 2016), (Colombo et al., 2019). The controlling parameter is the nature of the substrate. Alkaline pH is preferred for solid waste as substrate (8.0-10.0), while liquid waste fermentation performs better at acidic pH (5.0-6.0) (Section 1.4.1). Fermentation at acidic pH would lead to possible chain-elongation due to the availability of higher reducing equivalents (H^+). Similar is the case of uncontrolled pH set at initial acidic or alkaline pH and pH controlled at alkaline pH, where the acidification process increases the pH and H_2 evolution (Sarkar et al., 2021). Thus, there exists a high overlapping of H_2 and VFA production. However, both processes are fairly independent of each other. Many pieces of literature with pretreated inoculum sludge depict the peak of H_2 evolution to be different from VFA production. This might be due to many secondary pathways like acetogenesis (hydrogenoclastic), chain-elongation, photo fermentation (Dahiya and Mohan, 2019),(Venkata Mohan et al., 2008),(Lee et al., 2014),(Saady, 2013). Therefore, anaerobic inoculum sludge pretreatment can be used as an effective VFA production system, which is otherwise extensively investigated for H_2 production. Therefore, a proper combination of pretreatment methods can effectively inhibit methanogens, restrict the consumption of organic contents leading to stable and higher VFA production inclined towards even-numbered fatty acids. However, the response to a particular treatment and its outcomes also depend on the origin or nature of the anaerobic inoculum and the waste feedstock. Therefore, the inoculum pretreatment could positively impact the PHA production and its property downstream. The implementation of such schemes will always weigh on the balancing of economic outlays in hydrogen production and subsequent polymer production.

1.4.1.2. Strategies employed for acidogenic bioconversion of waste residue for PHA production

Every investigation on acidogenic fermentation in the three-step methodology tried to refine the VFA content and distribution in anaerobic fermentation by tuning feedstock, pH, temperature, sludge retention time, hydraulic retention time, pretreatments, etc., with the sole purpose of influencing the PHA production, composition, and property. Shen et al. (Shen et al., 2014) had analyzed the impact of feedstock on VFA production and PHA composition with five different feedstock-waste starch, molasses, glycerol, protein, and waste sludge. Anaerobic fermentation revealed higher VFA content in starch, followed by molasses, glycerol, waste sludge, and protein. The feedstock conversion yield and overall conversion of substrate to PHA (9.5-24.3 %) followed in the order of waste glycerol, starch, molasses, waste sludge, protein. But regardless of the waste type, conversion of feedstock to VFAs was 2-fold higher than VFA to PHA. ADF can improve the conversion by enriching the activated sludge. Acetate was the prominent VFA produced in all the feedstock's fermentate except for waste glycerol, with propionate as prominent VFA (90 %). Butyrate was insignificant in waste glycerol fermentate, while it was higher in other fermentate after acetate. This difference in the VFA profile gets reciprocated in the composition of PHA. Therefore, under stable fermentation conditions, the feedstock selection dictates the PHA characteristics.

Similar to section 1.4.1, the nature of the substrate determined the operational condition for the AF-ADF process. Primary sludge and WAS were chiefly investigated, abundant, and cheaper carbon sources for short-chain fatty acid production. Most of the enhancement studies concentrated on elevating the SCOD content of sludge liquor by varying physical and chemical parameters. Alkaline pH aids in the hydrolysis of the sludge whereby proteins, carbohydrates, cells, etc., are ruptured or broken into soluble and simpler carbon molecules, easily taken up and converted to acids. Alkaline pH decreases the availability of reducing equivalents (H^+), reducing chain elongation, and promoting acetate/propionate production. This type of fermentation corresponds to copolymer production (PHBV) during PHA accumulation (Mengmeng et al., 2009). Similarly, WAS acidification at pH 10.0 resulted in the highest SCOD, VFA/SCOD of 62 %, and the highest acetate content (Liu et al., 2020). A batch co-fermentation of food waste and dewatered excess sludge was examined for VFA production at pH 5.0, 7.0, 9.0, 11.0. The anaerobic fermentation at an alkaline pH of 9.0 led to a VFA/SCOD ratio of 61 % and a VFA content of 25.9 $gCOD_{VFA}$/L (Chen et al., 2013). Similarly, the ratio of even-numbered to odd-numbered VFA was influenced by pH over food-waste composition and organic loading rate (directly proportional). The even to odd numbered VFA ratio is 44.91, 43.87 at pH 6.0, 7.0, respectively, with a better VFA/SCOD of 0.43 at pH 7.0 (Zhang et al., 2014). This ratio plays a vital role in the composition and physicochemical properties of PHA produced. However, some studies point out the influence of sludge composition on the VFA profile. A dewatered sludge with a comparatively higher proportion of protein (compared to the reported) fermented without pH control (varied between 6.8-7.1) at 55 ˚C produced a valeric acid dominant sludge hydrolysate (Hao et al., 2017). A pilot-scale investigation for the batch fermentation of thickened waste sludge initiated at a municipal wastewater treatment plant produced a hydrolysate with a higher VFA content of 9.4 $gCOD_{VFA}$/L, a yield of 0.9 $gCOD_{VFA}$/ $gCOD_{sol}$, and a higher proportion of acetate and butyrate at 42 ˚C and pH at 5.0-6.5 (Morgan-Sagastume et al., 2015). Thus, all these parameters have varying effects on the WAS properties and hydrolyzate characteristics, respectively, to drive the carbon consumption towards PHA.

Investigations on primary sludge utilization as a carbon source for PHA production are relatively less. Pittmann and Steinmetz (Pittmann and Steinmetz, 2013) found that a semicontinuous fermentation of PS at a pH 6.0, the temperature of 30 °C, and retention time of 4 d produced a higher VFA mass flow rate 1.9 gVFA/L*d. An increase in propionate and a decrease in acetate resulted in ascending pH 6.0-8.0, while both got reduced at pH 10.0. However, butyrate contributed the least. However, diluting primary sludge (70 %) with food waste (30 %) at pH 10.0 led to higher acetate content (76 %) and caproic acid content (43.5 %) without pH control (Perez-Zabaleta et al., 2021). Similarly, another method employed for modifying the acidogenesis of WAS or PS is high pressure thermal hydrolysis (HPTH). In the HPTH, pre-dewatered sludge is fed with steam at high pressure of 6-12 bar and heated to 155-175 ˚C in the hydrolytic reactor. The pretreated sludge is subsequently cooled to 100 ˚C and then to 35 ˚C in the flash tank and heat exchanger, respectively, before passing into the acidogenic fermenter. Due to thermal hydrolysis,

Table 1.1: Pretreatment of waste feedstocks through acidogenic fermentation (1st stage) to improve volatile fatty acid content for PHA production from enriched activated sludge.

Waste residue	Organic content (gCOD/L)	Organic loading rate (gCOD/L *d)	T (°C)	pH	HRT (d)	SRT (d)	DA %	VFA Conc. (gCOD/ L)	VFA profile	Reference
Sugar Cane Molasses	220[d]	0.34[b]	30	6	0.42	n.a	70[b]	0.24[c]	60/9/25/6	(Albuquerque et al., 2011)
	n.a	16.3	37	6	1	2.3	80	13.2	24/34/16/ 24-0/0/2	(Duque et al., 2014)
	220[d]	0.34[b]	30	6	0.42	n.a	70 [b]	n.a	HAc:HPr :HBu:HVa	(Oehmen et al., 2014)
	336	n.a	35	4.8	0.4	n.a	71	n.a	HAc:HPr :HBu:HVa	(Chen et al., 2017)
Cheese Whey	n.a	13.7 13.5	37	6	1	2.6 2.9	65- 74	9.7 10.6	61/13/12/ 1-4/0/8 65/9/13/2 -1/0/9	(Duque et al., 2014)
	n.a	n.a	n.a	6	8-12	n.a	95- 99[a]	31-32	44/2/50/1 -0/3	(Valentino et al., 2015)
	n.a	n.a	37	5.5	4	4	40[b] 60[b]	n.a	16/26-58 58/19/13/ 6-4	(Colombo et al., 2016)
	n.a	n.a	30	6	7	7	n.a	40	35/4/49/4 -0/8	(Janarthanan et al., 2016)
	n.a	15[d]	30	6	1	3	56	0.29[c]	52/9/15/5 -8/14	(Oliveira et al., 2017)
	83.3 136.2	15.2	n.a	5.5- 5.8	2	n.a	n.a	0.20 [c] 0.17 [c]	55/0/36-7 48/0/49	(Colombo et al., 2019)
Brewery Wastewater	n.a	3.3	37	6.2	1.4	15	60	n.a	69/14/9/8	(Ben et al., 2016)
	n.a	n.a	n.a	n.a	n.a	n.a	77	3.9	48/31/6.2 /11/0.8/0. 5-0/2.5	(Tamang et al., 2019)
Candy Factory	7.8 (avg.)	n.a	40	4.5	0.17-4	4	64	n.a	32/14/33/ 5	(Tamis et al., 2014)
Wood Mill Effluent	11.11	5.6 2.9	30	5.5	1 1.5	n.a	42	n.a	67/28/2/0 51/39/7/3	(Ben et al., 2011)
Paper Mill Effluent	26.5	n.a	15- 25	6	12 21	12 21	60 64[a]	6.7 8.9	60/26/5- 0/9 40/40/17- 0/3	(Jiang et al., 2012)
	n.a	n.a	30	5.0	0.125	n.c	78	≈6	HAc:HPr :HBu:HVa	(Tamis et al., 2018)
Hardwood sulfite spent liquor	267	11.8	30	n.c	1.76	1.76	28	5.5	53/22/19- 5.7	(Pereira et al., 2020)
Paperboard mill wastewater	0.92	1.5	n.c	n.c	0.75	n.a	n.a	0.25	54.1/3.3/ 38.7-3.8	(Farghaly et al., 2017)

Olive Mill Effluent	52.7	n.a	25	7	30	30	56	15.5	43/21/20	(Campanari et al., 2017)
	13.2	11.7	30	5.2	1.13	n.a	19	1.6	7.5/2.5/85	(Ntaikou et al., 2014)
Palm Oil Mill Effluent	65	n.a	28-30	n.c	n.a	7	n.a	5-8	n.a	(Salmiati et al., 2007)
Crude glycerol	14.7	10[h]	37	5	0.5-4.8	n.a	n.a	11.6	3/12/24/61[†]	(Burniol-Figols et al., 2018)
Bio-Oil	742	1[d]	30	5.5	2	2	n.a	0.17[c]	37/19/43	(Fidalgo et al., 2014)
Mussels Cooking Wastewater	n.a	1.48	37	4.6	6.25	6.25	43	0.72	n.a	(Roibás-rozas et al., 2021)
Dairy Manure	n.a	8.8[f]	22-25	n.a	4	4	7	5.1	47/24/19/9-0/0/1	(Coats et al., 2016)
Composite Food Waste	5150	17.6	30	n.c	n.a	n.a	57[a]	4.6	51/21/23/2/3	(Amulya et al., 2015)
Olive pomace	n.a	15	20-25	n.a	4	4	13	1.9	HAc:HPr:HBu:HVa	(Waller et al., 2012)
Organic Fraction of Municipal Solid Waste	n.a	44 (Kg) 42.7 43.7	55	n.a	21	21	6[i] 15[i] 14[i]	7.5[h] 7.8 5.4	39.8/40.3/15.3/0/0 37.1/46.1/10.2/2.7/2.2 45/20/29.4/2.8/1.1/1.7	(Colombo et al., 2017)
	n.a	n.a	n.c	n.a	7	7	52[a]	6.2	73/19.4/8.1	(Korkakaki et al., 2016a)
	47[i]	6[f]	55	n.a	20	n.a	6[i]	9.9	11/89	(Papa et al., 2020)
Excess Sludge	28[g]	n.a	42	n.c	4-5	4-5	0.13-0.22	6-9.4	28-38/13-23/15-26/4-11/6-9/12-18-0/0/1	(Morgan-Sagastume et al., 2015)
	n.a	23.8[e] 13.5[e]	40 50	n.c	2	2	55 65	4.9 3.9	50/16/14/1/12/6 42/13/15/2/8/20	(Jia et al., 2014)
	8.9[g]	n.a	55	n.c	1	2.5	n.a	4.3	51/10/9/8/1/8/22	(Hao and Wang, 2015)
	2.7[e]		30	10.0	1	1	62	1	46.1/31.5/20/2.4	(Liu et al., 2020)
Organic Fraction of Municipal Solid Waste +Excess sludge	51[i]	12-15[f]	37	n.c	6	6	86	31	24-34/10-20/30-40/0/0/0-0/25-35	(Moretto et al., 2020)
	n.a	20[f]	55	n.a	3.3	n.a	73	20	n.a	(Valentino et al., 2020)

Primary sludge	43 (TS)	n.a	30	6	4	4	15	7.6[h]	49/38/13	(Pittmann and Steinmetz, 2013)
Dewatered Sludge + Food Waste	n.a	4.2[e]	35	9	9	9	61	25.9	46/10/33/11	(Chen et al., 2013)
	n.a	4 (gVSS/L*d)	35	6.5	8	8	90	18.1 (gVFA/gCOD)	2.5 (even to odd ratio)	(Zhang et al., 2014)
	n.a	n.a	n.a	n.c 10	n.a	n.a	n.a	n.a	31/0/19/0/0/6.5-0/43.5 76/16/3.3/0.2/0/3.8-0/0.2	(Perez-Zabaleta et al., 2021)
Pimary sludge+excess sludge	42.5[g]	10.6-11.5[f]	35	n.c	n.a	4	22	9.6	28/16/30/26	(Zhang et al., 2019)
	59[g] 49[g]	20 30	42	n.c	1 2	1 2	50[a] 60[a]	19.9 18.8	27/17/23/5/4/17-0/7 32/17/18/8/9/14-0/2	(Morgan-Sagastume et al., 2010)

: HAc:HPr:HBu:HVa: iHBu:iHVa-HLa/HCa/EtOH- %w/w or % mol/mol or COD/COD basis, n.c : not controlled, n.a: not available, †: 1,3-propanediol , a: /$COD_{effluent}$ or gVS, b: C-mol /C-mol, c: C-mol/L, d: g sugar /L e: gVSS /L, f: gVS/L*d, g: gVS/L, h: g/L, i: Kg kg−1 $OFMSW_{w.w.}$, Units specifically used are given in brackets, Acetate-HAc, propionate-HPr, butyrate-HBu, isobutyrate: iHBu, Valerate-HVa, isovalerate:iHVa, HLa: lactic acid, HCa: caprolic acid, EtOH: ethanol, DA: Degree of acidification

the biomass breaks down, leading to an increase in the SCOD that can boost biogas production by 50-100 %. Cambri™ and Exelys™ are the two of the largest commercial HPTH process that can treat a combined 1 million tonnes annually. In most processes, the substrate used was a mixture of PS and WAS. Fermentation of pretreated sludges resulted in a 2-6 fold increase in VFA yield and VFA production rate compared to raw sludge with a shift in SCOD: COD ratio from 0.04-0.4. The pretreated sludge's product spectrum depended on sludge's composition than the operating condition (Morgan-sagastume et al., 2011). But the VFA yield of pretreated sludge was 44.6 % (0.22 gVFA/gVS) higher than that of raw sludge when semi-continuously acidified at 35 ˚C (mesophilic). Meanwhile, the fermentation at 55 ˚C (thermophilic) led to a 15.7 % improvement in the VFA yield for the raw sludge and an inhibitory effect on the pretreated sludge (Zhang et al., 2019). The fermentation of thermally hydrolyzed sludge produced acetate followed by butyrate dominated fermentate with higher valerate content.

Many studies exploited the dual capacity of acidogenic fermentation, H_2, and PHA production. The acidogenic effluent from hydrogen production in a periodic discontinuous (sequencing) batch reactor using composite food waste with VFA content of 4g/L which was used for sludge enrichment and subsequent PHA accumulation (Amulya et al., 2015). The effect of HRT (7.5-60 h) on simultaneous H_2 and PHA production was investigated with OME in a mesophilic-continuous acidification reactor. VFA yield and propionate content increased with HRT, but a lower HRT of 14.5 h and 24 h resulted in higher acetate and butyrate yield and better H_2 yield, respectively (Ntaikou et al., 2009).

Another study investigated the interactive effect of pretreatment, dilution ratio, pH, and carbon to nitrogen ratio (C/N) on the anaerobic digestion for enhancing multistage OME valorization. Pretreatment by centrifugation of the pH adjusted OME (7.0 or 9.5 with CaO) had no significant effect on the OME conversion rate and yield. At the highest dilution (1:5.2), the VFA content was higher (6.8 gCOD/L) for the fermentation with uncentrifuged OMW than with centrifuged. Meanwhile, the lowest dilution (1:1.6) had the highest VFA content for both pretreatments (indicating no significant inhibition), although the conversion yield is lower. However, no substantial influence on acidogenesis was observed with uncentrifuged OME (1:1.6) and increasing pH (7.0-9.5) and C/N ratio (100:0.2-100:1.6). At the same time, OME acidification at 35 °C and 55 °C resulted in higher VFA content (19 gCOD/L) and conversion yield (57 %) compared to 25 °C, but with high instability (Campanari et al., 2017). The potential of olive pomace, left out after oil extraction for PHA production was also explored through pomace pretreatment and varying the sequential batch fermentation parameters. Pretreatment through buffering the feedstock to pH 10.0-10.5 before addition to fermenter derived better organic acid production compared to other pretreatment methods (centrifugation with OMW, combined centrifugation-buffering) and a 76 % increase in production compared to untreated pomace. The influence of HRT was prominent over all the tested pomace concentrations. An HRT of 4 d and 75 g/L pomace had the highest pomace conversion yield of 0.0147 g organic acid/ g pomace (Waller et al., 2012)

Anaerobic digestion of palm oil mill effluent in a fixed bed reactor with palm fiber as bed material revealed the effect of SRT on VFA production. The acidification increased the VFA content till SRT of 6 d to about 10.1 gCOD/L followed by a decline at 7 d SRT. Methanogenesis prevailed at SRT ≥7 d (Salmiati et al., 2007). Another major effluent investigated for the AF-ADF route is paper mill effluent. The effluent is nutrient-deficient wastewater that requires nitrogen and phosphorus sources to support fermentation and subsequent PHA production. Therefore, a study acidified paper mill effluent under nitrogen excess and limited condition without using any inoculum at pH 6. The acidogenic fermentation was faster in excess condition than limited condition, notwithstanding a similar 60 % acidification. The fermentate's VFA composition was butyrate >acetate > propionate for both the condition with acetate and propionate produced more in ammonia limitation. Hence, the fermentate's nutrient level needs to be set such that the PHA accumulation remains unaffected (Jiang et al., 2012). Similarly, Wood mill effluent generated from fiberboard manufacturing contains high COD with low biodegradability (BOD/COD: 0.16-

3.6) and nutrients. Continuous anaerobic fermentation of wood mill effluent at pH 5.5 and two HRTs of 1 d and 1.5 d revealed no significant influence over VFA content. Even then, the degree of acidification was higher at 1.5 d (43 %) than 1 d (37 %). Acetate and propionate were the major VFAs in the wood mill effluent fermentate. Butyrate and valerate increase with HRT at the expense of the other two. The low output from wood mill effluent points towards pretreatment before acidogenic fermentation (Ben et al., 2011).

Sugar cane molasses, the major industrial byproduct employed, contains 54 % (w/w cane molasses) of total sugars, which is comprised primarily of sucrose (62 %) and fructose (38 %). Molasses was fermented in a continuous acidification reactor by varying pH (5.0,6.0,7.0) and C/N ratio (16, 33). pH failed to show any significant influence over the molasses conversion yield and specific productivity. Acetate was the dominant VFA produced in all the pH evaluated. Acetate and propionate dominated at pH 7.0, while butyrate and valerate increased at pH 5.0. The higher reducing equivalence available at lower pH might have catered to converting sugars into longer chain fatty acids. A C/N ratio of 33 left low residual nitrogen (0.1 Nmmol/L**)**, while the much higher residual nitrogen (10-12 Nmmol/L) was observed at a C/N:16. The influence of C/N ratio change on the VFA profile was insignificant, while yield and productivity got depreciated (Albuquerque et al., 2007).

Cheese whey is the dairy industry's primary waste product produced from precipitation and draining of milk casein amid cheese making. A recent study carried out the fermentation using the raw cheese whey alone, or the substrate is sterilized and mixed with sporogenous acidogenic mix culture at 37 ˚C and pH 5.5. The fermentation of raw cheese whey alone produced lactate, acetate, and butyrate. In contrast, acetate, propionate, and butyrate (in the order) occupied the major share of dominant VFAs in the presence of mix culture. Fermentation of cheese whey with sporogenous acidogenic culture possessed a much higher conversion yield than raw substrate alone (Colombo et al., 2016). An additional step of enzymatic hydrolysis of cheese whey or whey permeate converting lactose into fermentable sugars can further enhance the fermentation process (Colombo et al., 2019). Also, cheese whey having a relatively complex matrix gave proper response with mix culture under a feedstock shift from cheese whey to sugar cane molasses and then back to cheese whey. The fermentation system reacted immediately, reaching stable production within 3 SRTs (9 days) when the feedstock shifted from cheese whey to sugar cane molasses. Similarly, the system was able to counteract the shift from molasses to cheese whey with stable fermentation within 12 days. The fermentation content was higher for molasses than cheese whey (13.2 and 9.7–10.6 gCOD-FP/ L, respectively). This was due to the difference in the feedstock matrix and the availability of nutrients. But the conversion yields for both substrates were similar (0.8, 0.65, and 0.75 gCOD-FP/ gCOD sug.). Cheese whey fermentate was dominated by acetate, followed by propionate and butyrate. On the contrary, propionate, valerate, acetate, and butyrate in order prevailed in molasses fermentate. Thus acidogenic fermentation is quite stable to feedstock shifts in terms of fermentation products of the particular feedstock, assuring stable feed composition for PHA production (Duque et al., 2014).

The organic fraction of municipal waste treatment through leaching generates leachate with high organic content. In an OFMSW to PHA study, the leachate directed to an upflow anaerobic sludge blanket (UASB) ensures organic content removal through biogas production. The leachate recirculation supplies anaerobic mix culture for acidification of the OFMSW bed in the hydrolytic and acidogenic tunnel maintained at SRT 7 days. This leachate had a conversion yield >50 %, with acetate and propionate as the principal acids. The leachate was pretreated to remove phosphate by continuous aeration for 5 h to control the leachate's nutrient level, necessary for PHA accumulation. Continuous aeration ensured CO_2 stripping, an increase in pH to 9, and 90 % phosphate removal without any substantial loss of SCOD (Korkakaki et al., 2016a). Another attempt used the leachate from the continuous irrigation of OFMSW with digestate from an anaerobic continuous stirred tank reactor (CSTR) in a continuous anaerobic percolation biocell reactor (APBR). Three trials with different OFMSW/digestate ratios (adjusting the digestate flow rate) lasting 21 days generated percolate/leachate. The collected percolate was pretreated by centrifugation and ammonia stripping. The total organic acid content increased with the digestate flow rate, maximum (7831mg/L) being at the ratio of 1:0.9 kg/kg (digestate flow rate 0.04 L/h kg $OFMSW_{w.w}$). Further increase in the digestate flow rate resulted in a decrease in organic acid content. Meanwhile, pretreatment reduced the suspended organic content in the percolate and C/N ratio to 10. All the pretreated percolates were diluted and adjusted to the requirement of enrichment and accumulation. In all the pretreated percolates, even-numbered acids dominate over odd-numbered acids, (65.1/34.9) highest at ratio 1:0.9-1.8 kg/kg (Colombo et al., 2017). Tamis et al. (Tamis et al., 2014) tried to utilize candy factory effluent as feedstock for PHA production through two-step acidification. The effluent was first fed into a UASB, and the outlet of the UASB was the inlet of a second anaerobic liquid tank, both maintained at pH 4.5, 30-40 $^{\circ}$C, HRT of 4 h, and SRT of 4 days. The first stage had significant production of ethanol and VFA. In contrast, the ethanol got converted to mostly butyrate in the second stage resulting in an overall VFA/SCOD of 0.64 gCOD/gCOD with acetate and butyrate as major components.

Altering the anaerobic inoculum is one of the least discussed areas for influencing PHA production through acidogenesis. Some investigations combined inoculum pretreatment with other process parameters to improve acidogenesis (Zhang et al., 2019)(Colombo et al., 2016). Pretreatment of anaerobic inoculum effectively inhibits methanogens, enhances the activity of acidogens, and accumulates VFAs. Pretreatment of inoculum concentrates on enriching the anaerobic sludge with hydrogen-producing mix cultures. Higher production of acetate is a property of hydrogen production that can aid in downstream PHA production. Selecting the right combination of seed anaerobic sludge and waste feedstock can deliver fermentate-rich even-numbered VFA (higher even to odd ratio) that can influence PHA composition (Colombo et al., 2019). In one such study, heat-shock pretreated anaerobic inoculum fermented crude glycerol resulting in butyrate and 1,3-propanediol dominated the fermentate.

The pretreatment and subsequent enrichment might have led to the emergence of 1,3-propanediol producing bacteria in the mixed culture when glycerol was available as a carbon source. The fermentation product's effect on PHA culture enrichment and accumulation was further studied (Burniol-Figols et al., 2018). The consequence of these processes on the interaction between seed inoculum and waste residue (substrate) decides the fermentative outcomes. PHA production through waste residues can be tuned through the optimization of these processes for effective carbon recovery. The effects of different inoculum pretreatment on PHA production are yet to be understood. The acidification performance of other kinds of waste residues remains a potential area for the investigation to expand the horizon of opportunity for efficient waste management through PHA production. Table 1.1 elaborates on the detailed operational parameters and outcomes of acidogenic fermentation of different feedstocks.

1.4.2. Enrichment of activated sludge with PHA accumulating mix culture

A broad spectrum of microbial fauna exhibit a basal level of PHA production. In the absence of an external carbon source, microbes utilize storage for their growth and maintenance. Activated sludge contains a variety of microbial communities from which PHA competent culture needs to be enriched. Aerobic dynamic feeding converges loose clusters of growth-oriented microbial communities into a restricted assemblage of storage-oriented communities. The mechanism of ADF is to expose MMC to a short period of excess carbon availability (feast) followed by an extended carbon starvation period (famine). MMC adapts, survives, and grows in this cyclic feast-famine process that causes a shift in the metabolic pathway where more carbon is diverted towards storage to survive famine. As shown in Fig. 1.3a,b, carbon and nutrient consumption with simultaneous PHA storage defines the feast phase, while consumption of stored PHA and remaining nutrient takes place during the famine phase to drive cellular maintenance and growth. The sequential batch reactor operation (SBR) ensures repeated exposure to the feast-famine cycle with daily replenishment of carbon and nutrient, thereby accelerating and cementing the effect of the selective pressure. Thus, a community with a competitive advantage with higher and faster accumulation develops that edges out less competent ones or non-accumulator. This enriched sludge with PHA storing phenotype is used to maximize PHA production (Serafim et al., 2004).

Parameters like OLR, pH, temperature, substrate, feast/famine ratio, etc., direct the top competitors to overcome other communities (Table 1.2). Many species dominate the enrichment system that can direct fermented VFA towards maximum PHA production depending on conditions persisting during enrichment and accumulation (Morgan-Sagastume, 2016).

The principle of ADF is to impose a growth limitation via the feast-famine cycle, nutrient availability, SRT, etc. The growth limitation results in reduced synthesis of growth-related proteins, enzymes that restrict the movement of acetyl-CoA into the TCA cycle (tricarboxylic acid) and improve the flux towards the PHA synthesis pathway. Fermented waste residue supplies acetyl-CoA for PHA production primarily through the β-oxidation pathway and other catabolic pathways. Thus, the available CoASH reduces due to the formation of acetyl-CoA from the supplied VFAs/fermented waste residue, thereby uplifting the inhibition of the PHA synthesis pathway imposed by CoASH. Also, the increased acetyl-CoA coupled with growth-limitation inhibits pantothenate kinase activity inhibiting CoASH synthesis. The improved PHA synthesis generates NADPH (Nicotinamide adenine dinucleotide phosphate) by the reduction of acetoacetyl-CoA to 3-hydroxybutyryl-CoA by NADPH:acetoacetyl-CoA reductase. NADPH and NADH inhibit citrate synthase, further reducing the TCA cycle activity (Kessler and Witholt, 2001),(Tan et al., 2014), (Vallari et al., 1987).

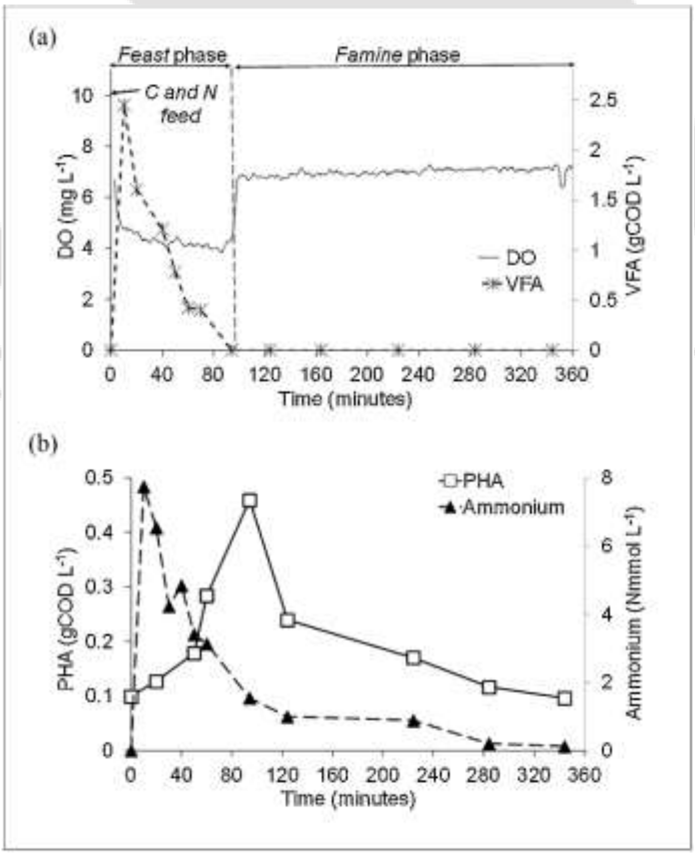


Figure 1.3: The mechanism of a aerobic dynamic feeding strategy during (a) coupled carbon-nitrogen addition (conventional), (b) the subsequent PHA storage response (Silva et al., 2017). Reused with permission from Elsevier.

1.4.2.1. Inoculum

The dependence of PAP of activated sludge on the inoculum is yet to be adequately understood. The sludge collected from different unit operations from effluent treatment plants showed the reliance of origin on PHA accumulation under nitrogen-limited conditions without enrichment (Sakai et al., 2015). In feast/famine enrichment, higher PHA storage ensures better survival of a microbial community. This selective pressure excludes more non-accumulators and accumulators with lower storage. Thereby, the robustness of ADF depending on the top competitors' turnover and not on the stability of accumulating microbial community structure (Huang et al., 2018). Janarthanan et al. (Janarthanan et al., 2016) had noticed a reciprocal shift in community structure existing throughout ADF operation. A 63-day enrichment produced a shift between dominant species *Flavisolibacter and Zoogloea* with a stable PHA production profile. During the initial acclimatization phase, the relative abundance of top competitors selected by the feast/famine cycle indicates the PHA level. The top competitors largely converged and stabilized during the maturation phase, where the PHA fluctuation got reduced. Moreover, occasional turnover happens when the top competitors lose the edge and other potential accumulators fill the gap. This dynamic shift ensures high process stability and a higher PHA level (Huang et al., 2018). Thus, feast/famine enrichment imparts strong selective pressure on the MMCs in activated sludge from entirely different origins that can lead to a similar PHA storage response. Besides, initial biomass concentration's influence over storage performance was insignificant even though higher concentration caused unstable physical status (Chen et al., 2017).

1.4.2.2. Dissolved oxygen

Dissolved oxygen (DO) is an absolute requirement for the growth of microorganisms, indicating the real-time state of biomass growth. In the ADF, DO decreases during the feast phase due to active microbial growth. The downregulation of cellular metabolism prompted by the exhaustion of external carbon accompanied by the onset of internal carbon consumption results in the decline of growth with a concomitant increase in DO during the famine phase (Salmiati et al., 2007). However, varying the initial DO level had no significant influence over the enrichment process, given the oxygen starvation is avoided. A higher and lower DO had enriched MMC with high volumetric and kinetic PHA production (Wang et al., 2017). However, the possibility of a shift in the metabolic route during feast/famine enrichment under oxygen limitation is possible for particular substrates. Enrichment with glycerol diverted the metabolism towards polyglucose rather than PHA under oxygen limitation (Moralejo-Gárate et al., 2013). Similarly, the influence of aeration rate on the enrichment process with fermented dairy manure also showed no statistical relevance. Lower aeration rates had less resolution for the feast/famine pattern. ADF at higher aeration rates was related to carbon cycling, while nitrogen cycling sustains the enrichment under lower aeration (Coats et al., 2016). Earlier, the possibility of PHA enrichment with bulking sludge induced in activated sludge by providing low DO (1 mg/L in feast phase) got suggested. The bulking sludge enriched the MMC with a yield of 0.4 Cmol PHA/Cmol Ac (Wen et al., 2012). Therefore, DO level and aeration rates need to standardization according to the microbial growth and other operational condition with particular attention to avoid any oxygen limitation during feast-famine enrichment.

1.4.2.3. Feast to famine ratio

Feast to famine ratio (F/F) is considered as a boundary condition for enrichment. Microbial growth or optimal metabolic functioning and PHA storage occur during the feast phase, ensuring shorter feast length and prepares MMC for survival during the famine phase. The stored PHA degraded during the famine phase ensures the survival, growth, and maintenance of the MMC. The feast phase leads to a decline in storage unless the famine phase's length gets prolonged enough to induce growth limitation (Reis et al., 2011). An extended feast phase promotes growth over accumulation, leading to the domination of non-accumulators. Therefore, an F/F ratio of <0.33 is required to ensure the implementation of a strong ecological selective pressure (Hao et al., 2018). The F/F ratio can be controlled by either tuning the organic load or microbial growth. A nutrient stressed and uncontrolled pH condition led to growth limitation that prevented biomass concentration from increasing during enrichment with fermented molasses. Thus organic load becomes the determinant for the F/F ratio. Low biomass at higher substrate concentration reduces substrate uptake rate extending the feast phase with subsequent loss of internal growth limitation. Hence critical substrate concentration is required (Albuquerque et al., 2010). A higher F/F ratio of 0.5 enriched an effective PHA accumulating culture for a valerate-dominated hydrolysate due to the sequential adaptation of valerate-consuming culture under high organic loading (Hao et al., 2018).

Under a constant OLR, nutrient availability could tune the microbial growth and the F/F ratio. The F/F ratio remained below 0.4 under the nitrogen excess condition due to a higher biomass growth rate (0.96 g/L d), resulting in 17 % storage. But an increase in OLR shifts the F/F ratio higher, leading to reduced storage (10 %). Increasing cycle length will reduce the OLR with the subsequent reduction in accumulation. Thus, uncoupling carbon and nitrogen availability came up as a solution to enrich PHA accumulating culture under a high F/F ratio (Oliveira et al., 2017). Nitrogen limitation also lowers F/F for a given OLR. The competition between MMCs for faster uptake of nitrogen available in a limited quantity results in a higher substrate uptake rate. The PHA storage also gets consumed to maintain the uptake rate. In this way, nitrogen limiting condition can diminish the PAP of sludge when exposed for an extended period (Johnson et al., 2010b). Aerobic Dynamic Discharge (ADD) is an extension of ADF comprising two settling and withdrawal per cycle with the additional one at the end of the feast phase by replacing half the supernatant with carbon starved feed to induce famine. ADD enriched biomass had a PHA yield of 0.77 C molPHA/C molAc while ADF was 0.50 CmolPHA/C molAc after 30 days of operation with a 4 cycle/day (Chen et al., 2015a). Therefore, the F/F ratio is a critical factor for improving the PAP of activated sludge. A lower F/F ratio, coupled with other parameters, could elicit substantial selective pressure to derive higher PHA storing MMC.

1.4.2.4. Organic Load

The carbon supplied contributes to the enrichment process's efficiency besides VFAs implementing an additional selective pressure by coercing the microbial community to divert the extra carbon towards PHA production due to overlapping pathways and uplifting of inhibition (section 1.3). At low OLR, MMC readily removes substrate to storage and growth with smaller feast phase, and they will be further favored becoming predominant. High OLR (31.25 gCOD/L d) during the start-up phase imposes a competition for substrate consumption. Thereby biomass growth prevailed as a means to utilize substrate faster with less diversion for the slower storage. But the high OLR and consequent weaker storage conversion stabilized the enrichment on a longer feast phase. Prolonged exposure to shorter famine reduces the kinetic pressure leading to declined PHA yield and productivity. So neither biomass with higher storage response dominates nor other communities are coerced to increase their storage at higher OLR (Albuquerque et al., 2010). Campanari et al. (Campanari et al., 2014) also found the maximum storage response (0.5 gCOD/gCOD) to be at a moderate OLR (4.70 gCOD/Ld) than either the higher or lower OLR (8.4 or 0.24 gCOD/Ld) for MMC enrichment with fermented OME. Albuquerque et al. (Albuquerque et al., 2010) detected growth limitation at a high organic load of 60 Cmmol/L of fermented molasses, which lead to a lower storage capacity of MMC. Struvite precipitation of magnesium, ammonia, and phosphate at the high organic load added to the growth-limiting condition. This leads to lower biomass growth and PHA storage due to a higher F/F ratio in the range of 0.53-1.1 that enriched MMC with lower storage response. However, a moderate organic load of 45 Cmmol/L enriched activated sludge with better PAP as neither substrate nor F/F ratio was a limiting factor. Uncoupling the carbon and nitrogen availability was recently proposed to be a potential route to enrich culture with a better storage response than conventional ADF at high OLR, 8.5 gCOD/Ld (Oliveira et al., 2017). Extending the cycle length can also balance the impact of organic load on the F/F ratio. The cycle length (HRT also) was 4 times that of the feast phase at each organic loading (1-6 g/L) until a steady-state for enriching a PHA storing MMCs using valerate-dominated hydrolysate was achieved (Hao et al., 2017). The microbial community also shifts in storage response to OLR. The PHA yield corresponding to two OLR analyzed had a relative abundance of *Azoarcus, Thauera, and Paracoccus*. A moderate OLR incremented the population of *Azoracus*, resulting in better performance while a relative increase of weaker accumulator, *Thauera*, and their flanking population dominated at higher OLR (Carvalho et al., 2014).

Similarly, a *Lampropedia hyaline* dominated culture enriched under a high organic loading rate and 1 d residence time showed better PHA productivity during batch accumulation tests. Increasing the feeding rate also had improved the storage response (Valentino et al., 2014). Besides, a continuous supply of carbon can also improve the PHA storage of activated sludge. Dosing half of the carbon at the start of the cycle and the rest by continuous feeding (throughout the cycle) at 100 mL/d rate and 2 times per day feed frequency resulted in high PHB accumulation and domination of *Plasticicumulans acidivorans,* similar to conventional feast/famine enrichment. This improvement in storage indicates the necessity of a true feast phase over a true famine phase (Marang et al., 2018).

The VFA content and composition also regulate the effect of organic loading on storage response. Surplus VFA under a higher organic loading rate is an additional upregulation of the PHA synthesis pathway. Moreover, the acetate-propionate enriched culture had better substrate uptake, PHA yield, and HV fraction than acetate enriched (Wang et al., 2013). The VFA composition also selectively enriches the microbes that could readily metabolize the VFA present in the fermented waste residue. *Thauera* genera had clear domination in a feast/famine reactor fed with propionic acid, while *Paracoccus* and *Comamonadaceae* strain were the top competitors with acetic acid and butyric acid (Huang et al., 2018). Similarly, *Thauera* and *Azoarcus* prevailed for acetate and butyrate as the major VFA, while *Paracoccus* had a broader range of substrate for consumption with a higher specific uptake rate (Albuquerque et al., 2012). Segregated flux balance analysis of fermented molasses also observed a similar correlation. The study found higher VFA uptake and HB content associated with *Azoarcus* than *Paracoccus* related to higher HV production flux. *Thauera* had a slower substrate uptake rate but better biomass growth, especially with butyric acid. The VFA profile and the associated production fluxes of each community could predict HB:HV content (Pardelha et al., 2013). The selection of specific PHA accumulators could also influence the PHA storage as well as the operational stabilities. A higher abundance of *Thauera* genera affected the floc formation due to the hydrophilic saccharides formation leading to lower physical properties of the culture (Huang et al., 2018). A feedstock shift also affects storage response depending on the acidogenic fermentation outcomes. However, a pseudo steady-state established after each shift provides efficient performance in each fermentate (Duque et al., 2014).

Hence it is necessary to adjust parameters with respect to organic loading to enrich MMC with required biomass growth and substantial storage response.

1.4.2.5. Temperature

The temperature is a specific parameter that also impacts microbial community structure during enrichment. Long term temperature change has a better impact than short term changes. Long term exposure allows the selection of particular MMCs adapted to that temperature, while short term exposure can induce instability. Long term exposure to a higher temperature of 30 °C drove the culture towards PHB storage, while a lower temperature of 15 °C directed the carbon source towards biomass yield. This impact was due to the difference in the acetate uptakes at 30 °C (higher) and 15 °C (lower) (Johnson et al., 2010a). Similarly, in the enrichment of mix culture at 20 °C and 30 °C with sodium acetate as carbon source, *Zoogloea* spp. and *Plasticicumulans acidivorans*, respectively, dominated with an accumulation of ≥75 % (Jiang et al., 2010). Therefore, a longer-term temperature change reciprocates stable performance with higher or lower response respective to the temperature. In contrast, a short temperature change had elicited an unstable response caused by the incomplete PHB degradation for *P.acidivorans* at 20 °C leading to limited biomass growth, allowing *Zoogloea* spp. to outcompete (Jiang et al., 2010). Similarly, a short-term increase in temperature (1 cycle) of steady-state SBR from 20 °C to 30 °C did not affect biomass growth or PHB consumption during the famine. The reaction rates got negatively influenced during the feast phase without affecting maximum PHB storage due to sudden metabolic disturbances (Johnson et al., 2010a). A sequential enrichment with starch industry wastewater observed the PHA production to vary within a range related to the seasonal temperature fluctuation without loss in performance. Nonetheless, lower temperatures could more efficiently carry out PHA production through higher substrate consumption (De Grazia et al., 2017). Therefore, an ADF enrichment system can perform optimally under longer-seasonal temperature changes without much impact from sudden temperature changes. An ADF enrichment reactor integrated into municipal wastewater treatment had improved the storage capacity under an expected seasonal variation of 8.4 °C to 22.8 °C (Morgan-Sagastume et al., 2015).

1.4.2.6. Sludge retention time

Sludge retention time selects MMC, which could swiftly replicate and replace the lost amount due to sludge wasting within the stipulated time under the bioprocess conditions. In other words, SRT separates cultures based on fast growers or slow growers corresponding to the applied selective pressure. SRT can be kept constant by wasting a calculated volume of sludge by considering the reactor's biomass concentration and discharge (Chen et al., 2017). Under short SRT and feast-

Table 1.2: Bioprocess control and storage response of sequential batch enrichment of activated sludge through aerobic dynamic feeding (2nd stage) for PHA production from fermented waste feedstocks/VFAs.

Carbon Source	Organic loading rate (gCOD/L *d)	CL (h)	T (°C)	pH	HRT (d)	SRT (d)	Nutrient ratio (COD: N:P)#	PHA yield (gCOD/ gCOD)+	PHA storage (gPHA/g VSS)	Reference
Sugar cane molassess	0.045[b]	12	23-25	n.c	1	10	100:8:1	0.70[d]	0.25	(Albuquerque et al., 2010)
	0.057[b]	12	23-25	8.0	1	4	100:10:1	0.67[d]	0.23	(Duque et al., 2014)
	0.09[b]	12	23-25	8.0	1	10	100:8:1	0.73[d]	0.26	(Oehmen et al., 2014)
	2.5	12	20	n.c	1	5	100:6:1	0.8	0.31	(Chen et al., 2017)
Cheese whey	0.054[b]	12	23-25	8	1	4	100:10:1	0.81	0.26	(Duque et al., 2014)
	38[b] 39.7[b]	12	25	8.8	1	5	100:12.3:3 100:19.7:3.9	0.7[d] 0.9[d]	0.3 0.4	(Colombo et al., 2016)
	0.1[b]	12	23-25	n.a	1	4	100:4:0.6	0.72	0.2	(Oliveira et al., 2017)
	1.5	12	25	8.8	1	5	100:9:4	0.8 0.73	0.11 0.11	(Colombo et al., 2019)
Brewery Wastewater	0.068[b]	12	n.c	n.c	1	7	100:3.8	0.35	0.28	(Ben et al., 2011)
Candy Factory effluent	8	12	30	6.5-7.5	1	1	100:4	0.37 (gPHA/COD)	0.4	(Tamis et al., 2014)
Paper mill Effluent	4.5	24	30	7.0	2	2	100:6:1.5	0.80	0.79	(Jiang et al., 2012)
	n.a	12	30	6.6-7.2	1	1	100:3.3	0.4 (gPHA/COD)	0.5	(Tamis et al., 2018)
Hardwood spent sulfite liquor	7	12	n.c	n.c	1	5	n.a	0.42[d]	0.22	(Pereira et al., 2020)
Olive mill Effluent	7	6	25	7.6	0.5	1	100:3	0.2	0.11	(Campanari et al., 2017)
Bio-oil	2	12	20–23	7.2	1	5	100:5:1	0.37[d]	0.07	(Fidalgo et al., 2014)
Crude Glycerol	4.4	12	30	8.0	1	1	100:12:1.5 100:0:1.5	0.42[d]	0.2[e] 0.23[e]	(Burniol-Figols et al., 2018)
Mussels Cooking Wastewater	n.a	12	30	n.c	1	2	n.a	n.a	0.19	(Roibás-rozas et al., 2021)
Dairy manure	0.44	24	22-25	n.a	4	4	100:8.8-22:0.5-2.6	0.75[d]	0.14	(Coats et al., 2016)

Composite food waste	11[a]	12	n.a	8.0	n.a	n.a	100:8:1	0.110	0.16 (gPHA/g DCW)	(Amulya et al., 2015)
Olive Pomace	0.7	n.a	20-25	n.a	1	6	n.a	0.28	0.39	(Waller et al., 2012)
Organic Fraction of Municipal Solid Waste	1.3	12	25	8.8	1	5	100:13:0.4	0.51 0.54 0.42	0.34 0.38 0.23	(Colombo et al., 2017)
	6.8 (10 %) 7.7 (25 %)	12	30	7.0	1	1	100:4.6:1.5 100:7.5:1.1	0.66 0.62	0.39 0.53	(Korkakaki et al., 2016a)
	1	12	22	n.c	2	n.a	100:4.5:9.1	0.1	0.17	(Valencia et al., 2021)
Excess sludge	2.06	96	30	n.c	4	16	100:5.8:0.6	0.077[c]	0.42	(Hao et al., 2017)
Organic Fraction of Municipal Solid Waste +Excess sludge	4	12	25-28	n.c	1	1	100:1.9:1.6	0.46	0.2	(Moretto et al., 2020)
	2-4.4	6	15-34	n.c	1	1	100/3.4/0.6	0.45	0.15	(Valentino et al., 2020)
Municipal sewage [f]	3	2	8.4-22.8	n.a	0.13	1.9-1.7	100:10-12:1.3-1.5	n.a	0.04	(Morgan-Sagastume et al., 2015)
Primary sludge	0.6	24	20	7.0	2	2	≥ 100:3:0.8	0.28	n.a	(Pittmann and Steinmetz, 2014)
Dewatered sludge + food waste	2	12	21.2 21.5	8.0 8.2	1	n.a	n.a	0.22 0.20	n.a	(Perez-Zabaleta et al., 2021)
Pimary sludge+ Excess sludge	6	8	35	n.c	6	6	100:140	0.16[d]	0.21[e]	(Morgan-Sagastume et al., 2010)
	5[a]	96	35	n.a	n.a	n.a	100:7:0.24	0.35	n.a	(Zhang et al., 2019)
Acetate	4.08	11	15-28	9.5	1	10	100:2.9	0.4[d]	0.35	(Ben et al., 2016)
	2.2	12	30	7.5	1	n.a	1	0.65 [d]	0.32	(Tamang et al., 2019)
	1.5	12	n.a	7.0	1	10	100:8:1	0.5[d]	0.67	(Farghaly et al., 2017)

#: % w/w or % mol, + : based on soluble COD or COD_{VFA} consumed, n.a: not available, n.c: not controlled, a: gCOD/L, b: C-mol /L*d , c: $gCOD_{PHA}/gCOD_{Excess\ sludge}$, d: C-mol/C-mol, e: gPHA/gTSS, f: not fermented, g:C-mol PHA/C-mol X*h, CDW: Cell dry weight, CL: cycle length

famine selective pressure, PHA accumulating culture grows fast enough to replace the lost amount. The accumulators accelerate the storage rate during the feast phase with a sustained biomass growth rate during the famine phase. Meanwhile, slower growth of non-accumulators devoid of carbon source to replicate during the famine phase gets washed out quickly. Longer SRT tends to increase the redundant population's biomass concentration, leading to competition for the available substrate. Biomass growth gets promoted to increase the substrate uptake rate, whereby non-accumulators gain the upper hand. The abundant availability of carbon and restricted biomass growth encourages PHA accumulators to store further (Chua et al., 2003). Feast-Famine enrichment using fermented molasses at a short SRT of 5 d reduced microbial diversity, indicating better selective pressure. The kinetic advantage of the weeding effect on PHA accumulators aids higher PAP of activated sludge. Long-term physiological adaptation results in faster substrate uptake towards PHA production, balancing the F/F ratio imparting a stronger selection. In contrast, inert biomass accumulation with weaker storage and quicker growth leads to reduced PAP at a longer SRT of 10 d (Chen et al., 2017). Serafim et al. (Serafim et al., 2004) successfully enriched PHA accumulating culture (37 %) at SRT of 10 d due to the growth limitation imposed by nitrogen limiting conditions. However, a nitrogen limited enrichment under lower SRT eventually loses PAP due to a complete washout. A culture exposed to SRT of 0.5 d to 1 d and carbon limitation preferred biomass growth for faster substrate uptake but resulted in reduced storage due to the higher F/F ratio. Therefore, an SRT of 4 d under a medium C/N ratio could better be equipped for enrichment, inflicting a dual limitation (Johnson et al., 2010b). Jiang et al. (Jiang et al., 2010) proposed longer cycle length at an applied SRT (1 d) could impose stronger selective pressure. The growth limitation reduces biomass concentration due to the extended famine phase and increases the food to the microbe ratio during the feast phase culminating in higher PHA production. So MMC that could maximally utilize the substrate for storage at the start of the cycle could survive the weeding effect. Moreover, longer HRT/SRT (3 d/12 d) enriched sludge with better PHA yield, 0.368 gPHA/ L fermented olive pomace, while shorter retentions (1 d/6 d) derived better storage of 39 % (Waller et al., 2012). Hence, the optimization of SRT considering the operational conditions is outmost necessary to maintain a proper feast-famine cycle and improve the PAP of activated sludge.

1.4.2.7. Cycle length

The duration of a sequential operation also imparts significant influence during ADF. Moreover, the cycle length and volume exchange ratio decide the HRT of a sequential enrichment reactor. HRT becomes a controlling factor that determines the time given to different microbial communities to consume the substrate, reflected in the form of OLR for a given load. Varying the cycle length from 24 h to 12 h resulted in increased PHA production from fermented food waste with a lower feast-famine ratio. The increase in OLR altered the flux towards storage response and growth response resulting lower feast-famine ratio (Amulya et al., 2015). However, some studies adjusted the cycle length or volume exchange ratio to keep the HRT constant while evaluating the respective parameters' impact on the enrichment process. In order to ensure an HRT of 1d, the volume exchange ratio was increased (0.042-0.75 %) along with cycle length (1-18 h). The SBRs with a lower cycle length of 2 and 6 h (feed frequency 12 and 4 times per day, respectively) dominated by *L. hyaline* had a higher substrate removal rate than 8 h (3 times per day feeding frequency), resulting in better storage during batch accumulation. However, Jiang et al. (Jiang et al., 2010) had observed an increment in biomass PHA during enrichment to increase from 5.6 % to 71.3 % with cycle length 1 h to 18 h. A longer cycle length selects a population with higher storage to survive the famine phase, thereby reducing microbial diversity. Further increasing the volume exchange ratio to 0.83 % (20 h cycle length) had a negligible effect on storage response but induced operational instability. A high ratio causes microbes to increase the growth rate to consume external carbon sources, resulting in a larger flanking population (Marang et al., 2016). Hence, a shorter cycle length would require a long enrichment period to establish the proper feast-famine condition. A longer cycle length at a fixed SRT/HRT inflicts substantial selective pressure much quicker.

1.4.2.8. pH

pH is a selection criterion for each microbial community. Exposure to low pH had an adverse effect on the activated sludge process. However, the inherent neutralizing property of aerobic respiration results in a pH shift to a higher value due to bicarbonate formed with CO_2 evolution (Baldwin and Campbell, 2001). Chua et al. (Chua et al., 2003) found similar PHA production abilities of activated sludge at pH 7.0 and 8.0. Enrichment of MMC with a mixture of acetate: propionate resulted in higher PHA production at pH 7.5 than at 9.5 (Villano et al., 2010). For fermented sugar cane molasses, enrichment at pH 8.0 resulted in higher biomass without compromising polymer storage/ specific storage rate compared to pH uncontrolled (<9.0)(Oehmen et al., 2014). The enrichment of the top PHA accumulating communities might reason the undeterred PHA production at different pH analyzed. pH 8.0 increased the count of *Azoarcus* spp., while pH 7.5 gave dominance to *Lampropedia hyaline* (Villano et al., 2010)(Oehmen et al., 2014). Due to this effect, most of the investigations tried to control the pH during enrichment between 7.0-8.0. Recently, Montiel-jarillo et al. (Montiel-jarillo et al., 2017) reported higher PHA storing capacity (36 %) for pH uncontrolled (varying between 8.8-9.0) enrichment than pH-controlled at 7.5 (20 %) even though similar was the substrate uptake rate. Similarly, the storage response of activated sludge improved for fermented food waste initially adjusted to pH 8.0 and left uncontrolled at a 12 h cycle length (Amulya et al., 2015). Therefore, top competitors' turnover at different pH maintains a high and constant PHA storage during feast/famine enrichment when the pH is left uncontrolled. Activated sludge enriched without pH control for longer term selects top competitors adapted to the progressive pH shift. Therefore, an uncontrolled pH enrichment is economical without impacting productivity considering the process control and large-scale implementation of PHA production from activated sludge.

1.4.2.9. Nutrient availability

The optimum COD:N:P for an activated sludge system is at 100:5:1. The requirement of nitrogen for cellular machinery is more than phosphorus leading to faster nitrogen uptake. Tuning nitrogen availability can affect microbial growth more than phosphorus (section 1.4.3.3). Earlier research had depicted the modulation of nitrogen content to elicit a more substantial selective pressure over phosphorus content in elevating the storage response of activated sludge. Enrichment under nitrogen limitation improved storage (59 %) than phosphorus limitation (Wen et al., 2010). So most of the studies stressed the effect of nitrogen availability on culture enrichment and accumulation. The nitrogen limitation is when nitrogen gets completely consumed before carbon is exhausted. Similarly, in nitrogen excess (carbon limitation), nitrogen stays for the entire duration of the cycle while carbon gets consumed swiftly (Johnson et al., 2010b). Strong nitrogen limitation had been shown to increase the storage response of activated sludge with acetate as a carbon source (Suresh Kumar et al., 2004). Earlier research found the ADF enrichment of activated sludge under nitrogen limiting (100:2:2) condition to have a better yield than nitrogen excess (100:12:2) at 0.69 & 0.51 g COD PHA /g COD S, respectively (Basak et al., 2011). In nitrogen limitation, the production of proteins, nucleic acids, other essential enzymes, etc., reduces due to reduced growth, diverting more of acetyl-CoA towards PHA synthesis from the TCA cycle. Prolonged enrichment under nitrogen limitation diminishes the PAP gained during the initial run due to the extended growth limitation resulting in exhausted cell machinery. At short SRT, a low nitrogen amount initiates competition among MMC for faster uptake. Hence, the carbon uptake accelerates along with PHA's degradation to facilitate swifter nitrogen uptake and associated growth. Due to the growth preference, the internal CoASH increases due to the diversion of a substantial fraction of acetyl-CoA towards the Krebs cycle, leading to inhibitory effects on the PHA synthesis pathway and the total loss of storage. Added to this, sludge bulking becomes very prominent under nitrogen limitation that could affect mass transfer (oxygen, carbon, etc.) in the reactor due to excess filamentous growth, loss of settling characteristics, and loss of storage response (Johnson et al., 2010b) . Nitrogen available in the required amount at moderate SRT (4 d) ensures a balance between growth and storage, depending on other parameter influencing feast-famine cycling (Johnson et al., 2010b).

Most of the ADF modes of enrichment chose nitrogen excess over limitation (Valentino et al., 2016). At a fixed SRT, nitrogen excess increases the biomass yield on VFA and better substrate uptake rate resulting in carbon limitation. The feast phase reduces, imparting substantial selective pressure and growth limitation with an extended famine phase (Johnson et al., 2010b). Such dual limitation increases the volumetric PHA productivity for waste feedstocks (Oehmen et al., 2014). However, nitrogen excess enrichment poses a possibility for reduced storage response, especially using fermented waste feedstocks due to nonVFAs. In this case, uncoupled carbon and nitrogen (excess) feeding strategy can improve the selective pressure imposed by feast-famine enrichment even at higher organic loading. Here, nitrogen addition is delayed until the feast phase's termination, imposing a nitrogen deficiency that leads to comparatively higher accumulation and nitrogen availability during the famine, which ensures the survivability of cultures with better storage capacity (Fig 1.3c,d). The new strategy derived MMC with a higher production rate and storage (6.09 gPHA/Ld, 96 % CDW) compared to conventional ADF (2.55 gPHA/Ld, 86 % CDW) (Oliveira et al., 2017). A modified uncoupled scheme investigated for ADF enrichment with fermented crude glycerol had a nitrogen limitation during the feast phase and minimum required nitrogen during the famine phase. The PHA yield on 1,3-propanediol increased to 0.24 Cmol/Cmol with the new strategy, while the yield was negligible under nitrogen excess enrichment (Burniol-Figols et al., 2018). This uncoupled carbon and nitrogen strategy can increase the biomass concentration of an MMC enriched under conventional ADF strategy without losing the PAP of the sludge (Huang et al., 2017). The uncoupled strategy has shown its potential to enrich biomass with high storage response in a short period under nitrogen excess condition (Ahmadi et al., 2020). However, a progressive increase in the C/N ratio reduced storage response (Silva et al., 2017). Similarly, enrichment with OFMSW leachate at a C/N ratio of more than 23.3 led to a reduction in PAP of the activated sludge (Valencia et al., 2021). Hence, the nitrogen excess condition is an ideal strategy to enrich MMC with substantial storage response, while nitrogen limitation is suitable for maximizing accumulation. Thus, ADF can enrich a culture with high polymer accumulation capacity under unlimited carbon and nutrient conditions by adopting suitable matrix and conditions (Jia et al., 2013).

1.4.2.10. Possible routes to implement additional selective pressure

PHA production from waste residues and a mix culture has the inevitable chance of establishing non-PHA producers that affect the process's overall polymer yield. The availability of nonVFA carbon provides an upper hand to non-accumulators due to the preference of growth over accumulation even though ADF imposes a limitation. Additional measures in this direction are necessary to increase the overall storage response of the ADF process.

Some improvements were initiated in recent years to improve the understanding and process of ADF. Selective removal of non-PHA producers was shown using the acetate-methanol mixture by installing the pre-settling phase after the feast phase and decanting of supernatant containing 0, 12 %, and 60 % methanol. This operational sequence selected a population that consumed acetate faster, depicted by increased PHB storage 49 %, 58 %, and 70 % (CDW). Higher time of settling caused methanol consumption diverting carbon source to growth, severely reducing PHA accumulators. The settled biomass contained flocks of PHA accumulators due to a higher rate of settling velocity owing to higher density and cell volume. At the same time, non-accumulators were found around these flocks or suspended. Thus a pre-settling after feast phase can ensure higher enrichment from industrial effluents (Korkakaki et al., 2016b). The magnetic property of metallic cofactors in enzymes also was explored to influence PHA production. The magnetic field stepped up HB and HV production at 21 and 11 milli tesla (mT) during the famine period than in the feast phase. The remarkable promotion of PHA degradation under the magnetic field could facilitate PHA synthesis, improving the storage response. Some stimulatory effects of the magnetic field on synthesis and degradation enzymes could also be involved in enhancing the storage response (Zhu et al., 2012). Enriching specific groups in the microbial community had been a subject of investigations using ADF. Halophilic mix culture was enriched with PHA accumulators using sodium acetate as a carbon source with 64.7 % storage in ADF. The halophilic nature of microbes could also simplify the downstream process (Cui et al., 2016).

1.4.3. Polymer accumulation leading to final production

The ‘accumulation step’ provides the constant process condition to utilize the PHA accumulating potential of the enriched MMC fully. The enriched activated sludge drives the maximum conversion of carbon source into PHA. The biocatalyst is restricted to the feast phase without advancing into the famine period in the accumulation step. Hence it runs for a shorter period. This step ensures a higher polymer density and volumetric productivity required for efficient downstream processing. Fluctuations in dehydrogenase and phosphatase activity can be utilized to monitor the accumulation process's robustness to ensure a higher conversion (Amulya et al., 2016). Many factors influence the PHA accumulation, like nutrient availability, pH, feeding pattern, etc., that imparts additional selective pressure to enhance storage (Table 1.3).

1.4.3.1. Temperature

All microorganisms have an optimum temperature of maximal biomass growth yield, thereby deciding the particular community's dominancy. However, PHA accumulation is a short-term process that does not provide enough time for a specific microbial community to dominate.

Therefore, the temperature during enrichment influences more than the temperature during accumulation. The temperature of 30 °C had higher storage and yield achieved during enrichment and fed-batch accumulation. Culture enriched under 20 °C showed similar storage response irrespective of the temperature at fed-batch (15 °C, 20 °C, 30 °C). PHA accumulation at 15 °C showed the least performance of all (Johnson et al., 2010a). For an MMC enriched without temperature control, a temperature of 30 °C favored biomass growth while 15 °C lead to better storage response during PHA accumulation using fermented brewery effluent. However, enriched biomass accumulated maximum PHA without temperature control (Ben et al., 2016). Besides, maintaining the temperature of huge reactors draw a substantial share of capital. To integrate into the existing waste management system, PHA production without temperature control during enrichment and accumulation could render higher productivity and economic returns.

1.4.3.2. pH

As in the case of culture enrichment, pH≥7.0 provides a conducing environment to maximize the conversion. In this range, allowing the pH to fluctuate results in better PHA storage. Unregulated pH (8.8-9.2) enables the biomass to regulate the exterior condition according to its growth requirement (Montiel-jarillo et al., 2017). Besides, the culture enriched with pH control at 8.0 and uncontrolled pH (≤9.0) showed similar storage capacity under pH uncontrolled batch accumulation with fermented molasses (Oehmen et al., 2014). Even under nutrient deficient conditions, uncontrolled pH set initially at 6.9 produced better storage with 66.4 % (Kourmentza and Kornaros, 2016). An uncontrolled pH also resulted in maximum accumulation for fermented brewery effluent with similar enriched culture (Ben et al., 2016). The pH shifts within this alkaline range also can affect the monomer composition. A pH shift of 8.5 to 9.5 from enrichment to accumulation increased the HV content without decreasing the PHA production rate. Microbes quickly adapt to increasing alkalinity by adjusting the cytoplasmic pH for optimal functioning or electro-neutrality, requiring higher energy. The oxidation of acetyl-CoA (VFA uptake or PHA oxidation) meets this energy requirement that can lower PHA accumulation. Under such pH shifts, the presence of propionyl-CoA (propionic acid) could maintain storage response by condensing with acetyl-CoA forming HV (Villano et al., 2010). Therefore, maintaining a similar pH during enrichment and accumulation will energetically favor PHA production. Moreover, an uncontrolled pH accumulation can allow the enriched MMCs to adjust and perform maximally, leading to higher accumulation and economic production.

1.4.3.3. Nutrient availability

The nutrient level (nitrogen/phosphorus) determines the flux of carbon towards growth or accumulation. Nutrient stress induces PHA production. The nutrient stress disturbs the normal growth (TCA cycle) and metabolism, thereby diverting more of acetyl-CoA and uplifting an inhibitory effect of CoASH towards the PHA synthesis pathway (Section 1.4.2). This increase in acetyl-CoA production due to nutrient stress posttranslationally activates PHB synthase by regulating acetyl phosphate synthesis by phosphotransacetylase (Kessler and Witholt, 2001). Thereby, a part of available carbon gets diverted in storage form in order to survive without an external carbon supply. From the available literature, nitrogen (ammoniacal nitrogen) and phosphorus (phosphate phosphorus) impart this nutrient stress in the way of starved or limited conditions. Many studies have shown high biomass PHA content for accumulation studies conducted with nutrient starvation (Basak et al., 2011). However, the nutrient limitation could edge over nutrient starvation depending on the operational condition. Nutrient limitation increases PHA storing biomass yield without the risk of overtaking by growth response leading to higher productivity. Meanwhile, biomass content and substrate consumption declined over time during nitrogen starvation rendering higher biomass PHA content but reduced yield and productivity. Growth response prevails over storage in the nutrient excess condition (Valentino et al., 2015). Moreover, environmental samples as carbon sources contain excess to limited content of nutrients. Hence, the preferred route for maximum PHA accumulation is through nutrient limitation than starvation.

A C:N:P ratio of 100:5:1 provides a condition for activated sludge growth (Basak et al., 2011). Thus, biomass requires a higher nitrogen level than phosphorus to grow optimally. Cells primarily utilize phosphorus for nucleic acid (ribonucleic acid, RNA) and energy production, while nitrogen for proteins, enzymes, nucleic acids, etc. Thus nitrogen's requirement is higher than phosphorus (Cooney et al., 1976). A fed-batch PHB production system had high storage of 70 % under optimal phosphorus limitation (Cavaillé et al., 2013). The optimal nitrogen and phosphorus limitations provided consistent productivity with fermented cheese whey permeate (Valentino et al., 2015). However, recent work showed that limited nitrogen with excess phosphorus had better storage, yield, and storage rates than dual or phosphorus limitation (Montiel-jarillo et al., 2017). Nitrogen limitation can effectively impair proper growth by restricting the synthesis of essential cell machinery. However, phosphorus present in the required quantity augments biomass growth and thereby PHA productivity as the growth rate is proportional to the nucleic acid content. Moreover, the limitation concentration for phosphorus is very low to implement effective stress (Cooney et al., 1976). Hence, the majority of the research work implemented a nitrogen limited accumulation system for higher carbon recovery.

The nutrient condition during enrichment also influences the MMCs storage response in accumulation. Basak et al. (Basak et al., 2011) observed higher PHA storage and yield for nitrogen deficient (limited) conditions during the enrichment and accumulation stage compared to nitrogen excess conditions. However, another study had shown that sludge enriched under the nitrogen excess condition had the maximum storage response when exposed to nitrogen limiting conditions during batch accumulation. Nitrogen limited enrichment weakens MMC due to prolonged exposure to severe growth limitation resulting in comparatively lower PAP (Johnson et al., 2010b).

Many of the fermented waste feedstocks contain a certain amount of nutrients. Fermented waste feedstocks with high organic load improve storage response without adding nitrogen sources (Amulya et al., 2015). Besides the effective ecological selective pressure imparted to activated sludge, VFA influences the PHA accumulation more than the presence or absence of nutrients (Valentino et al., 2015). A high storage yield of 1.0 $gCOD_{PHA}/gCOD_{VFA}$ resulted from the fed-batch PHA accumulation from fermented OME without nutrient addition (Campanari et al., 2017). Activated sludge enriched in fermented crude glycerol attained maximum PHA yield (0.55 $gCOD_{PHA}/gCOD_{total}$) when accumulated without nitrogen addition to the fermentate (Burniol-Figols et al., 2018). At the same time, struvite precipitation is a feasible option to remove excess nutrients from fermented excess sludge to establish a nutrient limitation condition (Mengmeng et al., 2009).

1.4.3.4. Oxygen limitation

Oxygen limitation in the form of DO is yet another kind of stress that can drive accumulation. Anoxic environment, DO limited, increased PHA storage with fermented and un-fermented food waste compared to the aerobic environment. The availability of oxygen directs the metabolism towards assimilative activities such as protein and carbohydrate synthesis, which get partially restricted in anoxic conditions (Reddy and Venkata Mohan, 2012). Furthermore, the anoxic (microaerophilic) condition converts glucose to VFA (4.256 g/l) with simultaneous cell growth (9.3 g/l), leading to higher storage as to aerobic condition (Amulya et al., 2016). Thus speculations about the use of dual nitrogen and oxygen limitation came up. However, dual limitation seriously affected the accumulation with lowered PHA storage by enriched culture for both the synthetic medium (18.2 %) and acidified OME (3.2 %). In comparison, nitrogen limitation showed better storage with 64.4 % and 8.8 %, respectively. This study showed the unsuitability of dual limitation for PHA accumulation and exemplified the high accumulation under nitrogen limitation by enriched culture (Kourmentza et al., 2015). Similarly, DO influences the substrate uptake rate with acetate and propionate, requiring higher DO than butyrate and valerate as the uptake of longer

VFAs requires less ATP. So a DO limited accumulation can be made efficient with butyrate and valerate enriched fermentate (Wang et al., 2017). Therefore, DO content can influence the storage response even though other parameters have higher implications on PHA accumulation.

1.4.3.5. Feed and its characteristics

PHA accumulators also exhibit substrate preference at appropriate conditions. The overall PHA yield was better for acetate than a mixture of acetate:propionate for an acetate enriched MMC regardless of the nutrient condition of enrichment. Noticeably, biomass enriched in nitrogen deficient conditions adapted better to this shift in carbon source. The HB yield was relatively lower than HV with acetate: propionate compared to acetate (Basak et al., 2011). However, butyrate or butyrate: acetate mixture induced better storage for mix VFA enriched MMC than other VFAs or combinations under nitrogen deficient and uncontrolled pH conditions (Kourmentza and Kornaros, 2016). So the substrate composition and culture conditions during enrichment impact substrate uptake and resulting growth/storage response during accumulation (Wang et al., 2013). If the carbon source and composition (VFA) remain unchanged, distinct microbial populations in non-enriched activated sludge could produce PHA with a similar average composition (Janarthanan et al., 2014). *Acidobacteria* and *Burkholderiales* were the dominant organisms during accumulation using mixed VFA acclimatized culture and acetate: propionate in the ratio 1:1, generating 47.7 % PHA with 48 % HV fraction (Wang et al., 2013). Besides, substrate composition dictates PHA composition and its physicochemical properties (Arcos-Hernández et al., 2013). This was also evident from the acidification of various waste feedstocks. Shen et al. (Shen et al., 2014) found VFA composition than yield influences the accumulation step's outcome.

Substrate composition being fixed, organic loading has a similar effect as in enrichment. The storage response increases with an increase in organic loading with a subsequent drop from the peak due to biomass and storage saturation. The trend is irrespective of nutrient availability (Basak et al., 2011). Fermented waste feedstocks also show a similar dip in storage yield beyond a particular organic load corresponding to the organic content during enrichment (Zhang et al., 2014). An increase in fermented brewery effluent load from 57 to79 Cmmol/L increased the PHA yield from 0.35 to 0.43 CmmolPHA/CmmolVFA with a subsequent decrease to 0.39 CmmolPHA/CmmolVFA at 109 achieved at Cmmol/L (Ben et al., 2016). Influencing salinity was also investigated to tune PHA production and composition from feast/famine enriched sludge. Storage performance was negatively impacted by increased NaCl concentration (7g/L) as PHA got diverted to balance the increased substrate uptake in response to the salinity stress. Propionate was preferably consumed to generate energy for cellular maintenance over other VFAs, reducing the HV content by nearly two folds (Palmeiro-Sánchez et al., 2016).

1.4.3.6. Feeding strategy

Feeding strategy is a contributing factor for polymer yield and composition with mix culture. Batch, sequential batch, and fed-batch are the most reported mode of administering VFAs to maximize accumulation (Table 3). The batch operation ensures ease of handling, requiring lower capital to maintain conditions for maximizing accumulation. However, the batch operation can result in a lower specific uptake rate and storage rate due to substrate inhibition (Serafim et al., 2004). Meanwhile, appropriate nutrient availability, pH, and substrate concentration can accumulate PHA as high as 86 % (Kourmentza and Kornaros, 2016). The sequential batch operation involves running the accumulation reactor in a feed-draw mode for a short period under a particular cycle length. Accumulation can be run at a higher organic loading by step by step increase through this kind of operation. A suitable feast/famine ratio maintained can also ensure a better storage response. Fermented excess or primary sludge are suitable candidates for such a mode of operation due to their very high organic content or fermentate matrix. Excess sludge-food waste fermentate had a PHA yield of 0.56 $gCOD_{PHA}/gCOD_{VFA}$ by a sequential increase in load from 0.36-4.6 gCOD/L under a feast/famine ratio of 1:3 (Zhang et al., 2014).

The fed-batch operation for PHA production employs pulse or continuous feeding. Pulse feeding involves the supply of a calculated or fixed amount of substrate to maintain a particular organic load in the reactor at a fixed or varying interval of time. Substrate consumption from a previous batch operation or fluctuation in DO decides the time interval of a pulse. Pulse feeding controlled using DO is termed as feed on-demand. This feed on-demand strategy supplies pulses near the utilization of substrate to maintain optimum respiration. The increase in DO (after the initial decline with substrate addition) indicates substrate exhaustion and time for the next pulse until the saturation of storage/growth. It also ensured a repeated period of near carbon limitation imposing stringency that sustained PHA production by promoting storage-phenotypic biomass growth, thereby sustaining the high PHA production rate long after the maximum storage yield. Feed on-demand strategy with or without nutrient restriction accumulates significant PHA (Valentino et al., 2015). Such a feed on-demand operation under phosphate limitation improved accumulation capacity to 70 % (Cavaillé et al., 2013). This strategy enables accumulation at higher organic load without any possible inhibition with optimum respiration. PHA accumulation of enriched culture with fermented candy factory effluent under feed on demand had increased the storage to 0.9 gPHA/gVSS (Tamis et al., 2014). Another type of fed-batch mode includes a short period of settling and discharging a designated supernatant volume before every alternating pulse feeding, termed as Aerobic dynamic feeding and discharge (AFD) (Jiang et al., 2009). In AFD also, fluctuations in DO decide the duration of each pulse. A recent study had shown that introducing a sedimentation step after the feast phase during enrichment selectively removed non-accumulators through supernatant discharge, thereby constricting the species diversity to PHA producers (Korkakaki et al., 2016b). Hence for waste feedstocks, AFD imposes additional pressure on enriched MMC by restricting COD consumption by flanking populations and improving PHA accumulation. Accumulation high as 72.9 % was attained in 10 h of operation with alkaline fermented WAS (Jiang et al., 2009). AFD accumulation with fermented molasses led to a higher PHA yield of 0.68 gCOD/gCOD (Chen et al., 2017).

Table 1.3: PHA accumulation performance of feast/famine enriched activated sludge and it's process condition (3rd stage) using fermented waste feedstocks

Waste feedstock	Reactor	Feeding pattern	Organic Loading (gCOD/L)	pH	T (°C)	Nutrient condition (COD: N:P)#	PHA storage (gPHA/gVSS)	PHA yield (gCOD/gCOD)+	PHA profile (HB:HV)#	Reference
Sugar Cane Molasses	Fed Batch	Pulse	1[a]	n.c	23-25	100:0	0.56	0.80[d]	85:15	(Albuquerque et al., 2011)
	Fed batch	Pulse	0.15[a]	n.c	23-25	N-limited	0.56	0.85	48:52	(Duque et al., 2014)
	Fed batch	Pulse	n.a	n.c	20	N-limited	0.61	0.68	n.a	(Chen et al., 2017)
	Fed batch	Pulse	0.48-0.56[a]	n.c	23-25	100:6.7-7.5	0.58	0.65[d]	n.a	(Oehmen et al., 2014)
Cheese Whey	Fed Batch	Pulse	111[a]	n.c	23-25	N-limited	0.65	0.67	81:19	(Duque et al., 2014)
	Fed Batch	Pulse	200 (each pulse)	n.a	30	1:10:0.5	0.66	n.a	n.a	(Valentino et al., 2015)
	Fed batch	Pulse	39.7 38 (C-mmol /L*d)	8.8	25	100:12.3:3 100:19.7:3.9	0.66 0.81	0.7[d] 0.8[d]	100:0 60:40	(Colombo et al., 2016)
	Fed batch	Pulse	100	n.c	30	100:1:0.5	0.59	0.52	76:34	(Janarthanan et al., 2016)
	Fed batch	Pulse	20.1 (3x)	n.c	23-25	100:0.4:0.1	n.a	0.96[d]	87:13	(Oliveira et al., 2017)
	Fed batch	Pulse	7.5	n.a	21		0.62 0.55	0.75 0.69	100:0	(Colombo et al., 2019)
Brewery Wastewater	Batch	Batch	0.11[a]	n.c	15-30 (n.c)	100:1.2	0.39	0.39[d]	n.a	(Ben et al., 2016)
	Fed batch	Pulse	2.1 (TOC)	n.a	n.a	100:0.2	0.45	0.76[d]	59:41	(Tamang et al., 2019)

Candy Factory effluent	Fed batch	Pulse	8.0	controlled	30	n.a	0.76	n.a	84:16	(Tamis et al., 2014)
Wood Mill Effluent	Batch	Batch	0.044[a] 0.048[a]	n.c	n.c	100:6 100:1	0.22 0.27	0.23[d] 0.35[d]	83:17 81:19	(Ben et al., 2011)
Paper Mill Effluent	Fed batch	Continuous	24	7.0	30	N-limited	0.77	0.8	n.a	(Jiang et al., 2012)
	Fed batch	Pulse	n.a	6.6	30	N-deficient	0.7-0.8	0.2-0.4	99.75:0.25	(Tamis et al., 2018)
Paperboard mill wastewater	Batch	Batch	1.5 (gCOD/Ld)	7.0	30	100:5.3	0.59	0.46	92:8	(Farghaly et al., 2017)
Olive Mill Effluent	Fed batch	Pulse	26.8	n.c	25	N-limited	n.a	1.0	n.a	(Campanari et al., 2017)
	Sequential batch	Sequential	12.9	7.0	27	N-Excess/ Limited cycle	0.25	n.a	n.a	(Ntaikou et al., 2014)
Palm Oil Mill Effluent	Fed batch	Pulse	32	7.0	28-30	Nutrient Limited	0.4[c]	n.a	n.a	(Salmiati et al., 2007)
Bio-Oil	Fed batch	Pulse	0.06[a]	n.c	20-23	Nutrient limiting	0.17	0.53[c]	73:23	(Fidalgo et al., 2014)
Crude Glycerol	Batch	Batch	0.045[a]	8.0	30	100:0:1.5	n.a	0.55	n.a	(Burniol-Figols et al., 2018)
Mussels Cooking Wastewater	Fed-Batch	Pulse	0.015-0.07 [a] (each pulse)	n.c	30	n.a	0.42	n.a	n.a	(Roibás-rozas et al., 2021)
Dairy manure	Fed batch	Pulse	5.1	n.a	22-25	100:8.8-22:0.5-2.6	0.91	1.1[d]	27:73	(Coats et al., 2016)
Organic Fraction of Municipal Solid Waste	Fed batch	Pulse	7.6 7.9 7.3	8.8	25	100:0.37:0.22-0.43	0.48 0.47 0.41	0.44 0.52 0.5	57:43 54:46 57:43	(Colombo et al., 2017)
	Fed batch	Continuous	11.9	7.0	30	100:16:0.09	0.78 0.77	n.a	n.a	(Korkakaki et al., 2016a)
	Fed batch	Pulse	1.5	n.a	n.a	N-starvation	0.15	0.94	29:71	(Papa et al., 2020)
Composite Food Waste	Batch	Batch	11	8.0	n.a	100:0.5:0.1	0.24	0.17	n.a	(Amulya et al., 2015)
Excess Sludge	Batch	Batch	8.7 5.9	7.0	30	100:17.7:0.5 100:13.5:0.4	0.24[c] 0.60[c]	0.12[e]	n.a	(Jia et al., 2014)
	Fed Batch	Continuous	9	7.8-8.2	20-29	100:11:5.5	0.37	0.37	66:34	(Morgan-Sagastume et al., 2015)
	Batch	Batch	0.65	n.a	25	100:6.3	0.63	0.52	98.3:1.7	(Liu et al., 2020)

Organic Fraction of Municipal Solid Waste +Excess sludge	Fed batch	Pulse	3 (per pulse)	n.c	25-28	100:1.9:1.6	0.62	0.59	n.a	(Moretto et al., 2020)
	Fed batch	Pulses	27	n.c	15-34		0.48	0.47	n.a	(Valentino et al., 2020)
Primary Sludge	Batch	Batch	1.2	7.0	20	100:2:0.5–100:3:0.8	0.28	n.a	n.a	(Pittmann and Steinmetz, 2014)
Dewatered Sludge + Food Waste	Fed batch	Continuous	0.72	n.a	25	100:1.2:0.13	0.65	n.a	n.a	(Chen et al., 2013)
	Sequential batch reactor	Batch	3.6	n.a	25	100:2.5:1	0.48[c]	0.54	38:10	(Zhang et al., 2014)
	Batch-fed batch	Pulse	2	8	21	N-limited	0.31 0.44	n.a	97.1:2.9 94.8:5.2	(Perez-Zabaleta et al., 2021)
Pimary sludge+excess sludge	Fed batch	Pulse	6 ($gCOD_{VFA}$/L*d)	6.3	35	100:20:4	0.19[b]	0.28[d]	74:26	(Morgan-Sagastume et al., 2010)

w/w or mol/mol basis, +: based on soluble COD or COD_{VFA} consumed, n.c: not controlled, n.a: not available, N: nitrogen source, a: C-mol/L, b: gPHA/gTSS, c: gPHA/gCDW, d: Cmol/Cmol, e: gPHA/gCOD, HB:HV-hydroxybutyrate:hydroxyvalerate. All the pulse feeding pattern are based on feed-on-demand strategy.

Another fed-batch mode uses continuous feeding, where the shorter pulse or flow rate modulates the substrate concentration in the reactor. Continuous pulsed feeding of fermented sludge liquid in short intervals (90s) created a micro feast-famine condition with better accumulation than the batch pulse feeding (Chen et al., 2013). Meanwhile, Chen et al. (Chen et al., 2015b) found that the continuous feeding strategy had a superior accumulation capacity than feed on demand or pulse addition with a self-balancing pH effect. Continuous feeding ensured complete substrate uptake by mix culture as the external substrate was undetected during most operations. Continuous feeding ensures higher PHA productivity since the number of pulses and the considerable feed volume of each pulse in pulse feeding reduces waste feedstocks' productivity as a carbon source (Albuquerque et al., 2011). Fed-batch accumulation from acidified OFMSW at a continuous flow rate of 2.3 mL/min led to maximum storage of 78.4 % (Korkakaki et al., 2016a). For that reason, the possibility of a combined enrichment and accumulation step can be a reality in the near future. The feeding pattern also influences PHA composition. Acetate: propionate mixture at various ratios supplied alone or combined for different durations according to feed on-demand strategy impacted PHA composition than PHA storage. The feeding pattern synthesized random copolymers, diblocks, or triblock and repeating multiblock polymers with a wide range of properties, indicating the possibility of a tailor-made PHA bioplastic (Arcos-Hernández et al., 2013). Waste feedstocks also exhibit a similar impact of feeding patterns. Continuous feeding of fermented molasses had increased HV content (8-9 %) compared to pulse feeding (Albuquerque et al., 2011). These investigations project the methodology as an improvement among the waste management strategies to be integrated with the existing infrastructure that is adaptable with fluctuating conditions to valorize waste to a biopolymer.

1.5. Conclusion

Polyhydroxyalkanoate production directly from waste feedstocks using mix microbial community present in the ecological niche through the three-step methodology is one of the most significant contributions towards mitigating anthropogenic activity's impact on the biosphere. Observing the developmental stages of the process, one can understand the power vested in nature, where the non-committed microbial community is trained to divert the major portion of carbon into an industrially valuable polymer.

Activated sludge is the ideal source of microorganisms to synthesize PHA from renewable sources of carbon like waste feedstocks. The non-axenic PHA synthesis from activated sludge is highly resilient to environmental conditions and easily adaptable to various feedstock. Activated sludge exposed to ADF reduces the species diversity to storage phenotype through strong ecological selective pressure effected by the feast-famine cycle. Sequential ADF with optimized SRT/HRT, the minimum required DO, and nitrogen excess condition without controlling pH/temperature efficiently enriches PHA accumulators in the MMC. Life cycle analysis and pilot-scale studies propose mix culture PHA accumulation as an economically and environmentally viable process. Waste feedstock, high organic load, renewable energy source raises mix culture PHA production above biogas production with reduced energy consumption, GHG emission than conventional plastics. In addition, bioconversion of the waste feedstocks to VFAs through acidogenic fermentation improves the carbon recovery to storage form. Several operational factors and waste feedstocks decide the VFA yield and composition. These two steps combined bulks the activated sludge with PHA suitable for downstream extraction and purification. The properties of PHA synthesized from mix culture improved by tuning VFAs content or fermentation of waste residue, feeding pattern, etc., to such an extent that PHAs can substitute petroleum-derived polymer. The outcomes of these studies show the feasibility and advantage of a scaling-up and integration of the AF-ADF process with existing treatment facilities. Future studies focusing on the perspective of improved and reproducible production by incorporating different sources of renewable carbon, energy, and MMCs with calculated functional aspects of PHA targeting a spectrum of application could extend the reach of the three-step process and general acceptance by public/private stakeholders.

References

Ahmadi, F., Zinatizadeh, A.A., Asadi, A., 2020. The effect of different operational strategies on polyhydroxyalkanoates (PHAs) production at short-term biomass enrichment. J. Environ. Chem. Eng. 8, 103531.

Albuquerque, M.G.E., Carvalho, G., Kragelund, C., Silva, A.F., Crespo, M.T.B., Reis, M.A.M., Nielsen, P.H., 2012. Link between microbial composition and carbon substrate-uptake preferences in a PHA-storing community. Int. Soc. Microb. Ecol. 7, 1–12.

Albuquerque, M.G.E., Eiroa, M., Torres, C., Nunes, B.R., Reis, M.A.M., 2007. Strategies for the development of a side stream process for polyhydroxyalkanoate (PHA) production from sugar cane molasses. J. Biotechnol. 130, 411–421.

Albuquerque, M.G.E., Martino, V., Pollet, E., Avérous, L., Reis, M.A.M., 2011. Mixed culture polyhydroxyalkanoate (PHA) production from volatile fatty acid (VFA)-rich streams: Effect of substrate composition and feeding regime on PHA productivity, composition and properties. J. Biotechnol. 151, 66–76.

Albuquerque, M.G.E., Torres, C.A. V, Reis, M.A.M., 2010. Polyhydroxyalkanoate (PHA) production by a mixed microbial culture using sugar molasses : Effect of the influent substrate concentration on culture selection. Water Res. 44, 3419–3433.

Albuquerque, P.B.S., Malafaia, C.B., 2018. Perspectives on the production, structural characteristics and potential applications of bioplastics derived from polyhydroxyalkanoates. Int. J. Biol. Macromol. 107, 615–625.

Amulya, K., Jukuri, S., Venkata Mohan, S., 2015. Sustainable multistage process for enhanced productivity of bioplastics from waste remediation through aerobic dynamic feeding strategy: Process integration for up-scaling. Bioresour. Technol. 188, 231–239. 2, 278-285

Amulya, K., Reddy, M.V., Rohit, M. V., Venkata Mohan, S., 2016. Wastewater as renewable feedstock for bioplastics production: Understanding the role of reactor microenvironment and system pH. J. Clean. Prod. 112, 4618–4627.

Arcos-Hernández, M. V, Laycock, B., Donose, B.C., Pratt, S., Halley, P., Al-luaibi, S., Werker, A., Lant, P.A., 2013. Physicochemical and mechanical properties of mixed culture polyhydroxyalkanoate (PHBV). Eur. Polym. J. 49, 904–913.

Baghchehsaraee, B., Nakhla, G., Karamanev, D., Margaritis, A., Reid, G., 2008. The effect of heat pretreatment temperature on fermentative hydrogen production using mixed cultures. Int. J. Hydrogen Energy 33, 4064–4073.

Balaji, S., Gopi, K., Muthuvelan, B., 2013. A review on production of poly β hydroxybutyrates from cyanobacteria for the production of bio plastics. Algal Res. 2, 278-285

Baldwin, D.D., Campbell, C.E., 2001. Short-term effects of low pH on the microfauna of an activated sludge wastewater treatment system. Water Qual. Res. J. Canada 36, 519–535.

Basak, B., Ince, O., Artan, N., Yagci, N., Ince, B.K., 2011. Effect of nitrogen limitation on enrichment of activated sludge for PHA production. Bioprocess Biosyst. Eng. 34, 1007–1016.

BBC, 2019. How plastic bags were supposed to help the planet [WWW Document]. URL https://www.youtube.com/user/bbcnews/featured (accessed 4.5.20).

Ben, M., Kennes, C., Veiga, M.C., 2016. Optimization of polyhydroxyalkanoate storage using mixed cultures and brewery wastewater. J. Chem. Technol. Biotechnol. 91, 2817–2826.

Ben, M., Mato, T., Lopez, A., Vila, M., Kennes, C., Veiga, M.C., 2011. Bioplastic production using wood mill effluents as feedstock. Water Sci. Technol. 63, 1196–1202.

Bengtsson, S., Hallquist, J., Werker, A., Welander, T., 2008. Acidogenic Feremntation of Industrial Waste Water: Effects of chemostat retention time and pH on volatile fatty acids production. Biochem. Eng. J. 40, 492–499.

BPI, 2015. Confused by the Terms Biodegradable and Biobased? [WWW Document]. URL http://www.bpiworld.org/Resources/Documents/Confused by the terms Biodegradable Jan 15.pdf

Burniol-Figols, A., Varrone, C., Daugaard, A.E., Le, S.B., Skiadas, I. V., Gavala, H.N., 2018. Polyhydroxyalkanoates (PHA) production from fermented crude glycerol: Study on the conversion

of 1,3-propanediol to PHA in mixed microbial consortia. Water Res. 128, 255–266.
Campanari, S., Augelletti, F., Rossetti, S., Sciubba, F., Villano, M., Majone, M., 2017. Enhancing a multi-stage process for olive oil mill wastewater valorization towards polyhydroxyalkanoates and biogas production. Chem. Eng. J. 317, 280–289.
Campanari, S., E Silva, F.A., Bertin, L., Villano, M., Majone, M., 2014. Effect of the organic loading rate on the production of polyhydroxyalkanoates in a multi-stage process aimed at the valorization of olive oil mill wastewater. Int. J. Biol. Macromol. 71, 34–41.
Carvalho, G., Oehmen, A., Albuquerque, M.G.E., Reis, M.A.M., 2014. The relationship between mixed microbial culture composition and PHA production performance from fermented molasses. N. Biotechnol. 31, 257–263.
Cavaillé, L., Grousseau, E., Pocquet, M., Lepeuple, A.S., Uribelarrea, J.L., Hernandez-Raquet, G., Paul, E., 2013. Polyhydroxybutyrate production by direct use of waste activated sludge in phosphorus-limited fed-batch culture. Bioresour. Technol. 149, 301–309.
Center for International Environmental Law, 2019. Plastic Proliferation Threatens the Climate on a Global Scale Plastic & Climate [WWW Document]. URL https://www.ciel.org/wp-content/uploads/2019/05/Plastic-and-Climate-Executive-Summary-2019.pdf (accessed 4.17.20).
Chae, Y., An, Y.J., 2018. Current research trends on plastic pollution and ecological impacts on the soil ecosystem: A review. Environ. Pollut. 240, 387–395.
Chaitanya, N., Sivaramakrishna, D., Kumar, B.S., Himabindu, V., Lakshminarasu, M., Vishwanadham, M., 2016. Selection of pretreatment method for enriching hydrogen-producing bacteria using anaerobic sewage sludge with three different substrates. Biofuels 7, 163–171.
Chang, S., Li, J.Z., Liu, F., 2011. Evaluation of different pretreatment methods for preparing hydrogen-producing seed inocula from waste activated sludge. Renew. Energy 36, 1517–1522.
Chen, G.-Q., Chen, X.-Y., Wu, F.-Q., Chen, J.-C., 2020. Polyhydroxyalkanoates (PHA) toward cost competitiveness and functionality. Adv. Ind. Eng. Polym. Res. 3, 1–7.
Chen, H., Meng, H., Nie, Z., Zhang, M., 2013. Polyhydroxyalkanoate production from fermented volatile fatty acids : Effect of pH and feeding regimes. Bioresour. Technol. 128, 533–538.
Chen, Z., Guo, Z., Wen, Q., Huang, L., Bakke, R., Du, M., 2015a. A new method for polyhydroxyalkanoate (PHA) accumulating bacteria selection under physical selective pressure. Int. J. Biol. Macromol. 72, 1329–1334.
Chen, Z., Huang, L., Wen, Q., Guo, Z., 2015b. Efficient polyhydroxyalkanoate (PHA) accumulation by a new continuous feeding mode in three-stage mixed microbial culture (MMC) PHA production process. J. Biotechnol. 209, 68–75.
Chen, Z., Huang, L., Wen, Q., Zhang, H., Guo, Z., 2017. Effects of sludge retention time, carbon and initial biomass concentrations on selection process : From activated sludge to polyhydroxyalkanoate accumulating cultures. J. Environ. Sci. 2, 76–84.
Chinthapalli, R., Skoczinski, P., Carus, M., Baltus, W., De Guzman, D., Käb, H., Raschka, A., Ravenstijn, J., 2019. Biobased Building Blocks and Polymers - Global Capacities, Production and Trends, 2018-2023, Industrial Biotechnology.
Choi, J., Lee, S.Y., 1999. Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. Appl. Microbiol. Biotechnol. 51, 13–21.
Chua, A.S.M., Takabatake, H., Satoh, H., Mino, T., 2003. Production of polyhydroxyalkanoates (PHA) by activated sludge treating municipal wastewater: Effect of pH, sludge retention time (SRT), and acetate concentration in influent. Water Res. 37, 3602–3611.
CNN, 2016. Plastic Island: How our throwaway culture is turning paradise into a graveyard [WWW Document]. URL https://edition.cnn.com/interactive/2016/12/world/midway-plastic-island/ (accessed 4.17.20).
Coats, E.R., Watson, B.S., Brinkman, C.K., 2016. Polyhydroxyalkanoate synthesis by mixed microbial consortia cultured on fermented dairy manure : Effect of aeration on process rates / yields and the associated microbial ecology. Water Res. 106, 26–40.
Colombo, B., Calvo, M.V., Sciarria, T.P., Scaglia, B., Kizito, S.S., D'Imporzano, G., Adani, F., 2019. Biohydrogen and polyhydroxyalkanoates (PHA) as products of a two-steps bioprocess from deproteinized dairy wastes. Waste Manag. 95, 22–31.
Colombo, B., Favini, F., Scaglia, B., Sciarria, T.P., D'Imporzano, G., Pognani, M., Alekseeva, A., Eisele, G., Cosentino, C., Adani, F., 2017. Enhanced polyhydroxyalkanoate (PHA) production from the organic fraction of municipal solid waste by using mixed microbial culture. Biotechnol. Biofuels 10, 1–15.

Colombo, B., Tommy, P.S., Maria, R., Barbara, S., Fabrizio, A., 2016. Polyhydroxyalkanoates (PHAs) production from fermented cheese whey by using a mixed microbial culture. Bioresour. Technol. 218, 692–699.
Cooney, C.L., Wang, D.I.C., Mateles, R.I., 1976. Growth of Enterobacter aerogenes in a chemostat with double nutrient limitations. Appl. Environ. Microbiol. 31, 91–98.
Cui, Y., Zhang, H., Lu, P., Peng, Y., 2016. Effects of carbon sources on the enrichment of halophilic polyhydroxyalkanoate-storing mixed microbial culture in an aerobic dynamic feeding process. Sci. Rep. 6, 30766.
Dahiya, S., Mohan, S.V., 2019. Selective control of volatile fatty acids production from food waste by regulating biosystem buffering: A comprehensive study. Chem. Eng. J. 357, 787–801.
De Grazia, G., Quadri, L., Majone, M., Morgan-Sagastume, F., Werker, A., 2017. Influence of temperature on mixed microbial culture polyhydroxyalkanoate production while treating a starch industry wastewater. J. Environ. Chem. Eng. 5, 5067–5075.
Dietrich, K., Dumont, M., Del Rio, L.F., Orsat, V., 2017. Producing PHAs in the bioeconomy — Towards a sustainable bioplastic. Sustain. Prod. Consum. 9, 58–70.
Din, M.F., Huey, S.C., Yunus, S.M., Ahmad, M.A., Ponraj, M., Ujang, Z., Shreeshivadasan, C., 2012. Raw material resource for biodegradable plastic production from cafeteria wastes. J. Sci. Ind. Res. 71, 573–578.
Dircks, K., Henze, M., Van Loosdrecht, M.C.M., Mosbæk, H., Aspegren, H., 2001. Storage and degradation of poly-β-hydroxybutyrate in activated sludge under aerobic conditions. Water Res. 35, 2277–2285.
Dobroth, Z.T., Hu, S., Coats, E.R., Mcdonald, A.G., 2011. Polyhydroxybutyrate synthesis on biodiesel wastewater using mixed microbial consortia. Bioresour. Technol. 102, 3352–3359.
Duque, A.F., Oliveira, C.S.S., Carmo, I.T.D., Gouveia, A.R., Pardelha, F., Ramos, A.M., Reis, M.A.M., 2014. Response of a three-stage process for PHA production by mixed microbial cultures to feedstock shift : impact on polymer composition. N. Biotechnol. 31, 5–11.
European Bioplastic, 2019. Bioplastics market data update [WWW Document]. URL https://www.european-bioplastics.org/market/ (accessed 4.20.20).
Farghaly, A., Enitan, A.M., Kumari, S., Bux, F., Tawfik, A., 2017. Polyhydroxyalkanoates production from fermented paperboard mill wastewater using acetate-enriched bacteria. Clean Technol. Environ. Policy 19, 935–947.
Feldman, D., 2008. Polymer history. Des. Monomers Polym. 11, 1–15.
Fidalgo, R.M., Ortigueira, J., Freches, A., Pelica, J., Gonçalves, M., Mendes, B., Lemos, P.C., 2014. Bio-oil upgrading strategies to improve PHA production from selected aerobic mixed cultures. N. Biotechnol. 31, 297–307.
Gameiro, D.N., 2019. Life in Bioplastic , it ’ s More Fantastic [WWW Document]. URL https://www.labiotech.eu/features/bioplastics-2019-feature/ (accessed 4.20.20).
Ghosh, S., Chakraborty, S., 2020. Production of polyhydroxyalkanoates (PHA) from aerobic granules of refinery sludge and Micrococcus aloeverae strain SG002 cultivated in oily wastewater. Int. Biodeterior. Biodegrad. 155, 105091.
Hamawand, I., 2015. Anaerobic digestion process and bio-energy in meat industry : A review and a potential. Renew. Sustain. Energy Rev. 44, 37–51.
Hao, J., Wang, H., 2015. Volatile fatty acids productions by mesophilic and thermophilic sludge fermentation: Biological responses to fermentation temperature. Bioresour. Technol. 175, 367–373.
Hao, J., Wang, H., Wang, X., 2018. Selecting optimal feast-to-famine ratio for a new polyhydroxyalkanoate (PHA) production system fed by valerate-dominant sludge hydrolysate. Appl. Microbiol. Biotechnol. 102, 3133–3143.
Hao, J., Wang, X., Wang, H., 2017. Overall process of using a valerate-dominant sludge hydrolysate to produce high-quality polyhydroxyalkanoates (PHA) in a mixed culture. Sci. Rep. 7, 1–11.
Hawkes, F.R., Hussy, I., Kyazze, G., Dinsdale, R., Hawkes, D.L., 2007. Continuous dark fermentative hydrogen production by mesophilic microflora: Principles and progress. Int. J. Hydrogen Energy 32, 172–184.
Huang, L., Chen, Z., Wen, Q., Lee, D.J., 2017. Enhanced polyhydroxyalkanoate production by mixed microbial culture with extended cultivation strategy. Bioresour. Technol. 241, 802–811.
Huang, L., Chen, Z., Wen, Q., Zhao, L., Lee, D.J., Yang, L., Wang, Y., 2018. Insights into Feast-Famine polyhydroxyalkanoate (PHA)-producer selection: Microbial community succession, relationships with system function and underlying driving forces. Water Res. 131, 167–176.

Ivanov, V., Stabnikov, V., Ahmed, Z., Dobrenko, S., Saliuk, A., 2015. Production and applications of crude polyhydroxyalkanoate- containing bioplastic from the organic fraction of municipal solid waste. Int. J. Environ. Sci. Technol. 12, 725–738.

Janarthanan, O.M., Laycock, B., Montano-herrera, L., Lu, Y., Arcos-hernandez, M. V, Werker, A., Pratt, S., 2016. Fluxes in PHA-storing microbial communities during enrichment and biopolymer accumulation processes. N. Biotechnol. 33, 61–72.

Janarthanan, O.M., Yu, Y., Laycock, B., Werker, A., Pratt, S., 2014. Fractionation of microbial populations in a PHA accumulating mixed culture and associated PHA content and composition. Int. J. Biol. Macromol. 71, 53–58.

Jia, Q., Wang, H., Wang, X., 2013. Dynamic synthesis of polyhydroxyalkanoates by bacterial consortium from simulated excess sludge fermentation liquid. Bioresour. Technol. 140, 328–336.

Jia, Q., Xiong, H., Wang, H., Shi, H., Sheng, X., Sun, R., Chen, G., 2014. Production of polyhydroxyalkanoates (PHA) by bacterial consortium from excess sludge fermentation liquid at laboratory and pilot scales. Bioresour. Technol. 171, 159–167.

Jiang, Y., Chen, Y., Zheng, X., 2009. Efficient polyhydroxyalkanoates production from a waste-activated sludge alkaline fermentation liquid by activated sludge submitted to the aerobic feeding and discharge process. Environ. Sci. Technol. 43, 7734–7741.

Jiang, Y., Marang, L., Kleerebezem, R., Muyzer, G., van Loosdrecht, M.C.M., 2010. Effect of temperature and cycle length on microbial competition in PHB-producing sequencing batch reactor. Int. Soc. Microb. Ecol. 5, 896–907.

Jiang, Y., Marang, L., Tamis, J., van Loosdrecht, M.C.M., Dijkman, H., Kleerebezem, R., 2012. Waste to resource: Converting paper mill wastewater to bioplastic. Water Res. 46, 5517–5530.

Johnson, K., Geest, J. Van, Kleerebezem, R., van Loosdrecht, M.C.M., 2010a. Short- and long-term temperature effects on aerobic polyhydroxybutyrate producing mixed cultures. Water Res. 44, 1689–1700.

Johnson, K., Kleerebezem, R., van Loosdrecht, M.C.M., 2010b. Influence of the C/N ratio on the performance of polyhydroxybutyrate (PHB) producing sequencing batch reactors at short SRTs. Water Res. 44, 2141–2152.

Karimi-Maleh, H., Kumar, B.G., Rajendran, S., Qin, J., Vadivel, S., Durgalakshmi, D., Gracia, F., Soto-Moscoso, M., Orooji, Y., Karimi, F., 2020. Tuning of metal oxides photocatalytic performance using Ag nanoparticles integration. J. Mol. Liq. 314, 113588.

Kessler, B., Witholt, B., 2001. Factors involved in the regulatory network of polyhydroxyalkanoate metabolism. J. Biotechnol. 86, 97–104.

Khardenavis, A.A., Vaidya, A.N., Kumar, M.S., Chakrabarti, T., 2009. Utilization of molasses spentwash for production of bioplastics by waste activated sludge. Waste Manag. 29, 2558–2565.

Kinhal, V., 2019. Biobased Polyster Plastics That Biodegrade [WWW Document]. URL https://greenliving.lovetoknow.com/Type_of_Biodegradable_Plastic (accessed 4.18.20).

Koller, M., Gasser, I., Schmid, F., Berg, G., 2011. Linking ecology with economy : Insights into polyhydroxyalkanoate-producing microorganisms. Eng. Life Sci. 11, 222–237.

Korkakaki, E., Mulders, M., Veeken, A., Rozendal, R., van Loosdrecht, M.C.M., Kleerebezem, R., 2016a. PHA production from the organic fraction of municipal solid waste (OFMSW): Overcoming the inhibitory matrix. Water Res. 96, 74–83.

Korkakaki, E., van Loosdrecht, M.C.M., Kleerebezem, R., 2016b. Survival of the Fastest: Selective removal of the side population for enhanced PHA production in a mixed substrate enrichment. Bioresour. Technol. 216, 1022–1029.

Kourmentza, C., Kornaros, M., 2016. Biotransformation of volatile fatty acids to polyhydroxyalkanoates by employing mixed microbial consortia : The effect of pH and carbon source. Bioresour. Technol. 222, 388–398.

Kourmentza, C., Ntaikou, I., Lyberatos, G., Kornaros, M., 2015. Polyhydroxyalkanoates from Pseudomonas sp. using synthetic and olive mill wastewater under limiting conditions. Int. J. Biol. Macromol. 74, 202–210.

Kourmentza, C., Plácido, J., Venetsaneas, N., Burniol-Figols, A., Varrone, C., Gavala, H.N., Reis, M.A.M., 2017. Recent advances and challenges towards sustainable polyhydroxyalkanoate (PHA) production. Bioengineering 4, 1–43.

Kumar, G., Ponnusamy, V.K., Bhosale, R.R., Shobana, S., Yoon, J.J., Bhatia, S.K., Rajesh Banu, J., Kim, S.H., 2019. A review on the conversion of volatile fatty acids to polyhydroxyalkanoates using dark fermentative effluents from hydrogen production. Bioresour. Technol. 287, 121427.

Lambert, S., Wagner, M., 2017. Environmental performance of bio-based and biodegradable plastics: The road ahead. Chem. Soc. Rev. 46, 6855–6871.

Lee, W.S., Chua, A.S.M., Yeoh, H.K., Ngoh, G.C., 2014. A review of the production and applications of waste-derived volatile fatty acids. Chem. Eng. J. 235, 83–99.

Lehninger, A.L., Nelson, D.L., Cox, M.M., 2008. Pathways for amino acid degredation, in: Lehninger Principles of Biochemistry. W.H Freeman, New York, pp. 687–702.

Lenz, R.W., Marchessault, R.H., 2005. Bacterial polyesters: Biosynthesis, biodegradable plastics and biotechnology. Biomacromolecules 6, 1–8.

Lin, C.Y., Chou, C.H., 2004. Anaerobic hydrogen production from sucrose using an acid-enriched sewage sludge microflora. Eng. Life Sci. 4, 66–70.

Liu, C.C., Zhang, L.L., An, J., Chen, B., Yang, H., 2016. Recent strategies for efficient production of polyhydroxyalkanoates by micro-organisms. Lett. Appl. Microbiol. 62, 9–15.

Liu, H., Wang, G., Zhu, D., Pan, G., 2009. Enrichment of the hydrogen-producing microbial community from marine intertidal sludge by different pretreatment methods. Int. J. Hydrogen Energy 34, 9696–9701.

Liu, H.Y., Hall, P. V, Darby, J.L., Coats, E.R., Green, P.G., Thompson, D.E., Loge, F.J., 2008. Production of polyhydroxyalkanoate during treatment of tomato cannery wastewater. Water Environ. Res. 80, 367–372.

Liu, Yuanjun, Guo, L., Liao, Q., Ran, Y., Hu, F., Gao, M., She, Z., Zhao, Y., Jin, C., Liu, Yiping, Wang, G., 2020. Polyhydroxyalkanoate (PHA) production with acid or alkali pretreated sludge acidogenic liquid as carbon source: Substrate metabolism and monomer composition. Process Saf. Environ. Prot. 142, 156–164.

Magdouli, S., Brar, S.K., Blais, J.F., Tyagi, R.D., 2015. How to direct the fatty acid biosynthesis towards polyhydroxyalkanoates production? Biomass and Bioenergy 74, 268–279.

Magrini, F.E., de Almeida, G.M., da Maia Soares, D., Fuentes, L., Ecthebehere, C., Beal, L.L., da Silveira, M.M., Paesi, S., 2020. Effect of different heat treatments of inoculum on the production of hydrogen and volatile fatty acids by dark fermentation of sugarcane vinasse. Biomass Convers. Biorefinery 1–14.

Marang, L., Jiang, Y., van Loosdrecht, M.C.M., Kleerebezem, R., 2014. Impact of non-storing biomass on PHA production : An enrichment culture on acetate and methanol. Int. J. Biol. Macromol. 71, 74–80.

Marang, L., van Loosdrecht, M.C.M., Kleerebezem, R., 2018. Enrichment of PHA-producing bacteria under continuous substrate supply. N. Biotechnol. 41, 55–61.

Marang, L., van Loosdrecht, M.C.M., Kleerebezem, R., 2016. Combining the enrichment and accumulation step in non-axenic PHA production : Cultivation of Plasticicumulans acidivorans at high volume exchange ratios. J. Biotechnol. 231, 260–267.

McKay, D., 2019. Fossil fuel industry sees the future of their product in hard-to-recycle plastic [WWW Document]. Conversat. URL https://phys.org/news/2019-10-fossil-fuel-industry-future-product.html (accessed 4.17.20).

Mengmeng, C., Hong, C., Qingliang, Z., Shirley, S.N., Jie, R., 2009. Optimal production of polyhydroxyalkanoates (PHA) in activated sludge fed by volatile fatty acids (VFAs) generated from alkaline excess sludge fermentation. Bioresour. Technol. 100, 1399–1405.

Metcalf, Eddy, 2003. Wastewater engineering : treatment and resource recovery. McGraw-Hill Higher Education, New York.

Montiel-jarillo, G., Carrera, J., Suárez-ojeda, M.E., 2017. Enrichment of a mixed microbial culture for polyhydroxyalkanoates production : Effect of pH and N and P concentrations. Sci. Total Environ. 583, 300–307.

Moralejo-Gárate, H., Kleerebezem, R., Mosquera-Corral, A., Van Loosdrecht, M.C.M., 2013. Impact of oxygen limitation on glycerol-based biopolymer production by bacterial enrichments. Water Res. 47, 1209–1217.

Moretto, G., Lorini, L., Pavan, P., Crognale, S., Tonanzi, B., Rossetti, S., Majone, M., Valentino, F., 2020. Biopolymers from urban organic waste: Influence of the solid retention time to cycle length ratio in the enrichment of a Mixed Microbial Culture (MMC). ACS Sustain. Chem. Eng. 8, 14531−14539.

Morgan-Sagastume, F., 2016. Characterisation of open, mixed microbial cultures for polyhydroxyalkanoate (PHA) production. Rev. Environ. Sci. Biotechnol. 15, 593–625.

Morgan-Sagastume, F., Hjort, M., Cirne, D., Gérardin, F., Lacroix, S., Gaval, G., Karabegovic, L., Alexandersson, T., Johansson, P., Karlsson, A., Bengtsson, S., Arcos-Hernández, M.V. Magnussona, P., Werker, A., 2015. Integrated production of polyhydroxyalkanoates (PHAs) with municipal

wastewater and sludge treatment at pilot scale. Bioresour. Technol. 181, 78–89.
Morgan-Sagastume, F., Karlsson, A., Johansson, P., Pratt, S., Boon, N., Lant, P., Werker, A., 2010. Production of polyhydroxyalkanoates in open, mixed cultures from a waste sludge stream containing high levels of soluble organics, nitrogen and phosphorus. Water Res. 4, 5196–5211.
Morgan-sagastume, F., Pratt, S., Karlsson, A., Cirne, D., Lant, P., Werker, A., 2011. Production of volatile fatty acids by fermentation of waste activated sludge pre-treated in full-scale thermal hydrolysis plants. Bioresour. Technol. 102, 3089–3097.
National Geographical society, 2019. Great pacific garbage patch [WWW Document]. URL https://www.nationalgeographic.org/encyclopedia/great-pacific-garbage-patch/ (accessed 4.17.20).
Ntaikou, I., Kourmentza, C., Koutrouli, E.C., Stamatelatou, K., Zampraka, A., Kornaros, M., Lyberatos, G., 2009. Exploitation of olive oil mill wastewater for combined biohydrogen and biopolymers production. Bioresour. Technol. 100, 3724–3730.
Ntaikou, I., Peroni, C.V., Kourmentza, C., Ilieva, V.I., Morelli, A., Chiellini, E., Lyberatos, G., 2014. Microbial bio-based plastics from olive-mill wastewater : Generation and properties of polyhydroxyalkanoates from mixed cultures in a two-stage pilot scale system. J. Biotechnol. 188, 138–147.
Oakes, K., 2019. Why biodegradables won’t solve the plastic crisis [WWW Document]. BBC. URL https://www.bbc.com/future/article/20191030-why-biodegradables-wont-solve-the-plastic-crisis (accessed 4.18.20).
Oehmen, A., Pinto, F. V., Silva, V., Albuquerque, M.G.E., Reis, M.A.M., 2014. The impact of pH control on the volumetric productivity of mixed culture PHA production from fermented molasses. Eng. Life Sci. 14, 143–152.
Ojumu, T. V., Yu, J., Solomon, B.O., 2004. Production of Polyhydroxyalkanoates, a bacterial biodegradable polymer. African J. Biotechnol. 3, 18–24.
Oliveira, C.S.S., Silva, C.E., Carvalho, G., Reis, M.A.M., 2017. Strategies for efficiently selecting PHA producing mixed microbial cultures using complex feedstocks : Feast and famine regime and uncoupled carbon and nitrogen availabilities. N. Biotechnol. 37, 69–79.
Pachapur, V.L., Kutty, P., Pachapur, P., Brar, S.K., Le Bihan, Y., Galvez-Cloutier, R., Buelna, G., 2019. Seed pretreatment for increased hydrogen production using mixed-culture systems with advantages over pure-culture systems. Energies 12, 1–26.
Pagliano, G., Ventorino, V., Panico, A., Pepe, O., 2017. Integrated systems for biopolymers and bioenergy production from organic waste and by-products: A review of microbial processes. Biotechnol. Biofuels 10, 1–24.
Palmeiro-Sánchez, T., Fra-Vázquez, A., Rey-Martínez, N., Campos, J.L., Mosquera-Corral, A., 2016. Transient concentrations of NaCl affect the PHA accumulation in mixed microbial culture. J. Hazard. Mater. 306, 332–339.
Papa, G., Pepe, T.S., Carrara, A., Scaglia, B., Imporzano, G.D., Adani, F., 2020. Implementing polyhydroxyalkanoates production to anaerobic digestion of organic fraction of municipal solid waste to diversify products and increase total energy recovery. Bioresour. Technol. 318, 124270.
Pardelha, F., Albuquerque, M.G.E., Carvalho, G., Reis, M.A.M., Dias, J.M.L., Oliveira, R., 2013. Segregated flux balance analysis constrained by population structure/function data: The case of PHA production by mixed microbial cultures. Biotechnol. Bioeng. 110, 2267–2276.
Patterson, T., Massanet-Nicolau, J., Jones, R., Boldrin, A., Valentino, F., Dinsdale, R., Guwy, A., 2020. Utilizing grass for the biological production of polyhydroxyalkanoates (PHAs) via green biorefining: Material and energy flows. J. Ind. Ecol. 1–14.
Pereira, J., Queirós, D., Lemos, P.C., Rossetti, S., Sera, L.S., 2020. Enrichment of a mixed microbial culture of PHA-storing microorganisms by using fermented hardwood spent sul fi te liquor. N. Biotechnol. 56, 79–86.
Perez-Zabaleta, M., Atasoy, M., Khatami, K., Eriksson, E., Cetecioglu, Z., 2021. Bio-based conversion of volatile fatty acids from waste streams to polyhydroxyalkanoates using mixed microbial cultures. Bioresour. Technol. 323, 124604.
Pietrini, M., Roes, L., Patel, M.K., Chiellini, E., 2007. Comparative life cycle studies on poly(3-hydroxybutyrate)-based composites as potential replacement for conventional petrochemical plastics. Biomacromolecules 8, 2210–2218.
Pittmann, T., Steinmetz, H., 2014. Polyhydroxyalkanoate production as a side stream process on a municipal waste water treatment plant. Bioresour. Technol. 167, 297–302.
Pittmann, T., Steinmetz, H., 2013. Influence of operating conditions for volatile fatty acids enrichment as

a first step for polyhydroxyalkanoate production on a municipal waste water treatment plant. Bioresour. Technol. 148, 270–276.
Prabhu, R., Altman, E., Eitemana, M.A., 2012. Lactate and acrylate metabolism by Megasphaera elsdenii under batch and steady-state conditions. Appl. Environ. Microbiol. 78, 8564–8570.
Pryde, S.E., Duncan, S.H., Hold, G.L., Stewart, C.S., Flint, H.J., 2002. The microbiology of butyrate formation in the human colon. FEMS Microbiol. Lett. 217, 133–139.
Ragsdale, S.W., 2008. Enzymology of the Wood-Ljungdahl pathway of acetogenesis. Ann. N. Y. Acad. Sci. 1125, 129–136.
Ravindran, A., Adav, S., Yang, S.S., 2010. Effect of heat pre-treatment temperature on isolation of hydrogen producing functional consortium from soil. Renew. Energy 35, 2649–2655.
Reddy, M. V, Venkata Mohan, S., 2012. Influence of aerobic and anoxic microenvironments on polyhydroxyalkanoates (PHA) production from food waste and acidogenic effluents using aerobic consortia. Bioresour. Technol. 103, 313–321.
Reis, M., Albuquerque, M., Villano, M., Majone, M., 2011. Mixed Culture Processes for Polyhydroxyalkanoate Production from Agro-Industrial Surplus/Wastes as Feedstocks, in: Moo-Young M (Ed.), Comprehensive Biotechnology. Academic Press, pp. 669–683.
Rodriguez, B.A., Stowers, C.C., Pham, V., Cox, B.M., 2014. The production of propionic acid, propanol and propylene via sugar fermentation: an industrial perspective on the progress, technical challenges and future outlook. Green Chem. 16, 1066–1076.
Roibás-rozas, A., Val, Á., Hospido, A., Mosquera-corral, A., 2021. Strategies for the valorisation of a protein-rich saline waste stream into polyhydroxyalkanoates (PHA). Bioresour. Technol. 124964.
Saady, N.M.C., 2013. Homoacetogenesis during hydrogen production by mixed cultures dark fermentation: unsolved challenge. Internaltional J. Hydrog. Energy 8, 13172–13191.
Sakai, K., Miyake, S., Iwama, K., Inoue, D., Soda, S., Ike, M., 2015. Polyhydroxyalkanoate (PHA) accumulation potential and PHA-accumulating microbial communities in various activated sludge processes of municipal wastewater treatment plants. J. Appl. Microbiol. 118, 255–266.
Salmiati, Ujang, Z., Salim, M.R., Md Din, M.F., Ahmad, M.A., 2007. Intracellular biopolymer productions using mixed microbial cultures from fermented POME. Water Sci. Technol. 56, 179–185.
Sarkar, O., Kumar, A.N., Dahiya, S., Krishna, K.V., Yeruva, D.K., Mohan, S.V., 2016. Regulation of acidogenic metabolism towards enhanced short chain fatty acid biosynthesis from waste : metagenomic pro fi ling. RSC Adv. 6, 18641–18653.
Sarkar, O., Rova, U., Christakopoulos, P., Matsakas, L., 2021. Influence of initial uncontrolled pH on acidogenic fermentation of brewery spent grains to biohydrogen and volatile fatty acids production: Optimization and scale-up. Bioresour. Technol. 319, 124233.
Sattar, A., Arslan, C., Ji, C., Sattar, S., Mari, I.A., Rashid, H., Ilyas, F., 2016. Comparing the bio-hydrogen production potential of pretreated rice straw co-digested with seeded sludge using an anaerobic bioreactor under mesophilic thermophilic conditions. Energies 9, 198.
Serafim, L.S., Lemos, P.C., Oliveira, R., Reis, M.A.M., 2004. Optimization of polyhydroxybutyrate production by mixed cultures submitted to aerobic dynamic feeding conditions. Biotechnol. Bioeng. 87, 145–160.
Shah, A.A., Hasan, F., Hameed, A., Ahmed, S., 2008. Biological degradation of plastics : A comprehensive review. Biotechnol. Adv. 26, 246–265.
Sharma, S., Chatterjee, S., 2017. Microplastic pollution, a threat to marine ecosystem and human health: a short review. Environ. Sci. Pollut. Res. 24, 21530–21547.
Shen, L., Hu, H., Ji, H., Cai, J., He, N., Li, Q., Wang, Y., 2014. Production of poly (hydroxybutyrate – hydroxyvalerate) from waste organics by the two-stage process : Focus on the intermediate volatile fatty acids. Bioresour. Technol. 166, 194–200.
Sikora, A., Błaszczyk, M., Jurkowski, M., Zielenkiewicz, U., 2013. Lactic acid bacteria in hydrogen-producing consortia: on purpose or by coincidence?, in: Kongo, D.J.M. (Ed.), Lactic Acid Bacteria - R & D for Food, Health and Livestock Purposes. InTech, pp. 488–514.
Silva, F., Campanari, S., Matteo, S., Valentino, F., Majone, M., Villano, M., 2017. Impact of nitrogen feeding regulation on polyhydroxyalkanoates production by mixed microbial cultures. N. Biotechnol. 37, 90–98.
Strong, P.J., Laycock, B., Mahamud, S.N.S., Jensen, P.D., Lant, P.A., Tyson, G., Pratt, S., 2016. The opportunity for high-performance biomaterials from methane. Microorgnisms 4, 1–20.
Suresh Kumar, M., Mudliar, S.N., Reddy, K.M.K., Chakrabarti, T., 2004. Production of biodegradable plastics from activated sludge generated from a food processing industrial wastewater treatment plant.

Bioresour. Technol. 95, 327–330.
Tamang, P., Banerjee, R., Köster, S., Nogueira, R., 2019. Comparative study of polyhydroxyalkanoates production from acidified and anaerobically treated brewery wastewater using enriched mixed microbial culture. J. Environ. Sci. 78, 137–146.
Tamis, J., Lužkov, K., Jiang, Y., van Loosdrecht, M.C.M., Kleerebezem, R., 2014. Enrichment of Plasticicumulans acidivorans at pilot-scale for PHA production on industrial wastewater. J. Biotechnol. 192, 161–169.
Tamis, J., Mulders, M., Dijkman, H., Rozendal, R., van Loosdrecht, M.C.M., Kleerebezem, R., 2018. Pilot-Scale Polyhydroxyalkanoate Production from Paper Mill Wastewater: Process Characteristics and Identification of Bottlenecks for Full-Scale Implementation. J. Environ. Eng. 144, 04018107.
Tampio, E.A., Blasco, L., Vainio, M.M., Kahala, M.M., Rasi, S.E., 2019. Volatile fatty acids (VFAs) and methane from food waste and cow slurry: Comparison of biogas and VFA fermentation processes. GCB Bioenergy 11, 72–84.
Tan, G.-Y., Chen, C.-L., Li, L., Ge, L., Wang, L., Razaad, I., Li, Y., Zhao, L., Mo, Y., Wang, J.-Y., 2014. Start a Research on Biopolymer Polyhydroxyalkanoate (PHA): A Review. Polymers (Basel). 6, 706–754.
Titz, M., Kettl, K.H., Shahzad, K., Koller, M., Schnitzer, H., Narodoslawsky, M., 2012. Process optimization for efficient biomediated PHA production from animal-based waste streams. Clean Technol. Environ. Policy 14, 495–503.
Ucisik, A.S., Henze, M., 2008. Biological hydrolysis and acidification of sludge under anaerobic conditions: The effect of sludge type and origin on the production and composition of volatile fatty acids. Water Res. 42, 3729–3738.
Valencia, A.I.S., Zamora, U.R., Rodríguez, M.M., Ramírez, J.Á., Peláez, M.L.S., Ortiz, C.F., 2021. Effect of C/N ratio on the PHA accumulation capability of microbial mixed culture fed with leachates from the organic fraction of municipal solid waste (OFMSW). J. Water Process Eng. 40, 101975.
Valentino, F., Beccari, M., Fraraccio, S., Zanaroli, G., Majone, M., 2014. Feed frequency in a Sequencing Batch Reactor strongly affects the production of polyhydroxyalkanoates (PHAs) from volatile fatty acids. N. Biotechnol. 31, 264–275.
Valentino, F., Karabegovic, L., Majone, M., Morgan-Sagastume, F., Werker, A., 2015. Polyhydroxyalkanoate (PHA) storage within a mixed-culture biomass with simultaneous growth as a function of accumulation substrate nitrogen and phosphorus levels. Water Res. 77, 49–63.
Valentino, F., Lorini, L., Gottardo, M., Pavan, P., Majone, M., 2020. Effect of the temperature in a mixed culture pilot scale aerobic process for food waste and sewage sludge conversion into polyhydroxyalkanoates. J. Biotechnol. 323, 54–61.
Valentino, F., Morgan-Sagastume, F., Campanari, S., Villano, M., Werker, A., Majone, M., 2016. Carbon recovery from wastewater through bioconversion into biodegradable polymers. N. Biotechnol. 37, 9–23.
Vallari, D.S., Jackowski, S., Rock, C.O., 1987. Regulation of pantothenate kinase by coenzyme A and its thioesters. J. Biol. Chem. 262, 2468–2471.
Venkata Mohan, S., Lalit Babu, V., Sarma, P.N., 2008. Effect of various pretreatment methods on anaerobic mixed microflora to enhance biohydrogen production utilizing dairy wastewater as substrate. Bioresour. Technol. 99, 59–67.
Venkata Mohan, S., Mohanakrishna, G., Goud, R.K., Sarma, P.N., 2009. Acidogenic fermentation of vegetable based market waste to harness biohydrogen with simultaneous stabilization. Bioresour. Technol. 100, 3061–3068.
Venkata Mohan, S., Nikhil, G.N., Chiranjeevi, P., Nagendranatha Reddy, C., Rohit, M. V, Naresh Kumar, A., Sarkar, O., 2016. Waste biorefinery models towards sustainable bioeconomy: critical review and future perspectives. Bioresour. Technol. 215, 2–12.
Villano, M., Beccari, M., Dionisi, D., Lampis, S., Miccheli, A., Vallini, G., Majone, M., 2010. Effect of pH on the production of bacterial polyhydroxyalkanoates by mixed cultures enriched under periodic feeding. Process Biochem. 45, 714–723.
Waller, J.L., Green, P.G., Loge, F.J., 2012. Mixed-culture polyhydroxyalkanoate production from olive oil mill pomace. Bioresour. Technol. 120, 285–289.
Wang, X., Oehmen, A., Freitas, E.B., Carvalho, G., Reis, M.A.M., 2017. The link of feast-phase dissolved oxygen (DO) with substrate competition and microbial selection in PHA production. Water Res. 112, 269–278.
Wang, Y., Cai, J., Lan, J., Liu, Z., He, N., Shen, L., Li, Q., 2013. Biosynthesis of poly (hydroxybutyrate-

hydroxyvalerate) from the acclimated activated sludge and microbial characterization in this process. Bioresour. Technol. 148, 61–69.

Wen, Q., Chen, Z., Tian, T., Chen, W., 2010. Effects of phosphorus and nitrogen limitation on PHA production in activated sludge sludge. J. Environ. Sci. 22, 1602–1607.

Wen, Q., Chen, Z., Wang, C., Ren, N., 2012. Bulking sludge for PHA production: Energy saving and comparative storage capacity with well-settled sludge. J. Environ. Sci. 24, 1744–1752.

Zhang, D., Jiang, H., Chang, J., Sun, J., Tu, W., Wang, H., 2019. Effect of thermal hydrolysis pretreatment on volatile fatty acids production in sludge acidification and subsequent polyhydroxyalkanoates production. Bioresour. Technol. 279, 92–100.

Zhang, M., Wu, H., Chen, H., 2014. Coupling of polyhydroxyalkanoate production with volatile fatty acid from food wastes and excess sludge. Process Saf. Environ. Prot. 92, 171–178.

Zhu, S.C., Xu, Z.L., Meng, H.J., Zhou, J., Chen, H., 2012. Effect of magnetic field on the accumulation of polyhydroxyalkanoates (PHAs) by microorganism in activated sludge. Bioprocess Biosyst. Eng. 35, 985–991.

Chapter 2

Experimental methods and materials

This chapter provides the comprehensive coverage of the materials and methods used in the research work. The list includes the inoculum used, the wastewaters fermented, the methodology employed for acidogenesis and feast-famine enrichment, the quantitative and qualitative analysis, equations and models describing the process. These procedures combined derived the outcome of the study examined in the subsequent chapters.

2.1. Biomass sludge used as inoculum

2.1.1. Anaerobic inoculum sludge

Anaerobic sludge performed as a biocatalyst for the conversion of organic content into volatile fatty acids (VFAs). Gobar gas plant sludge (GPS) was collected from a digester's outlet at a nearby location installed by IIT Guwahati. The plant was fed with a mixture of cow dung and duckweed. The plant was operational during sample collection. Another anaerobic inoculum sludge for the study was brewery anaerobic sludge (BAS), collected from a brewery near IIT Guwahati. BAS was taken from the lowest part of an up-flow anaerobic sludge blanket reactor treating brewery effluent continuously for an extended period. The sludge storage (1 L) was in a glass bottle, tightly sealed, and nitrogen purged at 4 °C until further use.

2.1.2. Activated sludge (Aerobic)

The activated sludge acted as the biocatalyst for carbon transfer into PHB, collected from two extremely different sources (Table 2.1). Petroleum refinery sludge (PRS) was collected from a petroleum refinery in Assam, India. The source of PRS was an aeration tank that received dissolved air flotation treated petroleum refinery effluent. The second sludge, brewery sludge (BS), was acquired from a brewery near IIT Guwahati, Assam, India. The outlet of the biomethanation reactor treating brewery effluent fed the aeration tank from which was the source of BS. Overnight aeration of collected sludge samples removed any available ammonia and soluble chemical oxygen demand (COD). The storage of extra samples is at 4 °C until further use. Initially, the aerated sludge samples were batch acclimatized for 20 days in once a day feed mode by replacing 50 % volume each day. The feed was synthetic wastewater with sodium acetate as the carbon source (COD 6 g/L) at C:N:P::100:7:1 and mineral salt media. The composition of synthetic wastewater ensured that the carbon and nitrogen are available for 24 h without pushing the cultures into any kind of stress. These acclimatized sludge samples are the inoculum for enrichment reactors.

2.2. Effluent streams used as fermentation medium

The substrate for acidogenic conversion was selected from the regionally available potential effluent sources. Rice mill Effluent (RME) was collected from a rice mill, while the brewery effluent (BE) was collected from a brewery located near IIT Guwahati. This accessibility dealt with the matter of sample collection and transportation. The effluent samples were collected and stored at 4 °C until further use.

2.3. Experimental layout

2.3.1. Batch acidogenic fermentation

All the experiments were carried out in 100 mL serum vial sealed with butyl rubber/aluminum cap with working volume 90 mL and 10 mL headspace. The sludge was washed with distilled water to remove the soluble COD prior to inoculation. The inoculum used initially for optimizing the food to microbe ratio (F/M) was the gobar gas plant sludge. Three F/M ratios of 0.5, 1.0, and 2.0 gCOD/gVSS were set by adjusting the sludge volume with RME. The reactors are incubated at 35 °C, 180 rpm, with pH adjusted to 6.0. The optimized F/M value was used for further studies.

2.3.1.1. Pretreatment of anaerobic inoculum sludge

Investigations to influence VFA content was done through pretreatment of the gobar gas plant and brewery anaerobic sludge. The pretreatment protocol used was a modified cyclic heat-acid shock (Venkata Mohan et al., 2009). The sludge transfer between heat shock at 100 °C for 2 h and acid shock at pH 3.0 (adjusted using 88 % orthophosphoric acid) for 24 h, repeated 3 times, ensured proper treatment. The sludge exposed to air during heat shock was nitrogen purged and sealed during acid shock. The pH of sludge was adjusted to 6.0, and volume made up after 3 cycles of pretreatment. Glucose was added to the pretreated sludge and kept for 24-48 h at 35 °C to revive the sludge. The revived-pretreated sludge and untreated sludge at F/M ratio 1.0 were transferred to the reactor with RME and BE (filtered through 0.45μm nylon filter paper, Axiva, India) and incubated at 35 °C with pH adjusted to 6.0. Sampling was carried out at an interval of 12 h for 10 days to analyze COD, and VFA content. Nitrogen purging at 24 h interval maintained anaerobic conditions where an outlet removes nitrogen purged along with gases produced within the interval. All the Experiments performed in the study were in duplicates.

2.3.1.2. Lab-scale batch fermentation for acidogenic bioconversion of RME

Acidogenic fermentation of RME (raw effluent) employed a batch reactor with 2 L working volume having a 200 mL headspace. The reactor was tightly sealed using a rubber cork with an inlet and outlet for purging nitrogen for an anaerobic condition or sampling. The gobar gas plant sludge was pretreated under cyclic heat and acid shock for three cycles and revived for 48 h using glucose as substrate (Section 2.3.1.1). The pretreated anaerobic inoculum was inoculated into RME at an F/M ratio of 1.0 in the batch reactor. The initial pH was set at 6.0 and left uncontrolled for the entire run. The reactor sealed using the rubber cork and parafilm film wrapped around was purged (bubbled) with nitrogen gas for 15 mins with constant stirring to create the anaerobic condition. The temperature was maintained at 30-35 °C with continuous stirring using a magnetic stirrer hot plate. Samples were withdrawn using a syringe for analyzing COD, VFA, and pH.

2.3.2. Sequencing batch reactor for mix culture enrichment

A batch reactor operated in a daily feed and draw mode is the ideal process for any kind of enrichment protocol. Aerobic dynamic feeding strategy implemented using such sequencing batch reactors (SBRs) exposed the sludge to a short period of carbon excess (feast) and an extended period of carbon starvation (famine) phase for an extended period.

2.3.2.1. Sequential enrichment of PRS and BS under nitrogen excess and limited conditons

PRS and BS were inoculated to four 2.4 L open/non-axenic glass reactors, fabricated in the lab using waste reagent bottles, with 2 L working volume at F/M:1. The chief operating conditions implemented in the 4 reactors are as shown in Table 2.1. The reactors operated under a 24 h cycle had a fill phase (10 min), aerobiosis phase (23 h), settling phase (40 min), decant phase (5 min), and idle phase (5 min). The SBRs ran solely with a volumetric exchange ratio of 50 % for the initial 30 days, after which a sludge wastage of 200 ml (mixed liquor) was included prior to decanting at the end of the cycle to maintain sludge retention time (SRT) at 10 d. The sludge wastage volume changed according to the volatile suspended solids (VSS) present in the decant phase. The synthetic media fed to the reactors comprises mineral salt medium with composition as follows (per liter): 0.2 g $MgSO_4.7H_2O$, 0.025 g $CaCl_2.2H_2O$, and 1.0 ml/L of trace element solution. The trace elements solution was of the following composition (per liter): 0.78 g $CuSO_4.5H_2O$, 5 g $FeSO_4.7H_2O$, 12.6 g $NaMoO_4.2H_2O$, 4.1 g $NiCl_2.6H_2O$, 4.4 g $ZnSO_4.7H_2O$, 2.4 g $CoCl_2.6H_2O$, 3 g H_3BO_3, 5 g $MnCl_2.4H_2O$, and 5 g EDTA. Sodium acetate, $CH_3COONa.3H_2O$, was supplied as a carbon source with chemical oxygen demand (COD) of SBRs maintained around 3.0 g/L. The nutrient level was maintained using K_2HPO_4, KH_2PO_4 (65 and 35 % respectively) as the source of phosphorus, and NH_4Cl as the source of nitrogen (ammoniacal nitrogen-N-NH_4), which varied according to C:N:P ratio maintained appropriately. The C/N ratio of SBR3 and SBR4 increased in 3 phases, phase I-C/N:25, phase II- C/N:50, phase III- C/N:100 was used to determine

Table 2.1: Operating condition for the sequencing batch reactor for sludge enrichment under aerobic dynamic feeding.

Operating Parameters	Sequencing batch reactors for enrichment			
	SBR1	SBR2	SBR3	SBR4
Inoculum	PRS	BS	PRS	BS
Inoculum concentration, VSS (g/L)	4	4.6	4.7	4.2
C:N:P	100:7:1	100:7:1	Phase I - 100:4:1 Phase II - 100:2:1 Phase III - 100:1:1	Phase I - 100:4:1 Phase II - 100:2:1 Phase III - 100:1:1
COD (g/L)	3	3	3	3
HRT (d)	2	2	2	2
SRT (d)	10	10	10	10
Cycle length (h)	24	24	24	24

HRT: hydraulic retention time

the limiting nitrogen value. The pH of the reactors was allowed to vary (≈ 8.2-9.7), while the temperature depended on the ambient condition. Continuous aeration managed dissolved oxygen (DO) using aquarium pumps to ensure airflow rate ≥ 2 L/min and a constant DO of 6-7 mg/L. Mixing in the reactor was provided using a magnetic stirrer so that the stirrers were switched off after the feast period to prevent any issue arising from the overuse of equipment. A sampling at an interval of 5 days was performed at the end of the feast phase and at the end of the cycle to determine the PHB storage, VSS, COD, DO, ammoniacal nitrogen (N-NH_4) during enrichment studies. The reactor's stability considered two consecutive readings of PHB storage, COD, and N-NH_4 consumption at the end of the feast to be within 5-10 % of each other.

2.3.2.2. Sequential enrichment of BS under different sludge retention time

The enrichment of MMC carried out in two sequential batch reactors (SBR) had a total volume of 3 L, working volume of 2 L, the headspace of 1 L, and sampling ports at 500 mL interval with an L/D ratio of 3.81. The SBRs made of acrylic tubes were mounted on magnetic stirrers and fastened to a steel frame using steel clamps. An air compressor provided the required aeration and stirring by the magnetic stirrers (Fig. 2.1).

The collected brewery sludge was acclimatized to the lab condition in a once-a-day draw and feed mode in a batch reactor for a week using synthetic media containing micronutrient and sodium acetate as a carbon source at a C:N:P ratio of 100:8:1. The acclimatized sludge was inoculated into the SBRs under an F/M ratio of 1.0. SBR operated in a sequential draw and feed mode with a 24 h cycle had 4 distinct phases, feeding (10 min), aerobic reaction (23 h), settling (40 min), and decant (10 min). The SBRs were fed with synthetic media having micro and macronutrient in the composition as given in Section 2.3.2.1. The carbon source in the media was sodium acetate (from

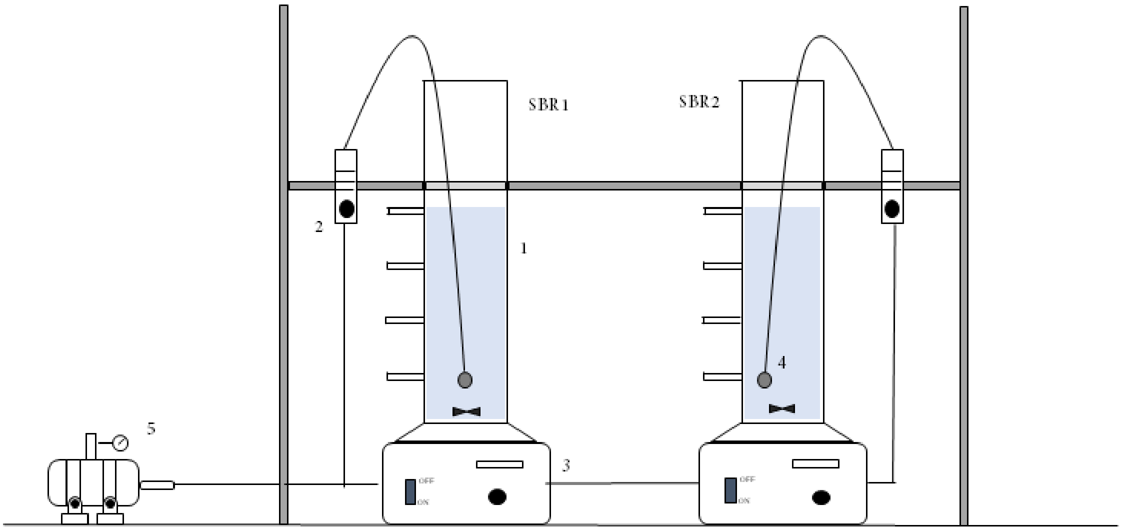


Figure 2.1: A schematic representation of reactor set up for brewery sludge enrichment. 1-sequential batch reactors made of acrylic tube, 2- Flow meter, 3-magnetic stirrers, 4- ceramic air diffusers, 5- air compressor.

here on simply acetate) with a soluble chemical oxygen demand (COD) maintained around 3 g/L and C:N:P ratio of 100:4-3:1. The hydraulic retention time (HRT) was fixed at 5 d with a volume exchange ratio of 25 %. The SBRs were maintained at two different SRTs. SBR1 was maintained at SRT of 5 d by wasting 400 mL and SBR2 at an SRT of 10 d by wasting 200 mL mixed sludge at the end of each cycle. The volume of sludge wasted was adjusted according to volatile suspended solid (VSS) content in the effluent decanted. The SBRs were run under two phases, Phase I without SRT control and Phase II with SRT control. The pH of synthetic media was around 7.0-7.5 while it allowed to vary in the reactor. The temperature fluctuated according to the ambient condition. The bioprocess monitoring was done for 60 days using samples taken at 5 day interval for VSS, COD, N-NH_4, and PHB analysis. The reactor stability was accessed with two consecutive sampling giving PHB content, COD, feast phase varying within 5-10%.

2.3.3. PHB accumulation assays for maximizing PHB accumulation

2.3.3.1. Batch accumulation studies with enriched PRS and BS under different nitrogen available conditions.

It is necessary to maintain a constant condition for evaluating the maximum PHB accumulated by the enriched sludge. Batch reactors are the simplest way to implement such a multi-component condition consistent for a long time. The batch assays in 4 open/non-axenic glass reactors had 1 L volume and 400 mL working volume. 200 mL of excess sludge wasted at the end of the cycle from SBR1, SBR2, SBR3, and SBR4 was inoculated into batch reactors BR1, BR2, BR3, and BR4, respectively (thereby maintaining the SRT and ensuring reactor performance). Three experimental runs executed finalized the optimal batch assay condition. Run 1- nitrogen excess condition (100:8:1), Run 2- nitrogen limitation (10:0.5:1), Run 3- nitrogen starvation (100:0.01:1). Maintaining nitrogen concentration at zero was impossible in nitrogen starvation as 1-3 ppm N-NH_4 was always found in the batch reactor. Preparation of feed was similar to that fed into SBRs, with COD maintained slightly higher to the tune of about 4 g/L. The aerobic reactions ran for 12 h with samples taken at four retention times (RT) each, RT1- 0 h sample, RT2- the point where the decrease in DO ceases, RT3- DO value reaches near the initial, RT4- the culmination of the batch assay. All reaction was carried out at an ambient temperature and without pH control. Mixing was provided using a magnetic stirrer and DO maintain through aeration using an aquarium pump. All experimental results are the mean of duplicates.

2.3.3.2. Accumulation studies with enriched BS under different feeding regimes

PHB accumulation assays were carried under different feeding regimes to identify the upper limit of storage for the enriched sludge. The accumulation reactor R1 inoculated with sludge from SBR1 and R2 with sludge collected from SBR2 had a volume of 1 L, a working volume of 600 mL, and a 400mL headspace. The sludge wasted at the end of the famine phase (end of cycle) is inoculated to the accumulation reactors without affecting the set SRT at an inoculum to feed ratio (I/F) ratio of 1:2 (v/v) by inoculating 200 mL of enriched sludge. The 400 mL collected from SBR1 is concentrated to 200 mL and inoculated to R1. The accumulation reactors had conditions similar to that of SBRs fed with synthetic media. All the assays were done to use RME as the media. Hence, the stock synthetic media prepared had COD of 3-3.5 g/L that get diluted 1.5 times similar to RME. No nitrogen source was added as the residual nitrogen from the SBRs gets diluted (3x dilution of inoculum), creating a nitrogen deficient-limited condition with a C:N:P ratio of 100:0.25-0.5:1.

Two principal accumulation reactor types used in the study were batch (BR1/BR2) and fed-batch (FBR1/FBR2). The aerobic reaction was monitored through dissolved oxygen (DO) measurement. In the batch assay, the reaction was stopped when there is no further increase in DO (saturation). Depending on the reaction time in the batch assay, the flow rate was set for the fed-batch assay with continuous feeding so that the working volume gets filled by the same time. The accumulation assays were also carried out in fed-batch with pulse-wise feeding as laid out by Chen et al. (Chen et al., 2017). The first pulse was fed at the beginning of the assay. After the reaction ended, 1 h settling and decant, the second pulse was fed. Each pulse and decanted volume was

400 mL (66 % of working volume). The pulse addition and duration of the reaction were according to the increase in DO (saturation). Sodium acetate was the carbon source for all the assays. Meanwhile, assays using RME and fermented RME was done in fed-batch reactor with pulse-wised feed addition. Magnetic stirrers were used for mixing, aquarium pumps for aeration, and pump for feeding in continuous mode. Samples were taken at the initial and final point of each reaction phase in all the accumulation assays for analyzing VSS, COD, acetate, N-NH_4, PHB.

2.4. Analytical methods and analysis

The COD analyzed by the closed reflux method, N-NH_4 by the phenate method, phosphorus phosphate (P-PO_4) by the stannous chloride method, total and volatile suspended solids were according to Standard Methods (APHA, 2005). The dissolved oxygen was measured in mg/L using a DO meter (Thermo Scientific, Massachusetts, U.S.A), while pH was analyzed using a pH meter (Metler Toledo, Ohio, U.S.A).

2.4.1. VFA analysis

The total volatile fatty acid (TVFA) composition was analyzed by a method adapted from Dahiya et al. (Dahiya et al., 2015). The analysis performed using high-performance liquid chromatography (HPLC) had C18 reverse-phase column (250 x 4.6 mm diameter; 5 µm particle size-Agilent) and UV–Vis detector at 210 nm (Agilent, U.S). A mobile phase consisting of 40 % methanol in 1 mN H_2SO_4, pH, 2.5–3.0 at a flow rate of 0.6 mL/min, and 40 µl sample injection volume achieved the sample separation. The summation of the individual VFA concentration gives TVFA content, and VFA_{max} gives the peak TVFA value (mg/L).VFA was monitored during the experiment using the Montgomery method (Siedlecka et al., 2008).

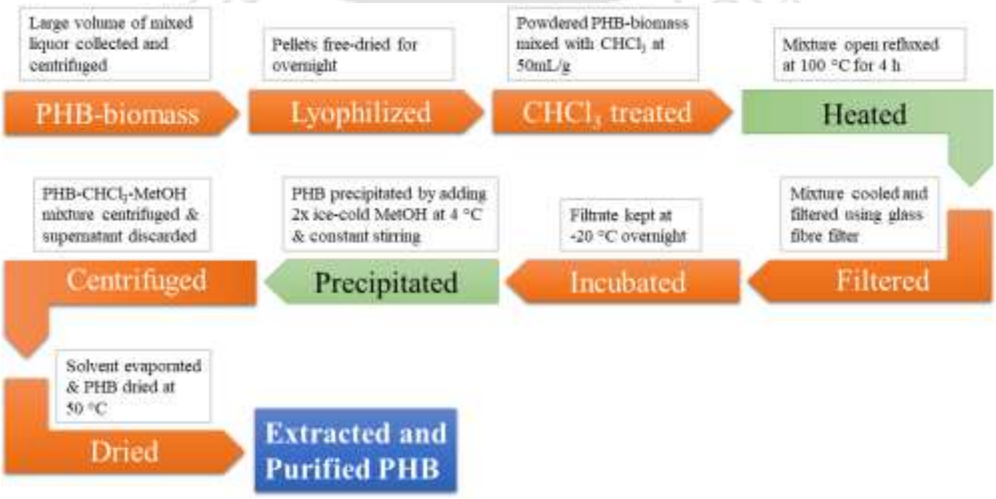


Figure 2.2: Flow chart of the downstream processing steps for PHB recovery

2.4.2. PHB estimation, recovery, purification

Thereby the monitoring of PHB production during the entire run of the SBRs was done through estimation of PHB extracted by a method standardized from the procedure given by Hahn et al. (Hahn et al., 1994). 7-8 mL of mixed liquor taken from the reactor was centrifuged at 7168 *x* g for 10 min, room temperature. The pellet was vortexed with an equal volume of 1:1 ratio of NaOCl:$CHCl_3$. The dispersion was transferred into a 100 mL screw-capped conical flask and kept at 37 °C for 3 h at 180 rpm. After the incubation period, the dispersion is centrifuged at 3000 *x* g for 20 min at room temperature resulting in three distinct layers. The bottom $CHCl_3$ phase gets separated from the top and middle NaOCl and the non-polymer cell-matrix layer, respectively, by pipetting out into glass tubes. The $CHCl_3$ is evaporated and subjected to crotonic acid assay by mixing with 10 ml of 18 M Conc. H_2SO_4 and subsequent heating at 100 °C for 10 min in a water bath. The digested solution is instantaneously cooled to stop the reaction, and the optical density measured at 235 nm using UV-VIS Spectrophotometer (Carybio 100, Agilent, California, U.S.A) (Amulya et al., 2016). The standards were prepared in a similar manner using P(3HB-co-2HV) (86:14 %, Sigma Aldrich, Missouri, U.S.A) (Law and Slepecky, 1960), (Nordeste et al., 2010).

Solvent extraction recovered the accumulated polymer through open refluxing mode for further analysis (Fig. 2.2). Lyophilized biomass was added to $CHCl_3$ (50 mL/g of biomass) and heated at 100 °C for 4 h (Kourmentza et al., 2015). The heating breaks the biomass releasing internal materials into solvent where soluble components get dissolved in the solvent. The mixture cooled to room temperature, filtered through a 0.45µm glass fiber filter (Axiva, Delhi, India) stored overnight at -20 °C. The dissolved components are precipitated at 4 °C, adding 2x ice-cold methanol under constant stirring and kept in -20 °C overnight to augment precipitation. The mixture obtained is centrifuged at 8000 rpm for 10min and the supernatant removed. Methanol was added to the pellet, vortex, centrifuged at the same condition, and dried at 50 °C till constant weight. This process eliminates impurities from the precipitate (now in white color). Crotonic acid assay of the precipitate had indicated the presence of PHB as a component in the mixture. The precipitate was submitted for characterization to deduce physiochemical properties, thereby confirming the presence of PHB in the mixture.

2.4.3. Qualitative analysis of PHB

Qualitative analysis of the extracted compound elucidates the chemical structure, evaluate the thermal and physical properties, thereby concluding and confirming the compound accumulated and extracted from enriched activated sludge. The analyses for functional group identification, deduction of chemical structure, molecular arrangement, thermal property, and molecular size are given below-

2.4.3.1. Functional group Identification: The production of PHB was confirmed simultaneously by identifying the functional groups using Fourier transform infrared radiation spectra (Spectrum series, Perkin Elmer, Massachusetts, U.S.A). About 20-40 µL of chloroform phase after the extraction applied on the diamond surface after cleaning with methanol/acetone, the chloroform was allowed to evaporate, after which the scan performed and spectra captured.

2.4.3.2. Deduction of Chemical structure: The chemical structure of the precipitate was deduced using nuclear magnetic resonance spectroscopy, thereby correlating the outcome with that of quantitative analysis to confirm the result of the PHB accumulation step. 1H and 13C spectra were recorded in 600 MHz NMR, Bruker ASCEND 600, Massachusetts, U.S.A at 20 °C. 0.5-2 mg of purified precipitate was dissolved in $CDCl_3$ in a quartz tube for the analysis with 1000 repeated scans for 13C. The spectra are processed in MestReNova.

2.4.3.3. Analyzing the molecular arrangement: Powder X-ray diffraction spectrometer (Agilent, California, U.S.A) equipped with Mo X-ray source, CCD detector (Eos), Oxford cryo system (80-500 K) and crystal alisPRO software and Autochem software was employed to identify the crystalline or amorphous structure of the precipitate (10-20 mg) at 2ϴ in the range 5-80 degree at 20 °C.

2.4.3.4. Evaluating the thermal property: Thermogravimetric analysis with simultaneous differential scanning calorimetry, TG/DSC was carried out under a temperature range of 20- 800 ˚C (TG/DSC STA449F3A00, Netzsch, Wunsiedel, Germany). 5-10 mg of the purified precipitate was taken in an aluminum pan for the analysis at a heating rate of 10 K/min under an inert atmosphere of argon (20-60 ml/min). TGA and DSC curve was plotted together and interpreted accordingly.

2.4.3.5. Molecular size estimation: Number average molecular weight (M_n), weight average molecular weight (M_w), and polydispersity index (PDI) were assessed here. 20-40 mg of purified precipitate dissolved in HPLC grade CH_3Cl, filtered and injected to gel permeation chromatography, using HPLC attached with refractive index detector (Shimadzu, Kyoto, Japan) (Bhagabati et al., 2019)

2.4.4. Microbial community analysis

The batch accumulation reactors with maximum output inoculated with PRS and BS were selected, and microbial community analysis performed for these two samples. The samples were collected at the end of the reactor run and stored at 4 °C. 16s rRNA gene amplicon analysis (Miseq) determined the microbial community distribution of the enriched sludge exposed to nitrogen availability to maximize PHB accumulation. The sequencing of samples was carried out by a private company (AgriGenome, India).

2.5. Calculations of bioprocess parameters

2.5.1. Volatile fatty acid production

The degree of acidification (DA) gives the percentage of influent converted to VFAs, calculated using Eq. 1. The TVFA content in terms of COD gives COD_{VFA} (mg/L). COD stability (ΔCOD) is the percent variation in COD between pretreated and untreated systems with RME (ΔCOD_{RME}) and BE (ΔCOD_{BE}) (Eq. 2). pH stability (ΔpH) shows the percent variation in pH between pretreated and untreated systems with RME (ΔpH_{RME}) and BE (ΔpH_{BE})(Eq. 3). ΔCOD and ΔpH, in a way, imply the efficiency of pretreatment. The yield coefficient calculated in the study is the ratio of VFA produced to nonVFA (the part of organic content contributed by other than VFA) consumed at the VFA_{max} (mg COD/L). In Eq. 4, $COD_{nonVFAmax}$ is the part of COD other than VFA at the peak, and COD_{VFAi}, or $COD_{nonVFAi}$ denotes the initial values in the expression (mgCOD/L). The yield for TVFA ($Y_{TVFA/nonVFA}$), HAc ($Y_{HAc/nonVFA}$), HPr ($Y_{HPr/nonVFA}$), HBu ($Y_{HBu/nonVFA}$), HVa ($Y_{HVa/nonVFA}$) is calculated from Eq.4 (mgCOD/mgCOD). Even to odd ratio (HB precursor/HV precursor) is the ratio of the sum of HAc and HBu content to the sum of HPr and HVa content (mg/mg).

$$DA = COD_{vfa}/COD_{influent} \tag{1}$$

$$\Delta COD = (COD_{pretreted} - COD_{untreated})/COD_{pretreated} \tag{2}$$

$$\Delta pH = (pH_{untreated} - pH_{pretreated})/pH_{untreated} \tag{3}$$

$$Y_{VFA/nonVFA} = (COD_{VFAmax} - COD_{VFAi})/(COD_{nonVFAi} - COD_{nonVFAmax}) \tag{4}$$

$$PR_{VFA} = (VFA_{max} - VFA_{initial})/T \tag{5}$$

The volatile fatty acid production rate (PR_{VFA}) differentiated kinetically between the pretreated and untreated systems concerning individual VFAs. The production rate is as given by Eq. 5, where VFA_{max} is the VFA content at the peak retention time (mg/L), $VFA_{initial}$ is the VFA content present at the start of the experiment (mg/L), and T is the peak retention time (h) (Dahiya et al., 2015).

2.5.2. PHB production

The stoichiometric and kinetic parameters show the effects of enrichment and accumulation on PHB production. PHB storage calculated as the fraction of VSS (cell dry weight) contributed by PHB (gPHB /gVSS) is expressed as % PHB. The calculation for the weight of active biomass is X (g) =gVSS - gPHB. The feast to famine ratio (f/f) was calculated as the ratio of the feast phase to the famine phase. The COD conversion values were 1.67 mgCOD/mgHB monomer, for PHB, 1.066 mgCOD/mgAc for acetate (Ac) and 1.42 mgCOD/mgX. The yield coefficient for PHB, $Y_{P/S}$, is expressed as the ratio of PHB produced to acetate consumed. The PHB produced from the two-pulse fed during fed-batch accumulation is expressed as overall $Y_{P/S}$. The PHB yield upon consumption of TVFA and acetate+butyrate (HAc+HBu) is $Y_{P/TVFA}$ and $Y_{P/HAc+HBu}$ ($mgCOD_{PHB}/mgCOD_S$). Meanwhile, $Y_{X/S}$ shows the active biomass growth from the consumption of acetate ($mgCOD_X/mgCOD_S$). Specific PHB production rate (q_p) is the ratio of the amount of PHB produced per unit of active biomass and the time taken to produce, expressed as $mgCOD_{PHB}/mgCOD_X h$. Specific substrate consumption ($-q_s$) rate is the ratio of the amount of acetate consumed per unit active biomass and the time taken to consume, expressed as $mgCOD_S/mgCOD_X h$. The rate of PHB production (r_p) is determined from the amount of PHB produced till the feast and the time taken to produce, expressed as $mgCOD_{PHB}$ /Lh. Similarly, the PHB consumption rate ($-r_p$) was calculated as the amount of PHB consumed until the end of famine and the time taken to consume, which, expressed as $mgCOD_{PHB}$/Lh. The amount of PHB produced per hydraulic retention time gives PHB productivity, $mgCOD_{PHB}$ /Lh (Colombo et al., 2016).

The distribution of molecular weight is obtained from the polydispersity index (PDI), which is the ratio of weight average molecular weight (M_w) and number average molecular weight (M_n) {PDI = M_w / M_n}. The weight-average degree of polymerization (DP_w) is determined as the ratio of weight average molecular weight (M_w) and molecular weight of monomer (M_o) {$DP_w = M_w / M_o$}. Crystallinity Index (CI) is obtained from the x-ray diffraction spectrum, calculated as the ratio of the area of crystalline peaks and total area of spectrogram peaks. The average crystalline size (L, nm) is derived from Scherer's formula, L= Kλ/βCosϴ where K=0.983, λ=0.154 nm, β= peak center of crystalline peaks, ϴ= half of X-ray diffraction angle (Kavitha et al., 2016).

The Eq. 6 was used to calculate and maintain the SRT (Liu and Tay, 2007). In the equation, X_r=VSS content in the reactor (mg/L), V_r= working volume of the reactor (L), t_c = cycle length (d), X_w= VSS content in the sludge wasted (mg/L), V_w= volume of sludge wasted (L), X_e = VSS content in effluent discharged (mg/L), V_e volume of effluent discharged (L).

$$SRT(d) = \frac{X_r V_r t_c}{(X_w V_w + X_e V_e)} \tag{6}$$

2.6. Kinetics and model fitting

2.6.1. Kinetics model for VFA accumulation

The modified Gompertz equation better explains the dynamic behavior of the VFA accumulation, Eq. 7. Gompertz equation gives the relation between cumulative acid concentration and retention time. In Eq.7, A is the cumulative TVFA accumulation at time t; A_{max} is the cumulative maximum of acid accumulated per liter; R_{max} is the maximum rate of acid accumulation; λ is the lag time to exponential acidification (Mu et al., 2006). The variables A and t were adjusted using the Solver program (Excel, 2010) to determine the best fit value of the biokinetic constants R_{max}, A_{max}, and λ.

$$A = A_{max} \exp\left\{-\exp\left[\frac{R_{max} \cdot e}{A_{max}} (\lambda - t) + 1\right]\right\} \tag{7}$$

2.6.2. Mass balance and kinetic model fitting for PHB production

The Eq 8 gives the material balance for the enrichment process (feast phase), where ΔY denotes the global yield of all the carbon recovered, and $Y_{CO_2/s}$ denotes the respiratory yield (C-mgCO_2/mgCOD_{Ac}), calculated from the cumulative CO_2 (in terms of carbon) evolved by respiration per substrate consumed. The CO_2 produced was calculated by integrating the curve under the experimental oxygen uptake rate (mg/Lh) over the time taken for substrate consumption such that 1 mol of O_2 consumed produces 1 mol of CO_2 (Albuquerque et al., 2010). The reduction in DO as a function of time gives the oxygen uptake rate (EPA, 2001).

The model proposed by Beun et al. (Beun et al., 2000) was employed to predict the kinetic behavior of PHB storage during MMC enrichment. Eq 9 denotes the linear equation for the feast period, where $-r_s$= acetate uptake rate (mgCOD/Lh), r_{xs} = active biomass growth rate by acetate consumption (mgCOD/Lh), r_p=PHB production rate (mgCOD/Lh), $Y_{X/S}$=active biomass growth yield obtained by the consumption of acetate (mgCOD/mgCOD), $Y_{P/S}$=PHB yielded by the consumption of acetate (mgCOD/mgCOD), m_s=maintainance coefficient during feast phase by acetate uptake (mgCOD/mgCODh), C_{XS}=active biomass concentration upon acetate consumed. Similarly, Eq 10 denotes the linear equation for famine period where $-r_p$=PHB consumption rate (mgCOD/Lh), r_{xp}=active biomass growth rate by PHB consumption (mgCOD/Lh), $Y_{X/P}$= active biomass growth yield obtained by the consumption of PHB (mgCOD/mgCOD), m_p=maintainance coefficient during famine phase by PHB uptake (mgCOD/mgCODh), C_{xp}=active biomass concentration upon PHB consumed. Plotting $-r_s$ against C_{XS} and $-r_p$ against C_{XP} determines m_s and m_p from the slope of the curve. The predicted PHB yield, $Y_{P/S}^{Pred.}$, is derived from the curve.

$$\Delta Y = Y_{X/S} + Y_{P/S} + Y_{CO_2/S} \tag{8}$$

$$(-r_s) = \frac{1}{Y_{X/S}}(r_{XS}) + \frac{1}{Y_{P/S}}(r_p) + m_s C_{XS} \tag{9}$$

$$(-r_p) = \frac{1}{Y_{X/P}}(r_{XP}) + m_p C_{XP} \tag{10}$$

References

Albuquerque, M.G.E., Torres, C.A. V, Reis, M.A.M., 2010. Polyhydroxyalkanoate (PHA) production by a mixed microbial culture using sugar molasses : Effect of the influent substrate concentration on culture selection. Water Res. 44, 3419–3433.

Amulya, K., Reddy, M.V., Rohit, M. V., Venkata Mohan, S., 2016. Wastewater as renewable feedstock for bioplastics production: Understanding the role of reactor microenvironment and system pH. J. Clean. Prod. 112, 4618–4627.

APHA, 2005. Standard Methods for the Examination of Water and Wastewater, 21st ed. American public health association /American water works association/ water environment federation, Washington DC.

Beun, J.J., Paletta, F., Van Loosdrecht, M.C.M., Heijnen, J.J., 2000. Stoichiometry and kinetics of poly-β-hydroxybutyrate metabolism in aerobic, slow growing, activated sludge cultures. Biotechnol. Bioeng. 67, 379–389.

Bhagabati, P., Hazarika, D., Katiyar, V., 2019. Tailor-made ultra-crystalline, high molecular weight poly(ε-caprolactone) films with improved oxygen gas barrier and optical properties: a facile and scalable approach. Int. J. Biol. Macromol. 124, 1040–1052.

Chen, Z., Huang, L., Wen, Q., Zhang, H., Guo, Z., 2017. Effects of sludge retention time, carbon and initial biomass concentrations on selection process : From activated sludge to polyhydroxyalkanoate accumulating cultures. J. Environ. Sci. 2, 76–84.

Colombo, B., Tommy, P.S., Maria, R., Barbara, S., Fabrizio, A., 2016. Polyhydroxyalkanoates (PHAs) production from fermented cheese whey by using a mixed microbial culture. Bioresour. Technol. 218, 692–699.

Dahiya, S., Sarkar, O., Swamy, Y.V., Venkata Mohan, S., 2015. Acidogenic fermentation of food waste for volatile fatty acid production with cogeneration of biohydrogen. Bioresour. Technol. 182, 103–113.

EPA, 2001. Specific Oxygen Uptake Rate in Biosolids (Method 1683).

Hahn, S.K., Chang, Y.K., Kim, B.S., Chang, H.N., 1994. Optimization of microbial poly(3-hydroxybutyrate) recover using dispersions of sodium hypochlorite solution and chloroform. Biotechnol. Bioeng. 44, 256–261.

Kavitha, G., Kurinjimalar, C., Sivakumar, K., Palani, P., Rengasamy, R., 2016. Biosynthesis, purification and characterization of polyhydroxybutyrate from Botryococcus braunii kütz. Int. J. Biol. Macromol. 89, 700–706.

Kourmentza, C., Ntaikou, I., Lyberatos, G., Kornaros, M., 2015. Polyhydroxyalkanoates from Pseudomonas sp. using synthetic and olive mill wastewater under limiting conditions. Int. J. Biol. Macromol. 74, 202–210.

Law, J.H., Slepecky, R.A., 1960. Assay of poly-beta-hydroxybutirate acid. J. Bacteriol. 82, 33–36.

Liu, Y.Q., Tay, J.H., 2007. Influence of cycle time on kinetic behaviors of steady-state aerobic granules in sequencing batch reactors. Enzyme Microb. Technol. 41, 516–522.

Mu, Y., Wang, G., Yu, H.Q., 2006. Kinetic modeling of batch hydrogen production process by mixed anaerobic cultures. Bioresour. Technol. 97, 1302–1307.

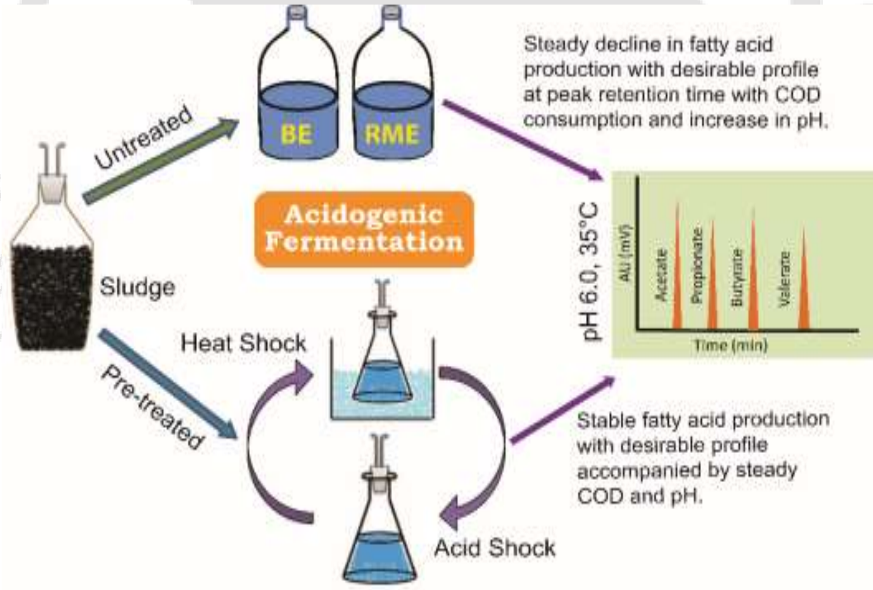
Nordeste, R.F., Trainer, M.A., Charles, T.C., 2010. Methods for the Isolation of Genes Encoding Novel PHB Cycle Enzymes from Complex Microbial Communities, in: Streit, W.R., Daniel, R. (Eds.), Metagenomics:Methods in Molecular Biology (Methods and Protocols). Humana press, Totowa, New Jersey, pp. 235–246.

Siedlecka, E.M., Kumirska, J., Ossowski, T., Glamowski, P., Gołębiowski, M., Gajdus, J., Kaczyński, Z., Stepnowski, P., 2008. Determination of volatile fatty acids in environmental aqueous samples. Polish J. Environ. Stud. 17, 351–356.

Venkata Mohan, S., Mohanakrishna, G., Goud, R.K., Sarma, P.N., 2009. Acidogenic fermentation of vegetable based market waste to harness biohydrogen with simultaneous stabilization. Bioresour. Technol. 100, 3061–3068.

Chapter 3

Acidogenic fermentation of agro-industrial effluents to enhance volatile fatty acid production through inoculum pretreatment



3.1. Background and Research gap

Management of waste generated from industrial processes is still a pressing issue concerning medium scale industries. In this aspect, a waste biorefinery approach could solve the problem of waste treatment and income generation through product formation (Venkata Mohan et al., 2016). Over the past 20 years, the research community investigated the suitability of integrating polyhydroxyalkanoate (PHA) production from activated sludge onto the existing waste management schemes. The bottlenecks for such integration were the carbon source and PHA accumulating potential of the activated sludge. Industrial effluents are ideal carbon sources for economizing PHA production that can serve the dual purpose of waste treatment and value addition. However, the low PHA specificity of raw effluents reduced the accumulating potential of activated sludge despite the growth limitation imposed on the culture (Liu et al., 2008).

PHA is the hydroxylated condensed chain of fatty acids, especially volatile fatty acids (VFAs). The metabolic investigations had revealed the overlapping regions of VFA and PHA metabolism (Magdouli et al., 2015). These led to speculation and final confirmation of the competitive advantage that VFAs provide to PHA accumulators and their storage performance (Cui et al., 2016). The acidogenic/acetogenesis stage (dark fermentation) during anaerobic digestion produces excess VFAs with the simultaneous evolution of H_2 and CO_2. Acidogenesis transforms the biodegradable organic contents of raw effluent into VFAs, thereby enhancing effluent conversion into specific intracellular lipophilic inclusions, PHA (Valentino et al., 2016). VFA profile comprising primarily of acetic acid (HAc), propionic acid (HPr), butyric acid (HBu), and valeric acid (HVa). VFAs direct the production of polyhydroxy(butyrate-co-valerate) (PHBV) copolymer (Shen et al., 2014). The presence of even-numbered fatty acids (HAc, HBu) improves the HB (3-hydroxybutyrate) fraction of PHBV, while odd-numbered fatty acid (HPr, HVa) corresponds to HV (3-hydroxyvalerate) fraction. A smaller HV fraction compared to HB fraction has better mechanical and satisfactory thermal properties for polymer applicability as bioplastics (Arcos-Hernández et al., 2013). Hence, it will be favorable to have an acidogenic fermentation platform with higher VFA content and even-numbered fatty acids.

Total VFA (TVFA) concentration influences PHA production to a great extent. Higher soluble chemical oxygen demand (COD) generally translates to higher total VFA concentration, while the VFA profile depends primarily on operating conditions. However, Morgan-Sagastume et al. (2015) proposed a fermentate with a VFA (in terms of COD) content of 500 mg/L and a high degree of acidification to suffice PHA accumulation. This projects medium strength effluents as a possible source of carbon. Rice mill effluent (RME) and brewery effluent (BE) are wastes from regional industries with medium soluble COD (1-4 gCOD/L), untapped for their acidogenic potential and to influence VFA composition. Starch and other carbohydrate moiety released during parboiling of rice dominate RME (Kumar et al., 2016). Meanwhile, the washouts from the fermenters generate BE majorly comprising fatty acids, ethanol, sugars, and other fermentative products left out due to yeast's action upon malted maize extract (Simate et al., 2011). Rice mills and breweries come under the orange and red category of highly polluting industries in Assam, India (Assam Govt./Environment and Forest, 2019). Hence, wastewater generating from these industries requires proper treatment before disposal. The effluent treatment could support the medium-scale industries to divert a portion of capital for waste management facilities through value addition. Mesophilic temperature and acidic pH of 5.0-6.0 improve the acidification of such effluent streams (Lee et al., 2014). Therefore, further study into strategies to improve the acidogenic potential of such medium-strength effluents is required to fill gaps in the available literature.

Various process parameters like pH, temperature, sludge retention time, hydraulic retention time, organic load, etc., were adjusted during acidogenic fermentation to influence VFA production. Pretreatment of seed anaerobic microbial mix culture is an effective method to improve acidification derived from the influence of process parameters. It depends on the complete inactivation or killing of methanogenic microbiota in the anaerobic inoculum. Most of the investigations tried to enhance hydrogen production using pretreatment methods like heat-shock, chemical, aeration, microwave, ultrasound, etc., with lesser stress on VFA production (Pachapur et al., 2019). Few studies have looked into the effect of pretreatment on VFA production. Fermentation of cheese whey with short heat-shock treated (120 °C, 20 min) inoculum resulted in a better conversion yield and 58 % HAc content compared to lower yield and 16 % HAc for fermentation with untreated inoculum (Colombo et al., 2016). For inoculum treated with longer heat-shock (110 °C for 1 h) and chemical treatment (2-bromoethanosulfonic acid sodium salt, BES), VFA content enhanced for anaerobic digestion of thermal hydrolysis treated sludge substrate compared to that raw sludge. However, the VFA profile remained more or less similar for raw and hydrothermally treated sludge (28/16/30/26: HAc/HPr/HBu/HVa) (Zhang et al., 2019). Acid pretreatment of anaerobic inoculum at pH 3.0 for 24 h resulted in a two-fold increase in VFA content and the relative yield of individual fatty acids with food waste as substrate (Sarkar et al., 2016). The mechanism of pretreatment depends on the physiological response of the microbial community towards the extreme condition. The exposure to extreme temperature (heat shock) triggers acidogenic H_2 producers to form spores for survival, while methanogens get inhibited or eliminated for their inability to sporulate. An extreme pH (acid shock) ensures the selection of acidogenic bacteria that can survive acidic conditions or form spores. This acid shock leads to the elimination of methanogenic activity of the anaerobic sludge as methanogens grow optimally in a narrow pH range of 6.8-7.2 (Sarkar et al., 2016), (Pachapur et al., 2019). Therefore, the type of pretreatment and substrate greatly influence the relative yield of individual fatty acids. Thus, an optimum pretreatment strategy to elevate VFA content and composition (specifically acetic acid) is required to improve PHA production and its property.

This study attempts to acidify two regionally available medium strength industrial effluents by locally acquired anaerobic sludge, gobar gas plant sludge (GPS), to improve its potential to be used as a fermentation medium in a downstream process to synthesize biodegradable plastic. The influence of such medium strength waste streams on acidification was analyzed by a comparative examination of acidogenesis by pretreated and untreated sludge. The outcome of the acidification process is analyzed considering the implication of the downstream synthesis of PHA.

Table 3.1: Characteristics of sludge and wastewater (RME and BE)

Parameter	GPS	RME	BE
pH	9	4.6	8
TSS (g/L)	70.9±8.2	0.31±0.02	0.74±0.03
VSS (g/L)	44.1±4.9	0.24±0.01	0.16±0.05
SCOD (g/L)	5.5±0.09	3.7±0.9	3.2±0.0
N-NH_4 (mg/L)	79.3±3.2	2.1±0.1	1.2±0.06
P-PO_4 (mg/L)	36.4±2.7	12.2±0.8	0.041±0.01
TVFA (g/L)	0.78±0.1	0.091±0.01	0.44±0.02

±SD

3.2. Results and discussion

3.2.1. Characteristics of the effluents

RME and BE are medium strength wastewaters having COD in the range of 3000 to 4000 mg/L). Nitrogen and phosphorus contents were 2.1 and 12.2 mg/L, respectively, for RME with very less TSS and TVFA. On the other hand, BE had a higher amount of TSS (0.74 g/L) and TVFA (0.44 g/L) with comparatively less nutrient value. GPS also had higher nutrient content owing to the source of the sludge. Hence, neither nitrogen nor phosphorus was required to be added from outside as wastewaters and methanogenic sludge provided the necessary nutrient to run the process with medium strength wastewater (Table 3.1). Besides this washing step, prior inoculation still retained considerable nutrients and VFA in the sludge.

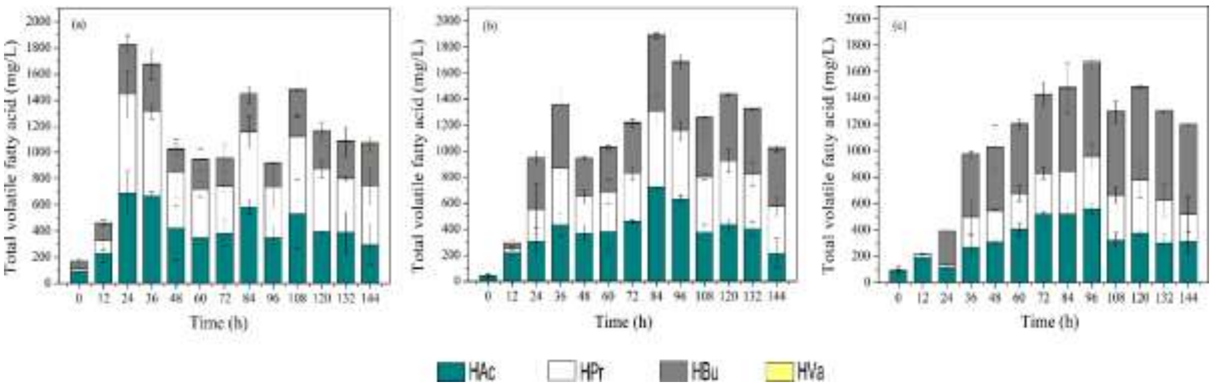


Figure 3.1: TVFA concentration for the acidification of RME at different F/M ratios of (a) F/M 0.5, (b) F/M 1.0, (c) F/M 2.0

3.2.2. Optimization of food to microbe ratio to obtain desired VFA production

Food to microbe ratio (F/M) denotes the proportion of COD available to the acidogens present for efficient derivatization of fatty acids. Acidification of RME followed different patterns at various F/M ratios (Fig 3.1). The TVFA peak (1823 mg/L) for F/M:0.5 was attained instantly at 24 h, mostly attributed to microbes dominating over the substrate (Fig. 3.1a). F/M:1.0 showed the highest TVFA concentration of 1897 mg/L at 84 h. The equilibrium between the microbial populations established as a result of the optimum level of soluble COD might have led to a maximum acidic conversion (Fig. 3.1b). F/M:2.0 exhibited a lower TVFA trend as compared to other F/M ratios. A TVFA concentration of 1679 mg/L was obtained at 96 h (Fig. 3.1c). An abundance of the substrate as to microbe might have led to an inhibitory effect similar to that report for higher organic loadings (Rincón et al., 2008). The F/M 0.5 and F/M 2.0 expose the sludge to probable carbon stress that affected VFA production. In all the ratios, TVFA starts to slide down from the peak depicting the consumption of fatty acids for methane production or other metabolic products (Fig. 3.1).

Table 3.2: Acidification of RME at different F/M ratio

F/M	Retention time (h)	TVFA conc. (mg/L)	Degree of Acidification (%)	VFA composition (HAc:HPr:HBu:HVa)
0.5	24	1823±0.2	70±0.25	37.8:41.6:20.5:0.1
1.0	84	1897±0.11	75±0.16	38:31:30.5:0.5
2.0	96	1679±0.07	69±0.08	33.2:23.7:42.7:0.4

± %SD

It is very crucial to define the VFA distribution of effluent to be put into use (Table 3.2). F/M:1.0 had a higher proportion of HAc followed by HBu and HPr. This corresponds more accurately to the optimal metabolic route for conversion of the organic compound towards acids. But HBu was the major fatty acid for F/M:2.0 as compared to HAc. Higher organic load compared to microbial population shifted acetyl-CoA towards HBu (Pryde et al., 2002). Meanwhile, F/M:0.5 was dominated by HPr, with HAc trailing behind. Such a high microbial density could have been the reason for the increased gas production (H_2) and accompanied an increase in NADH due to which metabolism stepped up HPr production to maintain the equilibrium between NADH/NAD^+ (Wang et al., 2006). It is interesting to note that HVa is at a minimal level in all the conditions, which can be attributed to the characteristics of the biocatalyst. Due to this variation in the VFA profile in each condition, there would be only minor variations in the respective degree of acidification. Nonetheless, F/ M:1.0 acidifies RME better. Considering the downstream application of acidified RME, F/M:1.0 can be considered the best condition among the three ratios due to a higher amount of TVFA and a balanced VFA profile in the order HAc>HBu>HPr. Higher TVFA concentration corresponds to higher PHA storage. Even numbered VFA (HAc, HBu) catalyzes for increased PHB proportion and directly links to the property of the final PHA biopolymer. F/M:2.0 would also reciprocate in the same way with lesser accumulation owing to lower TVFA concentration. An excess level of HPr at F/M:0.5 makes it less desirable for the downstream application as it will lead to an increase in PHV resulting in undesirable characteristics that could affect the applicability of PHA synthesized (Shen et al., 2014; Arcos-Hernández et al., 2013). Hence F/M:1 was used for further studies on acidification of RME and BE.

3.2.3. Total Volatile fatty acid content of acidified effluent prior and post pretreatment

A medium strength waste stream could also suffice the requirements for the downstream application through maintaining a higher DA, especially for polymer accumulation (Morgan-Sagastume et al., 2015). The VFA profile as well needs to be modulated for achieving desired polymer properties. One route for such improvement is through pretreatment. Methanogenic sludge from a gobar gas plant is dominated by obligate anaerobes like methanogens that restrict the activity of acidogens and readily consume acids produced into methane. Hence cyclic steps of heat shock-acid shock select spore-forming bacteria concomitantly removing methanogens (Sarkar et al., 2016). The acidogenic capacity of this pretreated sludge was assessed for RME and BE at F/M:1.0.

The TVFA concentration showed a marginal increase for acidification of RME with pretreated sludge (PT-RME) as compared to acidification of RME with untreated sludge (UT-RME). The maximum TVFA of 2437 mg/L was reached at 132 h for PT-RME, while at 84 h, UT-RME reached a peak TVFA of 1897 mg/L. The medium strength of RME imposed a constraint that limited the increase in TVFA in both cases (Table 3.3). TVFA concentration of PT-RME mostly remained above a particular value (1400 mg/L) from 24 h (Fig. 3.2b). This indicates that pretreatment effectively inhibited methanogenesis as methanation could have resulted in a continuous decrease of VFA. In UT-RME, there is a gradual increase in TVFA concentration that reaches a peak at 84 h with a subsequent decrease (Fig. 3.2a). Even though good acidification is obtained, the system suddenly shifts to methanogenesis. Nevertheless, the acidogenic effluent can still be of use provided the stringent condition for aiding the product formation downstream. Thus, PT-RME assures a high TVFA concentration throughout the period with a maximum at the peak, while UT-RME needs to be monitored closely to prevent the system from advancing into methanogenesis irrespective of the similar condition in both cases.

Table 3.3: Acidogenic fermentation of RME and BE using untreated and pretreated sludge

Effluent	Sludge	Retention time (h)	TVFA concentration (mg/L)	Degree of Acidification (%)	VFA composition#
RME	Pretreated	132	2437±0.03	86±0.13	58:6.2:29:6.8
RME	Untreated	84	1897±0.11	75±0.16	38:31:30.5:0.5
BE	Pretreated	168	2175±0.05	85±0.09	69.3:4.4:9.3:17
BE	Untreated	72	1697±0.13	71±0.14	59:9:6:26

± %SD, #: HAc:HPr:HBu:HVa

Acidification of BE with pretreated sludge (PT-BE) showed a similar increment in TVFA concentration from the initial hour as compared to the acidification of BE with untreated sludge (UT-BE) (≈ 40 %). TVFA concentration reached a peak value of 2175 mg/L for PT-BE at 168 h while that for UT-BE was 1697 mg/L at 72 h (Fig. 3.2c,d). Yet, the effect of pretreatment has influenced the acidification process. The acidification maintained a basic level of 1300 mg/L TVFA in PT-BE. In contrast, methanogenesis took over UT-BE after 72 h pointed out by the decrease in TVFA even though BE is composed of substantial VFAs. A COD/BOD:1.667 indicates the presence of hard to degrade components (Simate et al., 2011). This might be the reason that acidogens could only perform reduced bioconversion while methanogens easily volatilized COD to methane or growth (Fig. 3.2c,d). Yet, there exists an increment in VFA content for PT-BE than UT-BE. It can be inferred that pretreatment has enriched the sludge with acidogens which have converted the maximum possible non-VFA into VFA.

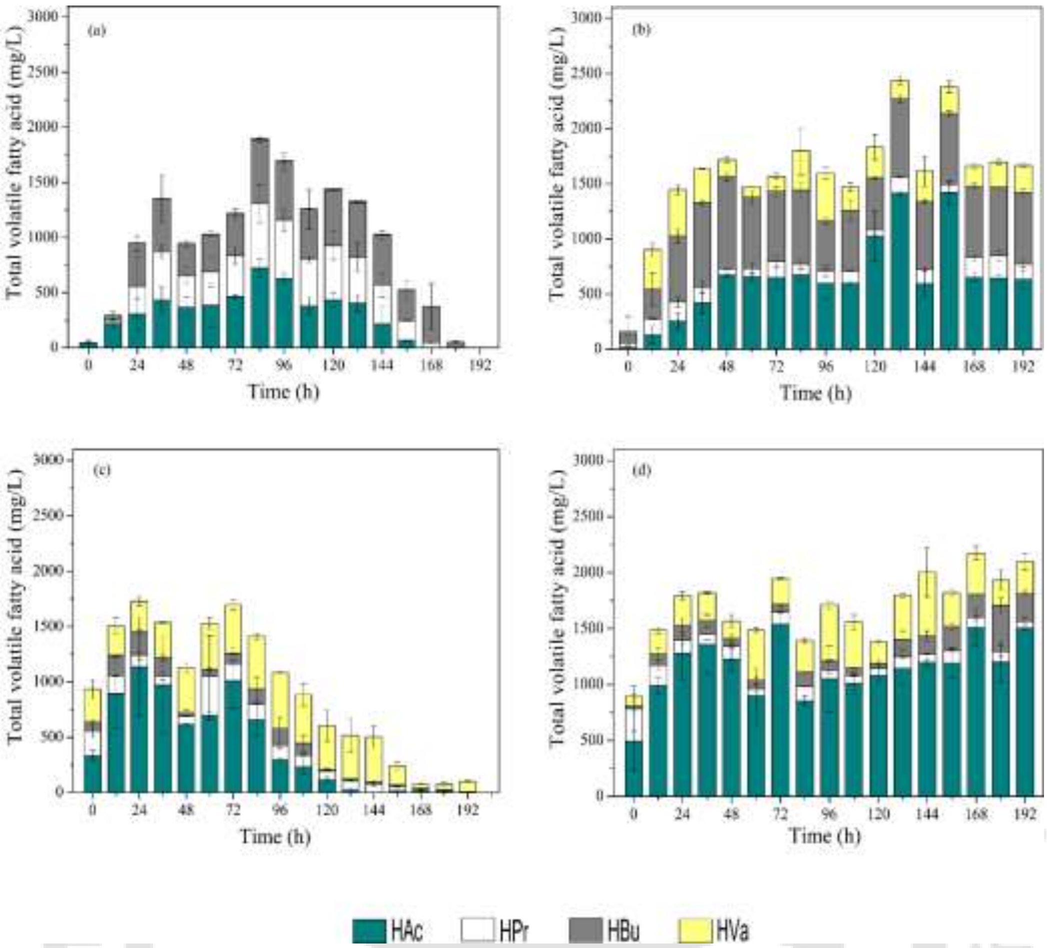


Figure 3.2: TVFA Concentration and VFA profile: Acidification of RME with (a) untreated sludge, (b) pretreated sludge; Acidification of BE with (c) untreated sludge, (d) pretreated sludge.

3.2.4. Volatile fatty acid profile of acidified effluent prior and post pretreatment

VFA profile shows a clear distinction between pretreated and untreated systems. The VFA profile (HAc:HPr:HBu:HVa) shifted from 38:31:30.5:0.5 at 84 h to 58:6.2:29:6.8 at 132 h after pretreatment (Table 3.3). PT-RME resulted in acidic effluent rich in HAc followed by HBu. This association of acetate and butyrate is a widely observed phenomenon that made acidification a suitable route towards bioplastic production. There exist a synergy between HAc and HBu. Acetate concentration escalates during the process and exists in an undissociated form enabling them to pass through the membrane overloading inside the cell. This affects the phosphoroclastic pathway that leads to HBu production through condensation of two acetyl CoA and subsequent reduction into HBu (Dahiya et al., 2015). A similar trend exists in UT-RME as well, but pretreatment has made this co-generation more prominent. The drastic change between PT-RME and UT-RME is in the production of HPr and HVa. HPr was the major component in UT-RME, while it is produced in very little quantity after pretreatment. HVa was absent or present in a minuscule amount in UT-RME, while HVa was in higher proportion than HPr in PT-RME. This probable shift in the odd number VFA can be best explained by the surfacing of acidogens earlier inhibited by the majority methanogenic microbial population. It could also maybe postulate that propionyl-CoA synthesized is getting condensed with acetyl-CoA leading to valerate production (Stadtman et al., 1949). HVa is correlated more with proteinous streams synthesized as a byproduct of amino acid catabolism. So it can be assumed that PT-RME enriched with acidogens could have stepped up protein catabolism. Thus pretreatment of sludge converted the effluent into a heterogeneous mixture worth to be explored for downstream use.

Similarly, PT-BE showed improvements in the VFA pattern compared to UT-BE (Fig. 3.2c,d). The composition of TVFA (HAc:HPr:HBu:HVa) for PT-BE at 168 h was in the ratio of 69.3:4.4:9.3:17 while for UT-BE was 59:9:6:26 at 72 h (Table 3.3). HAc concentration increased in both cases, but in UT-BE it declines and gets completely used up by 192 h. Meanwhile, HPr remained the lowest for both systems without any considerable improvement. HBu also improved after 120 h for PT-BE while get consumed entirely after 72 h for UT-BE. HVa improved from the initial hour in PT-BE while largely remained the same in UT-BE with a slight increase at 96 h. So it can be said that HVa/HPr producing acidogens or metabolic process remains unaltered even after pretreatment. As mentioned earlier, in PT-BE, an increase in acetate might have triggered butyrate to scale up. Methanogenesis took over UT-BE after 72 h, yet HVa remained the least consumed. This may be due to the relative ease of consumption of VFA as HAc got completely consumed followed by HPr and HBu. Brewing being a fermentation process of grains (barley, maize, etc.), the effluent contains a sizable amount of VFA making up the COD. The acidification of BE converts the non-VFA fermentable organic fraction into VFA. Pretreatment has improved and/or sustained VFA content as compared to untreated (Table 3.3). Therefore, through pretreatment RME and BE were refined to have the required VFA profile to ensure the accumulation of more of PHB than PHV when the acidified effluent is used as a carbon source for PHA accumulation. The type of monomer greatly influences the property and dictates the subsequent applicability of the polymer (Arcos-Hernández et al., 2013).

3.2.5. Chemical Oxygen Demand stability of the acidification processes

The chemical oxygen demand of the final acidified effluent plays a vital role in acidogenic fermentation, considering its further use. The most appropriate outlet condition would be to have less COD removal and a higher degree of acidification, i.e., COD should be diverted more to conversion than consumption indicated by percent variation in COD between both the system (ΔCOD). As shown in Fig. 3.3a, the reduction in COD in PT-RME is lower as compared to UT-RME. For RME, $\Delta COD_{RME} \leq 0.3$ indicates that gobar gas plant sludge has the innate capacity to interact with RME and acidify rather than consume the organic content. The organic content of RME comprises a large amount of starch and other higher molecular weight compounds (Kumar et al., 2016). The hydrolytic bacteria slowly break down these compounds along with acidogens converting them to acids till the peak at 84 h, after which methanogens took over the processing of UT-RME (Fig. 3.3a). But pretreatment further enriched the acidogens present in the sludge,

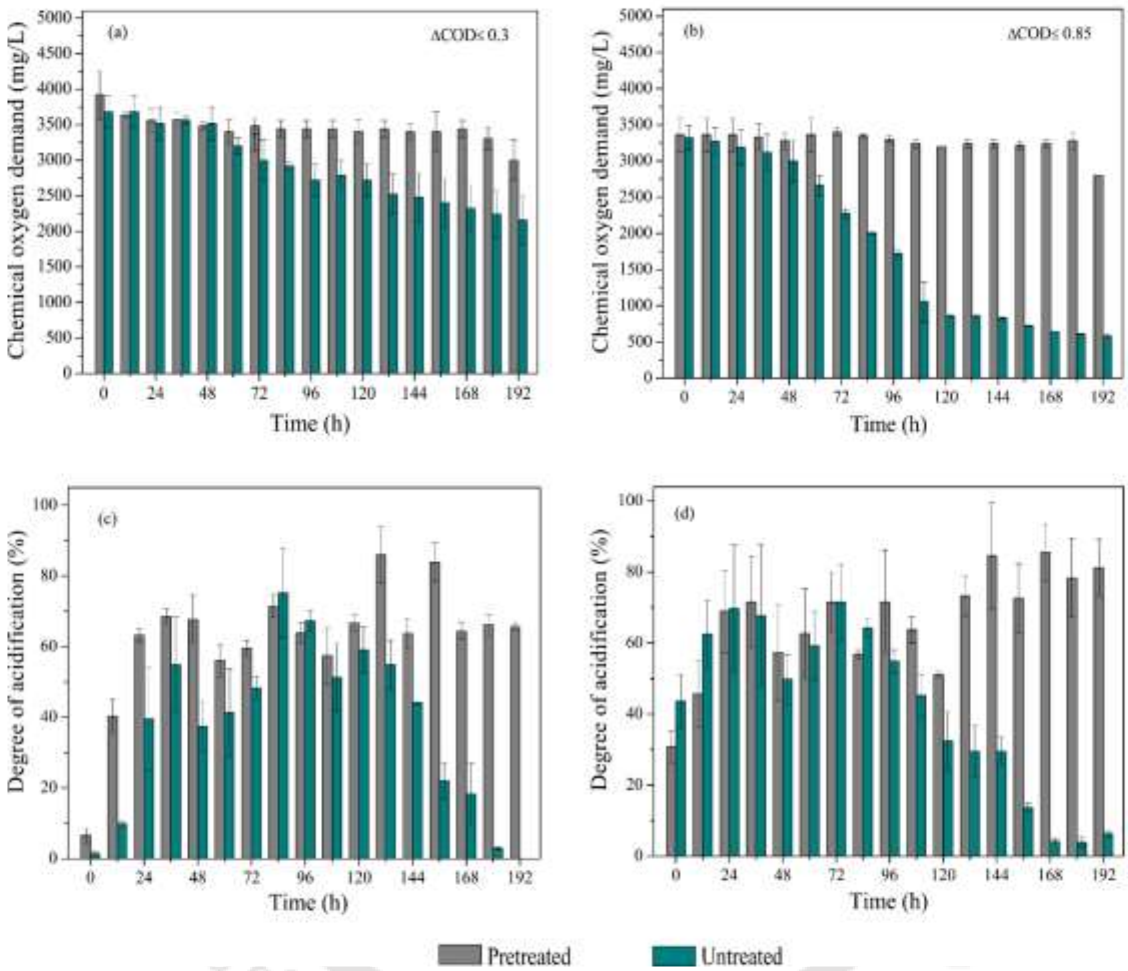


Figure 3: COD profile of pretreated and untreated system: (a) acidification of RME, (b) acidification of BE. The degree of acidification of pretreated and untreated system: (c) acidification of RME, (d) acidification of BE

thereby enhancing the hydrolysis and acidification. A similar trend was observed for PT-BE where pretreatment led to lower COD removal whereas UT-BE advanced into methanogenesis and COD plummeted down. BE contains a good amount of VFA and other simpler organic compounds (Simate et al., 2011). Methanogens took over the sludge and VFA was consumed after brief acidification till 72 h (the majority of COD was VFA). Meanwhile, the PT-BE devoid of a large proportion of methanogens maintained stable acidification. This is even highlighted by $\Delta COD_{BE} \leq 0.85$ that shows the gap between the pretreated and untreated system for BE (Fig. 3.3b). The matrix of effluent aided methanogenesis than acidogenesis as compared to rice mill effluent.

In both systems, nearly complete acidification is reached at respective peak retention times. But acidogenic effluent retrieved from the pretreated system at their respective peak retention time contains a high COD that comprises mostly VFA. As mentioned earlier, higher COD caters to higher polymer accumulation. Thus pretreatment effectively retains COD along with enriching the wastewater with VFA. Finally, wastewater treatment can be visualized here through the polymer production using effluent from acidogenesis using pretreated sludge due to higher COD available at the retention time with peak TVFA concentration. Thereby majority of the influent COD is removed as a value-added product.

Some part of the influent COD is used up for growth, while some might be converted to methane and VFA. The degree of acidification specifies the VFA yield, and a higher DA makes an acidification experiment successful (Fig. 3.3c,d). For PT-RME, the TVFA concentration reaches up to 2437 mg/L and degree of acidification at 86 %, while for UT-RME corresponding values are 1897 mg/L and 75 %. DA is the function of the VFA profile rather than VFA content. A higher TVFA concentration does not ensure higher DA. But here, pretreatment of sludge ensured improved VFA content and degree of acidification. A relatively higher amount of HVa and HBu present in PT-RME resulted in a higher COD (COD_{VFA}) and DA as compared to that of UT-RME (Dahiya et al., 2015). PT-BE results in TVFA concentration of 2175 mg/L and degree of acidification of 85 % at 168 h (Fig. 3.3c). The respective values for UT-BE are 1697 mg/L and 71 % at 72 h (Fig. 3.3d). The higher amount of HAc and HPr in PT-BE resulted in higher COD_{VFA} and DA compared to UT-BE (Dahiya et al., 2015). HBu and HVa more or less remained similar in both systems. Pretreatment has improved the overall acidification of RME and BE compared to untreated. The DA also depicts the acidification process to be successful for both effluents (Fig. 3.3c,d). For PT-RME, the acidification increased from 6 % at 0 h to 86 % at 132 h and it was 1-75% from 0-84 h for UT-RME. The long period of boiling released a large number of carbohydrates that underwent thermal hydrolysis converting starch and other similar compounds into simpler compounds like glucose, thereby improving acid production (Peixoto et al., 2012). This indicates the high biodegradability of RME due to the possible components that make up the organic content of COD. But for BE, DA is 42 % at 0 h, which improved to 85% at 168 h, while for UT-BE, the DA varied between 36-71 % in 0-72 h (Fig. 3.3c,d). Due to already available VFA in BE, untreated and pretreated sludge had to convert a smaller amount of non-VFA as compared to RME (Simate et al., 2011). Even though acidification of RME is better yielding than BE, both effluent streams are a viable source for acidogenic fermentation and subsequent downstream application concerning the degree of acidification.

3.2.6. Kinetics of the acidification process

Retention time is another point of concern in acidogenesis. The peak point of TVFA concentration for UT-RME is 84 h, UT-BE is 72 h, while for PT-RME and PT-BE is 132 h and 168 h, respectively. Pretreatment by heat/acid shock exposes microorganisms to high stress leading to spore formation. Under glucose as the sole carbon source, they regenerate quickly. But the growth is slower when exposed to wastewater as they tend to take more time to get acclimatized to the medium, so pretreated sludge takes a longer time to get a peak in its acid content. This drag in the process is effectively balanced with the improved degree of acidification (RME 75 to 86% and BE 71 to 85%, from untreated to pretreated system) and VFA profile. It is evident from Fig. 3.2 that pretreatment ensures a similar VFA profile after a certain retention time (24 h) for both effluent streams with HAc and HBu as the major fatty acids for RME, while HAc and HVa for BE. The pretreated system is found to stabilize with retention time. While the untreated system shows high variability with respect to time, major fatty acids change along the time with desired content primarily at peak. The observation is similar for TVFA concentration. Hence, for an untreated system, retention time becomes a crucial factor, while an acidogenic batch process can be brought independently of retention time through pretreatment (Fig. 3.2).

Table 3.4: Kinetic constants of Gompertz equation for pretreated and untreated systems

Effluent	Sludge	A_{max} (g/L)	R_{max} (g/Lh)	λ (h)	R^2	A (g/L)
RME	Pretreated	39.45	0.165	21.8	0.97	18.14
RME	Untreated	17.4	0.134	24.4	0.99	7.9
BE	Pretreated	45.7	0.158	8.7	0.97	24.89
BE	Untreated	16.11	0.139	1	0.99	10.14

In order to depict the impact of pretreatment on acidogenesis, the production kinetics of the TVFA was fitted to the modified Gompertz equation given in Eq. 7 (section 2.6.1), where A is the cumulative TVFA concentration at time t; A_{max} is the cumulative maximum of acid formed per liter; R_{max} is the maximum rate of acid formation; λ is the lag time to exponential product formation (Mu et al., 2006). The biokinetic constants such as A_{max}, R_{max}, λ were calculated by the Solver tool (Excel 2010) by adjusting variables in the equation. The cumulative TVFA production is shown in Fig. 3.4, which was used to fit the Gompertz equation.

The biokinetic constants are obtained from the equation is given in Table 3.4. The higher regression value (R^2>0.95) for all the systems illustrates the close fit of the study with Gompertz production kinetic model similar to that of Mu et al. (Mu et al., 2006). The pretreated system maintained a certain level of VFA production after a given time that has resulted in higher A_{max} of 39.5g/L and 45.7 g/L for RME and BE, respectively, while a decline in acidogenesis caused a lower value of A_{max} for untreated system (17.4 g/L, 16.11 g/L for RME and BE respectively). It is evident from R_{max} that the pretreatment process has accelerated the acidification process (0.165 g/Lh and 0.158 g/Lh for RME and BE) rather than impairing its bioconversion capability as compared to the untreated system (0.134 g/Lh and 0.139 g/Lh for RME and BE) (Table 3.4). As shown in Table 3.1, the BE contains some amount of acid initially, which might be the reason for a lower λ for UT-BE (1h), while pretreatment stress causes slightly longer λ for PT-BE (8.7 h). But λ value for RME shows that the pretreated system (21.8 h) takes less time than the untreated system (24.4 h) to jump-start the acidification process. Thus Gompertz equation also shows the improvement brought into the process through pretreatment of sludge and its economic viability over the untreated system.

Table 3.5: VFA production rate profile at maximum acid content

Effluent	Sludge	HAc (mg/Lh)	HPr (mg/Lh)	HBu (mg/Lh)	HVa (mg/Lh)	VFA_{max} (mg/L)
RME	Pretreated	10.6	0.81	4.61	1.25	2437±0.03
RME	Untreated	8.10	6.90	6.91	0.11	1897±0.11
BE	Pretreated	6.05	-1.17	1.07	1.68	2175±0.05
BE	Untreated	9.40	-0.87	0.17	1.90	1697±0.13

± %SD

Volatile fatty acid production rate (PR_{vfa}) throws light on the effect of pretreatment on individual acid production over the untreated system, which was calculated as per eq 5 (section 2.5.1). (Dahiya et al., 2015). Here, VFA_{max} is the VFA content at the peak retention time (mg/L), $VFA_{initial}$ is the VFA content present at the start of the experiment (mg/L), and T is the peak retention time (h). The production rates for both systems are shown in Table 3.5. The production was expeditious for HAc (10.6 mg/Lh) and HVa (1.25 mg/Lh) after pretreatment of sludge as compared to the untreated system (8.10 mg/Lh-HAc, 0.11 mg/Lh-HVa) while HPr and HBu showed a lag. HPr production in PT-RME (0.81 mg/Lh) was much slower than UT-RME (6.90 mg/Lh) when compared to HBu (4.61 mg/Lh for PT-RME and 6.91 mg/Lh for UT-RME). It is evident that after pretreatment of sludge, the COD shifted more to acetic acid, i.e., acetic acid metabolism was stepped up while retaining butyric acid (Fig. 3.2). As expected, HVa was produced the least. This can be correlated to be a function of the wastewater and the acidogens that became dominant after pretreatment. Therefore, the acidification of RME with pretreated gobar gas plant sludge is better and more affordable than acidification with untreated sludge (Table 3.5).

As observed earlier, the interaction of sludge with brewery effluent prior to or post-treatment remains more or less similar, as evident from Table 3.5. The production rate of HBu was 0.17 mg/Lh prior to treatment that improved to 1.07 mg/Lh after pretreatment. HPr gets consumed in both cases, with the pretreated system being faster. HAc, being the most vital for the study, production in PT-BE (6.05 mg/Lh) showed a slower rate than UT-BE (9.40 mg/Lh). HVa is produced here as the second major as compared to that of acidification of rice mill effluent. Still, the production remains similar for pretreated as well as the untreated system. Thus gobar gas plant sludge was able to acidify brewery effluent to some degree, but pretreatment didn't have an expected effect like that for rice mill effluent. Thus the kinetics of the acidification points out the positive impact of pretreatment and the suitability of PT-RME as a fermentation medium for downstream polymer accumulation.

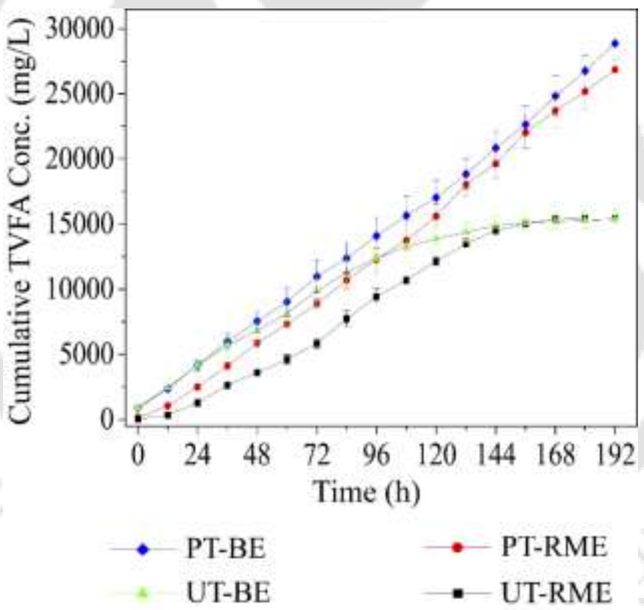


Figure 3.4: Cumulative TVFA concentration during the acidification of RME and BE with pretreated and untreated GPS

3.2.7. Impact of acidification on pH

pH is a factor that influences the economy and productivity of a process. The transition of pH from the initially set acidic condition towards alkaline is an indication of the system adjusting for methanation with a steady decrease in acid due to consumption. Fig. 3.5 depicts the difference that was brought into the system through pretreatment. The pH of PT-RME varied within the range 5.7-6.0 while the pH of UT-RME reached 7.6 with a related decrease in TVFA (Fig. 3.5a). The pH of PT-BE stretched between 5.9-6.0while UT-BE slowly but steadily increased to 7.5 (Fig 3.5b). The percent variation in pH between both the systems (ΔpH), $\Delta pH_{RME} \leq 0.25$ and $\Delta pH_{BE} \leq 0.23$ indicate the profound influence of pretreatment over acidogenesis as compared to untreated. pH stability reiterates the fact that pretreatment improved sludge's capacity for acidification irrespective of the medium. The pretreated sludge retained stable acidification while untreated sludge returned to its natural state of methanogenesis. The acidogens in untreated were outdone by methanogenic bacteria, which were mostly absent in pretreated. The untreated system continuously slipped into alkaline pH, indicating advance into methanation while with pretreated sludge pH stabilized at acidic pH after a particular retention time.

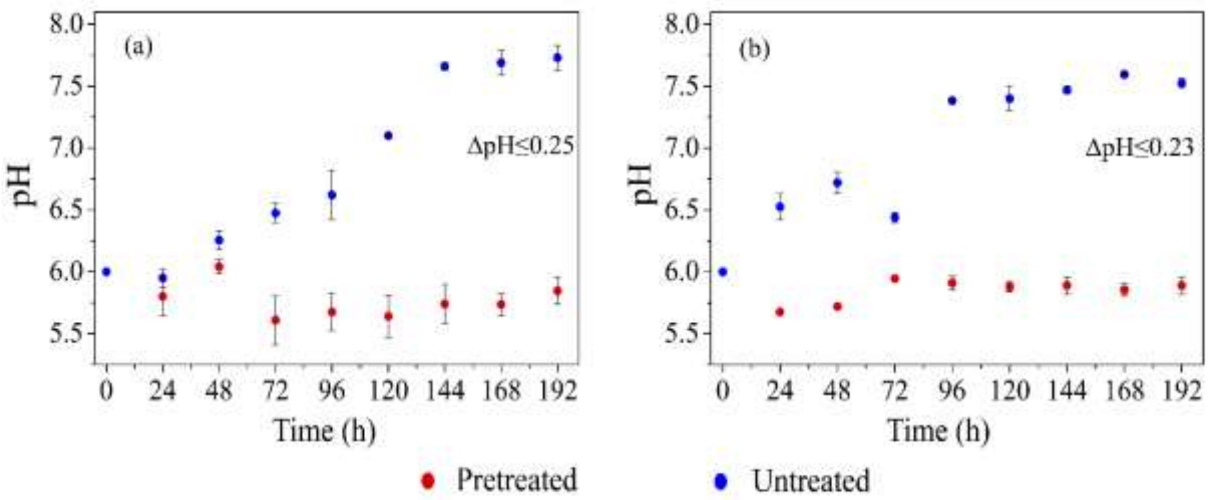


Figure 3.5: pH profile of pretreated and untreated system: (a) acidification of RME, (b) acidification of BE.

The narrow pH range also describes another feature of acidification with medium strength wastewater. A decrease in pH is an indication of acidogenic activity. Moreover, this decrease is characteristic of the type and amount of VFA produced. Thus a high TVFA concentration doesn't correspond to low pH. As the carbon length of fatty acids increases, their hydrophobicity increases. pH of the system is more influenced by acetic acid (pKa 4.75) than valeric acid. The pretreatment effectively enriched the microbial community of sludge, ensuring a VFA profile that maintained the pH in a stable range. The medium strength of the effluent streams restricted the TVFA concentration that assisted the VFA profile in attaining a stable pH. Acidification itself influences the buffering of the system through the formation of carbonate by CO_2 produced along with the VFA. Protein and macromolecules also tend to buffer the pH through their degradation, such as protein degradation leading to ammonia production that facilitates ammonium bicarbonate formation (Dahiya et al., 2015). Hence this study shows that the pretreated sludge could perform acidogenic conversion of medium strength wastewater without pH control. Thus the bioprocess control of the acidogenic system can be made economically and with process simplification.

3.2.8. Comparison between acidification of RME and BE

Many studies have shown that eliminating or restricting methanogens to be vital towards improving acidogenic fermentation and hydrogen production (Baghchehsaraee et al., 2008; Chang et al., 2011). Here the VFA content, COD, and pH show that pretreatment transformed the sludge into acidogens dominated inoculum, thereby improving acidification of RME and BE. But for RME, the TVFA concentration was increased by 93 % from the initial (0 h) while it was only 58 % for BE (Fig. 3.2). The degree of acidification also shows the same trend (Fig. 3.3c,d). This shows the better acidogenic bioconvertability of RME over BE. The effluent matrix might be responsible for this difference, even in untreated state sludge acidified rice mill effluent better than brewery effluent. The nutrient availability might be a reason that BE could not outperform RME (Table 3.1). Lower nitrogen and phosphorus could have slowed down the acidogenic activity of BE while nutrients were available in minimum required level in RME. The source of effluent might be another reason. The fermentation process in brewery might be releasing some hard to degrade components in to BE that would have interfered with acidogens. This might be reason that in UT-BE the system went into consumption of COD rather than production of VFA. RME on the other hand possess readily biodegradable components that acidogens metabolized easily as evident from pretreated and untreated system. These observation points out that RME is a better medium when gobar gas plant sludge used for acidification.

3. 3. Conclusion

It is evident from the study that gobar gas plant sludge possesses all the characteristics of a biocatalyst, either similar/better to that of reported sludge from effluent treatment plants. Here rice mill effluent is acidified for the first time along with brewery effluent. TVFA concentration, the degree of acidification, and the VFA profile of both effluent streams improved after pretreatment. COD consumption and pH fluctuation also stabilized by the end of the run in the pretreated system. The VFA profile completes the criteria of perfect fermentation media for PHA production. The production kinetics of the pretreated system also better compared to the untreated system. The higher degree of acidification substitutes for lower COD of the effluent stream to increase the polymer accumulation. Therefore, acidogenesis by pretreated sludge is preferable over untreated sludge, where the latter only serves the purpose at the optimum retention time. RME and BE are potent source for acidogenic bioconversion. Nonetheless, the RME has the upper hand in the process considering its bioconversion feasibility. The observation from this study raises the prospects of a pH uncontrolled acidogenic fermentation of medium strength effluent streams with pretreated sludge having stable acid production. This study showed a possible waste management strategy of these medium strength industrial effluents through acidogenic fermentation with potential for subsequent value addition.

References

Arcos-Hernández, M. V, Laycock, B., Donose, B.C., Pratt, S., Halley, P., Al-luaibi, S., Werker, A., Lant, P.A., 2013. Physicochemical and mechanical properties of mixed culture polyhydroxyalkanoate (PHBV). Eur. Polym. J. 49, 904–913.

Assam Govt./Environment and Forest, 2019. Category of Industries [WWW Document]. URL https://environmentandforest.assam.gov.in/information-services/category-of-industries-0 (accessed 8.24.20).

Baghchehsaraee, B., Nakhla, G., Karamanev, D., Margaritis, A., Reid, G., 2008. The effect of heat pretreatment temperature on fermentative hydrogen production using mixed cultures. Int. J. Hydrogen Energy 33, 4064–4073.

Chang, S., Li, J.Z., Liu, F., 2011. Evaluation of different pretreatment methods for preparing hydrogen-producing seed inocula from waste activated sludge. Renew. Energy 36, 1517–1522.

Colombo, B., Tommy, P.S., Maria, R., Barbara, S., Fabrizio, A., 2016. Polyhydroxyalkanoates (PHAs) production from fermented cheese whey by using a mixed microbial culture. Bioresour. Technol. 218, 692–699.

Cui, Y., Zhang, H., Lu, P., Peng, Y., 2016. Effects of carbon sources on the enrichment of halophilic polyhydroxyalkanoate-storing mixed microbial culture in an aerobic dynamic feeding process. Sci. Rep. 6, 30766.

Dahiya, S., Sarkar, O., Swamy, Y.V., Venkata Mohan, S., 2015. Acidogenic fermentation of food waste for volatile fatty acid production with cogeneration of biohydrogen. Bioresour. Technol. 182, 103–113.

Kumar, A., Priyadarshinee, R., Roy, A., Dasgupta, D., Mandal, T., 2016. Current techniques in rice mill effluent treatment: Emerging opportunities for waste reuse and waste-to-energy conversion. Chemosphere 164, 404–412.

Lee, W.S., Chua, A.S.M., Yeoh, H.K., Ngoh, G.C., 2014. A review of the production and applications of waste-derived volatile fatty acids. Chem. Eng. J. 235, 83–99.

Liu, H.Y., Hall, P. V, Darby, J.L., Coats, E.R., Green, P.G., Thompson, D.E., Loge, F.J., 2008. Production of polyhydroxyalkanoate during treatment of tomato cannery wastewater. Water Environ. Res. 80, 367–372.

Magdouli, S., Brar, S.K., Blais, J.F., Tyagi, R.D., 2015. How to direct the fatty acid biosynthesis towards polyhydroxyalkanoates production? Biomass and Bioenergy 74, 268–279.

Morgan-Sagastume, F., Hjort, M., Cirne, D., Gérardin, F., Lacroix, S., Gaval, G., Karabegovic, L., Alexandersson, T., Johansson, P., Karlsson, A., Bengtsson, S., Arcos-Hernández, M.V. Magnussona, P., Werker, A., 2015. Integrated production of polyhydroxyalkanoates (PHAs) with municipal wastewater and sludge treatment at pilot scale. Bioresour. Technol. 181, 78–89.

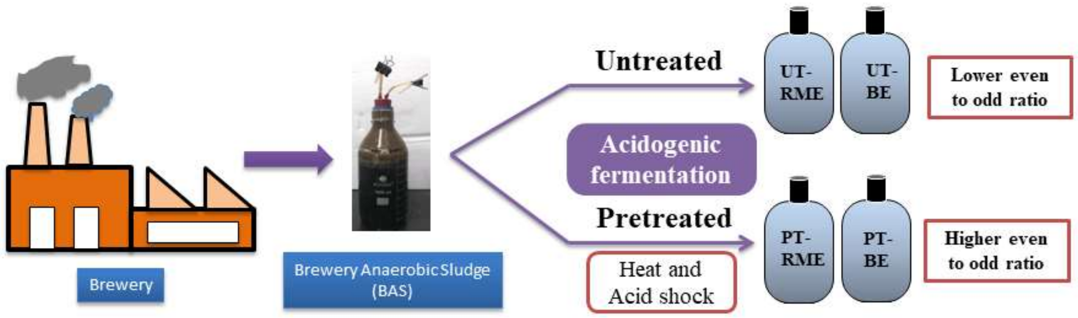
Mu, Y., Wang, G., Yu, H.Q., 2006. Kinetic modeling of batch hydrogen production process by mixed anaerobic cultures. Bioresour. Technol. 97, 1302–1307.

Pachapur, V.L., Kutty, P., Pachapur, P., Brar, S.K., Le Bihan, Y., Galvez-Cloutier, R., Buelna, G., 2019. Seed pretreatment for increased hydrogen production using mixed-culture systems

with advantages over pure-culture systems. Energies 12, 1–26.
Peixoto, G., Pantoja-Filho, J.L.R., Agnelli, J.A.B., Barboza, M., Zaiat, M., 2012. Hydrogen and methane production, energy recovery, and organic matter removal from effluents in a two-stage fermentative process. Appl. Biochem. Biotechnol. 168, 651–671.
Pryde, S.E., Duncan, S.H., Hold, G.L., Stewart, C.S., Flint, H.J., 2002. The microbiology of butyrate formation in the human colon. FEMS Microbiol. Lett. 217, 133–139.
Rincón, B., Sánchez, E., Raposo, F., Borja, R., Travieso, L., Martín, M.A., Martín, A., 2008. Effect of the organic loading rate on the performance of anaerobic acidogenic fermentation of two-phase olive mill solid residue. Waste Manag. 28, 870–877.
Sarkar, O., Kumar, A.N., Dahiya, S., Krishna, K.V., Yeruva, D.K., Mohan, S.V., 2016. Regulation of acidogenic metabolism towards enhanced short chain fatty acid biosynthesis from waste : metagenomic pro fi ling. RSC Adv. 6, 18641–18653.
Shen, L., Hu, H., Ji, H., Cai, J., He, N., Li, Q., Wang, Y., 2014. Production of poly (hydroxybutyrate – hydroxyvalerate) from waste organics by the two-stage process : Focus on the intermediate volatile fatty acids. Bioresour. Technol. 166, 194–200.
Simate, G.S., Cluett, J., Iyuke, S.E., Musapatika, E.T., Ndlovu, S., Walubita, L.F., Alvarez, A.E., 2011. The treatment of brewery wastewater for reuse: State of the art. Desalination 273, 235–247.
Stadtman, E.R., Stadtman, C.T., Barker, A.H., 1949. Tracer experiments on the mechanism of synthesis of valeric and caproic acids by Clostridium kluyveri. J. Biol. Chem. 178, 677–683.
Valentino, F., Morgan-Sagastume, F., Campanari, S., Villano, M., Werker, A., Majone, M., 2016. Carbon recovery from wastewater through bioconversion into biodegradable polymers. N. Biotechnol. 37, 9–23.
Venkata Mohan, S., Nikhil, G.N., Chiranjeevi, P., Nagendranatha Reddy, C., Rohit, M. V, Naresh Kumar, A., Sarkar, O., 2016. Waste biorefinery models towards sustainable bioeconomy: critical review and future perspectives. Bioresour. Technol. 215, 2–12.
Wang, L., Zhou, Q., Zheng, G.H., 2006. Comprehensive analysis of the factors for propionic acid accumulation in acidogenic phase of anaerobic process. Environ. Technol. 27, 269–76.
Zhang, D., Jiang, H., Chang, J., Sun, J., Tu, W., Wang, H., 2019. Effect of thermal hydrolysis pretreatment on volatile fatty acids production in sludge acidification and subsequent polyhydroxyalkanoates production. Bioresour. Technol. 279, 92–100.

Chapter 4

Regulation of volatile fatty acid accumulation from agro-industrial effluents through heat–acid shock pretreatment of anaerobic inoculum



4.1. Background and Research gap

In recent times, the waste biomass refinery approach is being actively explored as a sustainable waste management methodology. Polyhydroxyalkanoate (PHA) production from fermented waste feedstocks has been studied extensively to fabricate an economic waste treatment strategy. Various investigations tried to improve PHA production from waste feedstocks by improving the VFA (volatile fatty acid) content and profile, comprising acetate (Hac), propionate (HPr), butyrate (HBu), and valerate (HVa). A plausible route for this was the pretreatment of seed anaerobic sludge. Even though many pretreatment protocols are available, most of them emphasize the effect of pretreatment on hydrogen production. Meanwhile, the implications of a combination of pretreatment methods on acidogenic fermentation are also yet to be understood. The combined heat-acid shock was shown to be very promising in generating high VFA content. The cyclic heat-acid shock was successful in enhancing the TVFA (total VFA) content and refining the VFA profile for rice mill effluent (RME) and brewery effluent (BE) fermentate (Chapter 3). However, the dominance of even-numbered fatty acid is negligible. Higher even-numbered VFA corresponds to higher HB content than HV content that in turn influences PHA composition and property. Even to odd ratio indicates the possibility of HB or HV dominance in the final polymer accumulated (Zhang et al., 2014). Therefore, it is necessary to control the acidogenesis process to improve VFA production, at the same time reducing odd-numbered fatty acid proportion.

The heat-shock pretreatment is one of the well-documented pretreatment strategies. Ravindran et al. (Ravindran et al., 2010) observed that microbial communities in forest soil responded diversely to different heat-shock treatments. Similarly, waste activated sludge reacted differently to various heat-shock treatments (Baghchehsaraee et al., 2008),(Chang et al., 2011). In chapter 3, pretreatment of gobar gas plant sludge (cattle slurry) had enhanced TVFA production with RME as substrate. Meanwhile, the physical structure of the sludge could also impact the fermentative outcomes. Granular anaerobic sludge had acidified glucose better than slurry type sludge at a pH of 10.0 (Atasoy et al., 2019). Hence, the response of such anaerobic sludge of different origins/physical structures requires further study to understand the effect of pretreatment (heat/acid shock), VFA production, and profile. An approach to curtail the resurgence of methanogenic activity for heat-shock treated anaerobic seed sludge is also necessary (Saady, 2013). Therefore, the study examines the kinetic and volumetric impact of a combined heat-acid shock pretreatment of granular anaerobic sludge, brewery anaerobic sludge, and the two different effluents used on total VFA concentration and VFA composition. The effect of process conditions on even to odd ratio is also analyzed. Finally, we elucidate an acidogenic fermentation strategy to effect PHA production and composition.

Table 4.1: Characteristics of the fermentation media and biomass sludge

Parameter	BAS	RME	BE
pH	8.0	4.6	6.0
TSS(g/L)	132.2±12.7	0.31±0.02	0.79±0.06
VSS(g/L)	40.2±4.0	0.24±0.01	0.23±0.11
COD(g/L)#	0.55±0.01	3.7±0.9	4.3±0.2
N-NH_4(mg/L)*	418.5±16.9	2.1±0.1	185.9±13.1
P-PO_4(mg/L)+	8.2±0.2	12.2±0.8	3.1±0.3
TVFA (g/L)	0.026±0.007	0.091±0.010	1.6±0.1

±SD, * Ammonical nitrogen, + phosphorus in terms of phosphate, # soluble

4.2. Results and Discussion

4.2.1. Characteristics of biomass sludge and substrate

The inoculum sludge and effluent characteristics are as given in Table 4.1. The brewery anaerobic sludge (BAS) had low volatile suspended solids, VSS, content (40.2 g/L) as compared to total suspended solids with high ammoniacal nitrogen content (418.5 mg/L) and low soluble COD (0.55 g/L). Breaking of grains during fermentation might be releasing some non-biodegradable recalcitrant components or solids into BE that accumulate in the UASB, leading to higher solids content. For this reason, sludge was washed with distilled water prior to inoculation to minimize nutrient and organic content from the inoculum. In the fermentation medium, RME has higher phosphorus content (12.2 mg/L) compared to nitrogen (2.1 mg/L). The effluent is acidic (pH 4.0) and has a lower solids content. Meanwhile, the parameters were higher than usual for brewery effluent as it was in concentrated form. The number of fermenters operated was less, leading to a reduction in the dilution of effluent. A higher amount of nitrogen (185.9 mg/L), volatile fatty acid (1.6 g/L), and a higher COD (4.3 g/L) were present in the effluent. But the total suspended solid was lower as expected, 0.79 g/L (Table 4.1). BE diluted to bring COD near RME was used for acidification and the F/M ratio set ensures substrate availability without any potential inhibition or starvation.

4.2.2. Total Volatile fatty acid content of acidified effluent prior and post pretreatment

The methanogenic BAS catalyzes the bioconversion under the applied pH of 6.0. The acidification of RME attained a TVFA concentration peak swiftly. The acidification of RME with untreated BAS (UT-RME) produced a higher VFA_{max} (TVFA content at the peak) of 1855 mg/L compared to acidification of RME with pretreated BAS (PT-RME) at 1348 mg/L (Fig.4.1a,b). Similarly, the conversion yield of PT-RME declined (0.71 mgCOD/mgCOD) in comparison to UT-RME (0.82 mgCOD/mgCOD) (Table 4.2). The pretreatment was unable to increment VFA production in comparison to untreated sludge under the provided conditions. However, the untreated system lacked stability in the process as expected. From 152 h, the TVFA concentration declined by 43 % till 240 h due to the gradual domination of methanogens. Most probably, the method of pH control was insufficient to inhibit methanogens during UT-RME (Fig. 4.1a,4a). PT-RME, on the other hand, offers stable VFA production throughout the experiment. Other than a dip of 25 % from VFA_{max} at 144 h, the TVFA remained well above 1120 mg/L. The dip can be assumed to a momentary diversion to carboxylic acid other than VFA as there exists neither a drastic change in COD nor pH to indicate any consumption (Fig. 4.1b,2a,4a).

Table 4.2: Acidogenic bioconversion of RME and BE using untreated (UT) and pretreated (PT) sludge.

Effluent	Sludge	Peak (h)	VFA_{max}	DA	$Y_{VFA/nonVFA}$	VFA profile*	Even to Odd ratio
RME	PT	72	1348±0.00	55±0.04	0.71±0.01	54:2:41:3	20.97±0.08
RME	UT	72	1855±0.03	83±0.03	0.82±0.06	30:28:36:6	1.99±0.03
BE	PT	240	2222±0.01	79±0.03	0.56±0.03	81:13:6	6.43±0.02
BE	UT	96	2458±0.03	85±0.06	0.71±0.09	73:17:10	4.79±0.03

±SD% , *HAc:HPr:HBu:HVa, DA: Degree of acidification (%), $Y_{VFA/nonVFA}$: mgCOD/mgCOD, Even to odd ratio: mg/mg, VFA_{max}: mg/L

Brewery effluent contains a large amount of VFAs (Table 4.1). So the nonVFA (organic content other than VFA) COD available for acidification (1150 mg/L) is less compared to RME (3000 mg/L), as observed from Fig. 4.1,4.2. The conversion yield of BE is also comparatively inferior to RME (Table 4.2). Acidification of BE with untreated BAS (UT-BE) reached VFA_{max} of 2458 mg/L faster. In contrast, acidification of BE with pretreated BAS (PT-BE) resulted in decreased VFA content of 2222 mg/L (Fig. 4.1c,d). Thus, pretreatment failed to improve the TVFA concentration. The lower conversion yield for PT-BE (0.56 mgCOD/mgCOD) compared to UT-BE (0.71 mgCOD/mgCOD) points in the same direction. Also, acidification became slower as PT-BE reached VFA_{max} only by 240 h. Both the acidification system resulted in a stable TVFA production as methane production failed to initiate in UT-BE compared to UT-RME. The interaction of culture with wastewater and its matrix varies with the complexity of the substrate (Korkakaki et al., 2016). The brewery origin of BAS probably aided in the continuation of the acidogenesis process under pH control of 6.0 (24 h interval) as there was no sign of VFA consumption throughout 240 h in UT-BE (Fig. 4.1c,4.2b). Besides, pretreatment rendered VFA sinks inactive, leading to a constant level in VFA content for PT-BE.

Table 4.3: Biokinetic constants of Gompertz equation fitted to acetate, butyrate, propionate and valerate accumulation.

Effluent	Sludge	Acetate				Propionate			
		A_{max}	R_{max}	λ	R^2	A_{max}	R_{max}	λ	R^2
RME	PT	10716 ±0.05	48.56 ±0.05	32.13 ±0.06	0.97	678.90 ±0.14	8.21 ±0.08	0.0 ±0.0	0.99
RME	UT	9974 ±0.03	51.71 ±0.00	16.12 ±0.03	0.97	8982 ±0.12	42.11 ±0.07	11.34 ±0.18	0.96
BE	PT	49002 ±0.01	144.14 ±0.01	4.94 ±0.09	0.98	7967 ±0.01	22.86 ±0.01	3.21 ±0.30	0.98
BE	UT	46729 ±0.01	148.97 ±0.01	2.63 ±0.3	0.98	10273 ±0.02	33.76 ±0.02	0.0 ±0.0	0.99
Effluent	**Sludge**	**Butyrate**				**Valerate**			
		A_{max}	R_{max}	λ	R^2	A_{max}	R_{max}	λ	R^2
RME	PT	15214 ±0.01	54.81 ±0.02	29.07 ±0.02	0.98	6151 ±0.06	11.92 ±0.14	46.29 ±0.12	0.98
RME	UT	9923 ±0.1	41.19 ±0.10	19.63 ±0.10	0.98	3656 ±0.05	11.36 ±0.20	4.75 ±0.00	0.99
BE	PT	3658 ±0.04	12.02 ±0.01	0.0 ±0.0	0.99	-	-	-	-
BE	UT	4627 ±0.1	16.40 ±0.09	0.0 ±0.0	0.99	-	-	-	-

±SD% , PT: Pretreated, UT: Untreated, A_{max} : mg/L, R_{max}: mg/ Lh, λ: h

The heat-acid shock pretreatment selects spore-forming hydrogen producers, simultaneously removing acetoclastic methanogens. This gives acidogens an upper hand leading to VFA accumulation (Sarkar et al., 2016). However, the untreated system produced fermentate with higher TVFA concentration compared to the pretreated system. The pretreatment condition could have negatively impacted the acidogenic microbial community in BAS. Sludge pretreatment under acidic condition (pH 3.0 for 24 h) is a comparatively milder pretreatment that has reported significant VFA content for activated sludge and anaerobic sludge as inoculum with simple and complex substrates, respectively (Chang et al., 2011), (Sarkar et al., 2016). A higher temperature of 95-100 °C or longer duration (2 h) of heat exposure might point out to be a reason for the disparity. In an earlier study, an anaerobic sludge collected from a municipal water resource recovery facility produced lower TVFA content when pretreated at 95 °C for 0.5 h than untreated sludge (Baghchehsaraee et al., 2008). Similar to the study, dark fermentation of sugarcane vinasse with thermally pretreated (105 °C for 2 h) anaerobic inoculum collected from a UASB treating vegetable oil industry effluent produced the lowest TVFA concentration at pH 6.0. The same anaerobic inoculum pretreated at 120 °C for 20 min resulted in the highest TVFA conversion yield for sugarcane vinasse at pH 6.0 (Magrini et al., 2020). In another study, an anaerobic sludge from a dairy farm pretreated under 95 °C for 4 h showed the highest TVFA concentration than aeration or chemical pretreatment (Ghimire et al., 2015). Acidification of RME and BE using anaerobic gobar gas plant sludge pretreated at 100 °C for 2 h had enhanced VFA production (Chapter 3). Probably, non-sporulating acidogens dominate BAS that got inhibited, or spore-forming bacteria got affected. The spore-forming culture took a long time to germinate in the pretreated system, resulting in double the lag phase (λ) compared to the untreated system (Table 4.3). Single batch acclimatization might also have failed to activate the stressed mix culture properly. This might have affected the acidification of RME and BE with pretreated sludge. Baghchehsaraee et al. (Baghchehsaraee et al., 2008) reported that the type of sludge (anaerobic or aerobic) also influences the VFA production in response to heat shock pretreatment.

The study points towards a correlation between sludge (type and origin), temperature, and pretreatment time. An anaerobic digestate inoculum collected from an anaerobic plant that digests solid waste can be assumed to survive better under higher temperatures or time than wastewater treating sludge. A thorough screen of the microbial community could throw some light into this. Sourcing the anaerobic culture from the digestate coming out from the outlet valves of the anaerobic reactor could affect the inoculum's activity. Therefore, a preliminary screening of heat-shock conditions for a particular anaerobic sludge (keeping pH 3.0) is necessary before implementing such enrichment protocols. Acclimatization by the repeated batch process with the substrate to be used could balance any adverse effect of pretreatment and improve acidification (Sarkar et al., 2016). Further study on these minute aspects is necessary to avoid any downregulation of the pretreatment process. Acidification of RME and BE with untreated sludge improved the TVFA content, with RME having the upper hand (indicated by higher conversion yield). The effect of heat-acid shock also points in the same direction with implications on individual VFAs (Table 4.2). Also, the feeding mechanism in the PHA accumulation process, like feed on-demand, continuous feeding, etc., can minimize the effect of moderate TVFA content (Valentino et al., 2016). Therefore, the pretreated system can produce an ideal fermentate for PHA production if the VFA profile is enhanced with acceptable DA to balance the lower TVFA content.

4.2.3. Volatile fatty acid profiling of acidogenic effluent prior and post pretreatment

From earlier studies, we can consider that the VFA profile is more critical than TVFA concentration (Morgan-Sagastume et al., 2015), (Arcos-Hernández et al., 2013). VFA profile impacts the DA, pH, COD_{VFA} (total VFA in terms of COD) of the acidogenic effluent, and PHA composition and property. The heat-acid shock was able to influence VFA composition. Pretreatment refined VFA profile from HAc:HPr:HBu:HVa;30:28:36:6 for UT-RME to 54:2:41:3 for PT-RME at the respective VFA_{max} (Table 4.2). The HAc conversion yield for PT-RME (0.37 mgCOD/mgCOD) was nearly double that of UT-RME (0.20 mgCOD/mgCOD). Meanwhile, HBu yield improved for PT-RME at the expense of HPr and HVa (Table 4.4). The HPr and HVa yield was much higher for UT-RME compared to PT-RME. Due to this, even to odd ratio for PT-RME, 20.97 mg/mg, was ten times to that of UT-RME, 1.99 mg/mg (Table 4.2). The ratio is a clear indication of the shift in metabolic flux towards even-numbered VFA production when BAS got exposed to heat-acid shock. Therefore, acidogenesis of RME with pretreated sludge could derive an even-numbered VFA dominated fermentate.

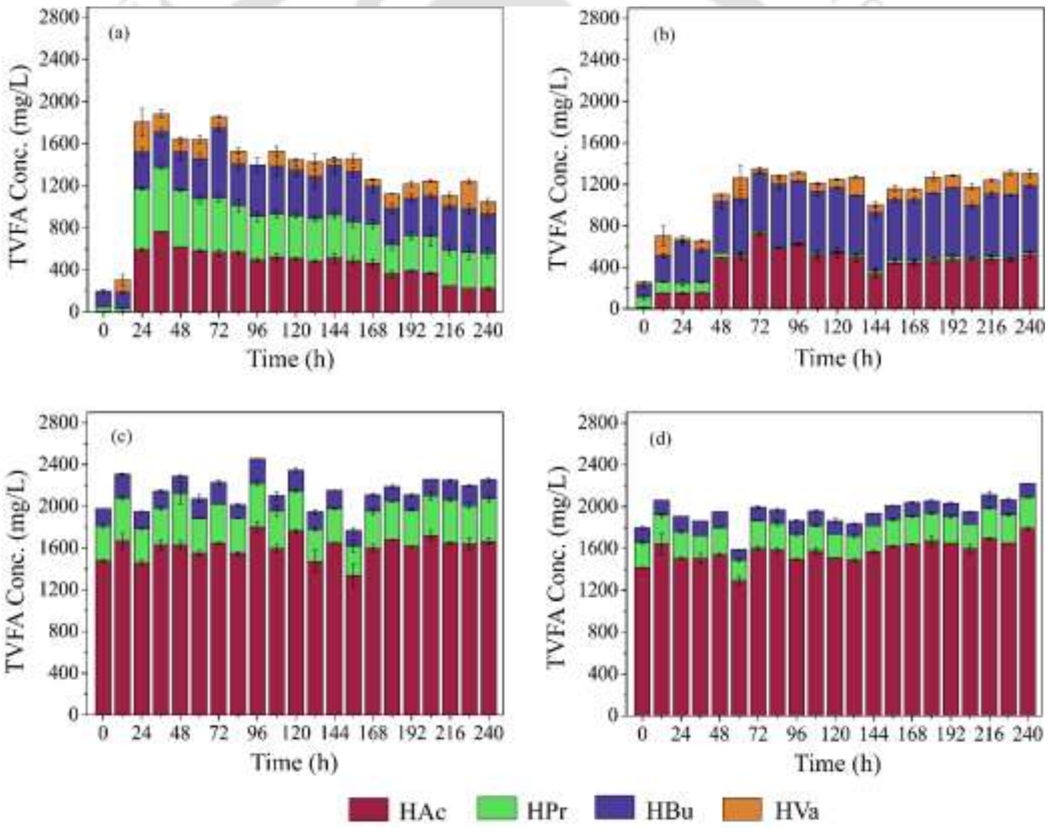


Figure 4.1: VFA content and profile of RME acidified with (a) untreated sludge, (b) pretreated sludge, and BE acidified with (c) untreated sludge, (d) pretreated sludge.

The VFA profile showed improvement during the acidification of BE as well. The VFA composition for PT-BE was HAc:HPr:HBu;81:13:6, while that for UT-BE was HAc:HPr:HBu; 73:17:10 at their respective VFA_{max} (Table 4.2). The metabolic shift that the pretreatment inflicted was very negligible. The HAc yield improved, 0.48 mgCOD/mgCOD, by a small fraction after

BAS pretreatment (Table 4.4). The yield of HPr and HBu was reduced due to the domination of acetate metabolism. Due to this, even to odd ratio was better for PT-BE than UT-BE but negligible compared to PT-RME. It is interesting to note that the VFA profile generally remained stable for UT-BE and PT-BE throughout the experiment. However, the VFA profile for PT-BE is advantageous over UT-BE in relation to the desired outcome due to a lower amount of HPr.

4.4: The yield coefficient and rate of production of individual VFA at peak.

Effluent	Sludge	Rate of production (mg/Lh)				VFA yield (mgCOD/mgCOD)			
		PR_{HAc}	PR_{HPr}	PR_{HBu}	PR_{HVa}	$Y_{HAc/nonVFA}$	$Y_{HBu/nonVFA}$	$Y_{HPr/nonVFA}$	$Y_{HVa/nonVFA}$
RME	PT	9.90 ±0.02	-1.08 ±0.07	6.14 ±0.01	0.16 ±0.17	0.37 ±0.02	0.39 ±0.01	-0.06 ±0.07	0.01 ±0.01
RME	UT	7.79 ±0.06	6.55 ±0.01	7.23 ±0.05	1.42 ±0.14	0.20 ±0.06	0.31 ±0.05	0.25 ±0.02	0.07 ±0.09
BE	PT	1.55 ±0.05	0.24 ±0.04	0.02 ±0.08	-	0.48 ±0.05	0.01 ±0.05	0.10 ±0.04	-
BE	UT	3.37 ±0.02	0.90 ±0.03	0.72 ±0.03	-	0.41 ±0.06	0.15 ±0.03	0.16 ±0.03	-

±SD%, PT: Pretreated, UT: Untreated

There are multiple attributes for the acceptable VFA composition of the pretreatment system. The interconnected network of many spontaneous reactions culminated in a higher even to odd ratio ($\Delta G<0$). Ravindran et al. (Ravindran et al., 2010) found heat-shock pretreatment (95-105 °C for 1 h) of forest soil to shift the metabolism from ethanol type to an acetate-formate type leading to higher H_2 production. The substrate also decides the relative dominance of the metabolic route along with pretreatment due to the macromolecules released (Ghimire et al., 2015). Heat pretreated inoculum led to HAc-HBu type fermentation for food waste while cattle slurry shifted metabolism to HAc-HPr type (Tampio et al., 2019). In the present study, the inclusion of acid shock with heat shock shifted fermentation towards acetate dominated HAc-HBu type with RME as a substrate. Similarly, BE induced a metabolic shift after the heat-acid shock treatment but comparatively weaker to RME (Fig. 4.1). The higher HAc yield (0.20-0.48 mgCOD/mgCOD) in the acidogenic system denotes the dominance of acetate production from carbohydrates. Acetyl-CoA produced from carbohydrates through the glycolytic pathway reduces into acetate through the β-oxidation pathway that gets accumulated under pretreatment (Saady, 2013). The removal of a large percentage of the acetoclastic community by pretreatment further incremented acetate accumulation (Table 4.4). An increase in HAc metabolism leads to an increase in hydrogen production. The purging of the reactor after a 24 h interval possibly led to a transient build-up of H_2 partial pressure creating a reducing environment. The reducing environment could have promoted homoacetogens to consume hydrogen and carbon dioxide into acetic acid through the Wood-Ljungdahl pathway as heat-shock cannot prevent homoacetogensis completely (Saady, 2013). This further maximized the acetate content in the fermentate (Fig. 4.1b). The momentary reducing environment and the lower pH (H^+) could have induced chain elongation. The higher accumulation of acetate resulted in the passage across the cell membrane due to the undissociated form at low pH. It influences the phosphoroclastic pathway leading to condensation of two acetyl-CoA and subsequent reduction into HBu under a reducing atmosphere (Dahiya et al., 2015). Similarly, the chain elongation of acetate by reacting with limited ethanol that may have formed also directs higher HBu production. At low pH, lactate oxidation might have led to butyrate formation (Agler et al., 2011). These might justify the higher yield of butyrate during the acidification of RME, especially PT-RME. The acidification of BE with untreated BAS had yielded negligible HBu content. Meanwhile, PT-BE shows neither production nor consumption of HBu, even though HAc got upgraded. The butyrate production from secondary fermentation seems to be absent when BE becomes the substrate.

Overall production of odd-numbered VFAs, HPr, and HVa, shows no significant improvement even after pretreatment. HPr yield was comparatively higher for UT-RME at VFA_{max} in the present study. HPr production in PT-RME reduced altogether after 48 h. The lactate reduction under H_2 partial pressure to HPr might have shifted to acetogenesis resulting in higher HAc with subsequent chain elongation to HBu at VFA_{max} (Fig. 4.1a,b). Pretreatment reduced HPr producers' competitiveness as these reactions have similar Gibbs free energy (-86 kJ/mol) (Agler et al., 2011). The production of HPr remained reduced for UT-BE and PT-BE alike, as the effect of reducing equivalents seems to promote the HAc pathway (Fig. 4.1 c,d). Higher HPr content in BE, among the fermentates, becomes undesirable for a polymer accumulating media. HVa yield was negligible or absent, possibly due to the low availability of amino acids in the substrate used, as HVa is produced mostly from amino acid catabolism. Hence, the mix culture could have favored the catabolism of available amino acids towards smaller chain VFA production. The acidogenic digestion of dewatered sludge with high protein concentration had resulted in a valerate-dominated hydrolysate (Hao et al., 2017). Also, chain elongation of HPr is an energetically favored route for HVa accumulation (Agler et al., 2011). Chain elongation could be the preferred route for HVa production during the acidification of RME, because of which HVa yield was higher for UT-RME compared to PT-RME. However, HVa was below the detectable amount for the acidification of BE, which can be considered an acetate-oriented fermentation process. Therefore, RME fermentate produced using pretreated BAS is a potential feedstock to drive desired PHA composition and property. These outcomes of the study could vary by a small fraction for different samples of both the effluents as the effluent quality greatly depends on the substrate used in the industry. However, different samples of effluents would follow a similar trend as in the study.

4.2.4. Chemical Oxygen Demand stability during the acidification processes

The acidification protocol in the study explores to lower the COD consumption and increment VFA accumulation. In this direction, pretreatment brought about stability in COD consumption compared to the untreated system for RME (Fig. 4.2a). The exposure to extreme conditions inactivated the primary carbon sink in BAS, i.e., methane production, and led to COD stability and VFA accumulation. COD of PT-RME stabilized swiftly by 48 h, while COD started declining from 152 h for UT-RME. BAS converted maximum possible organic content to VFA by VFA_{max}, after which UT-RME transcended slowly towards soluble COD consumption through biomethanation (Fig. 4.2a). COD stability, $\Delta COD \leq 0.32$, indicates the advantage that PT-RME attained to contain COD removal allowing maximal organic content available for bioconversion. The consumption of organic content got restricted effectively during the acidogenic fermentation of brewery effluent with BAS (Fig. 4.2b). As observed in PT-RME, the decelerated PT-BE obtained VFA_{max} by 240 h with stable production as the pretreatment restricted carbon removal. A $\Delta COD \leq -0.014$ signifies that at the employed operational condition, the methanogenic activity of BAS gets limited and reduces the COD consumption from BE with or without pretreatment.

The degree of acidification (DA) is the normalized value against COD that gives the efficiency of the process and the amount of nonVFAs left in the effluent (Eq. 1). DA is the function of the VFA profile. The contribution to VFA-COD increases with an increase in carbon chain length, ie. HAc<HPr<HBu<HVa. So, the peak degree of acidification (DA_{max}) is also a consideration for selecting the VFA_{max}. UT-RME attains a peak degree of acidification of 83 %, while it was 55 % for PT-RME (Fig. 4.2c). The gap in TVFA production between pretreated and untreated systems led to the difference in DA_{max} due to the negative effect of heat-acid shock. The HPr and HVa contents in UT-RME were also added to the COD_{VFA}. However, the relative concentration of VFAs causes the variation observed in the DA profile for UT-RME, whereas stability in PT-RME produces a steady DA profile (Fig. 4.2c). PT-RME had the upper hand concerning VFA profile and COD stability, while UT-RME had better DA_{max}. The DA_{max} for acidification of brewery effluent is at par for pretreated and untreated systems. UT-BE attains a DA_{max} of 85 %, while that for PT-BE is 79 %. DA_{max} also shows similar acidification ability for PT-BE and UT-BE as COD stability. However, the acidogens that were able to perform in the natural state became kinetically

weak after pretreatment (Table 4.4). The pretreatment might have attenuated acidogens as PT-BE attained near similar DA only by the 10th day (Fig. 4.2d).

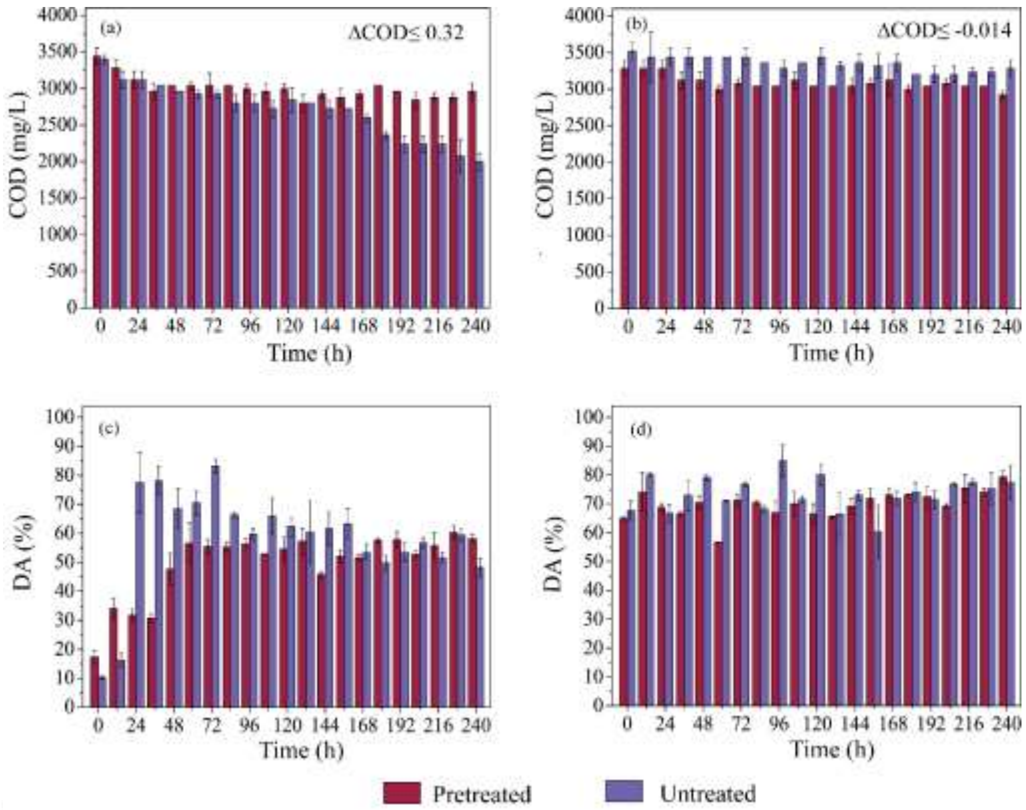


Figure 4.2: COD profiling for (a) acidification of RME and (b) acidification of BE with untreated and pretreated sludge. DA fluctuation for (c) acidification of RME and (d) acidification of BE with untreated and pretreated sludge.

The DA_{max} was high for UT-BE and PT-BE as the accumulation of HAc was strong enough to increase the COD_{VFA} even though HPr and HBu content was comparatively lower. Thus, acidification of BE was effective in retaining COD and maximally converted the COD into COD_{VFA}. BAS was able to acidify the major portion of nonVFA present in RME and BE. However, by considering the conversion yields and even to odd ratio, pretreatment enhanced VFA composition with adequate DA for PT-RME (Table 4.2).

4.2.5. Kinetics of acid production

Acidogenic fermentation of RME and BE with pretreated inoculum delivered a stable outcome (Fig. 4.1). Meanwhile, as indicated in Fig. 4.1a,4.4a, TVFA started a downward plunge from the VFA_{max} at retention time (RT) 152 h due to the domination of methanogens for UT-RME. However, BAS maintained acidogensis throughout the experimental run under the applied pH control for UT-BE (Fig. 4.1c, 4.4b). Hence, pretreatment of inoculum sludge is an effective protocol to transform the acidogensis process independent of RT.

Table 4.3 depicts the production rates for both systems. The production rate of HAc accelerated for PT-RME (9.90 mg/Lh) as compared to UT-RME (7.79 mg/Lh) at the respective VFA_{max}. HPr gets consumed faster for the pretreated system, while production occurs in the untreated system (Fig. 4.1 a, b). Even though the production rate for HBu was reasonable after pretreatment (6.14mg/Lh), it got accelerated for the untreated system (7.23 mg/Lh). The production rate for HVa was slower for both the system. Thus, pretreatment was able to kinetically improve the even-numbered VFA production while slowing down odd-number VFA effectively. The even-numbered VFAs lacked any temporal advantage over odd-numbered VFAs for UT-RME, which the even to odd ratio also reflects (Table 4.2,4.3). Brewery anaerobic sludge converted the nonVFA portion in BE to organic acid, unlike that one can be understood from Fig. 4.1c,d. The PR_{VFA} for PT-BE lags behind that of UT-BE. Even under better yield, the HAc production rate for PT-BE (1.55 mg/Lh) lags heavily behind that of UT-BE (3.37 mg/Lh) (Table 4.4). The HPr and HBu production from BE was much faster when BAS was untreated but slower than HAc. However, the HBu production rate was insignificant compared to HPr production for PT-BE. Thus, the pretreatment was unsuccessful in influencing the kinetics of neither TVFA nor the even-numbered VFA production during BE fermentation. BAS has the kinetic superiority to acidify BE without any stress from external stimuli compared to heat-acid shock. Therefore, the pretreatment of BAS improved the kinetics of production and accumulation of even-numbered VFA for RME as a substrate than BE.

The modified Gompertz equation provided depicted the dynamic behavior of VFA accumulation. The close-fitting nature of the experimental value (R^2>0.95) with the Gompertz equation is evident from Fig. 4.3, as reported by Mu et al. (Mu et al., 2006). Table 4.3 gives the values of the biokinetic constants adjusted for the model fitting done. The pretreatment exposes BAS to very harsh conditions. The spores formed takes time to germinate when exposed to non-defined substrates like RME and BE (Ghimire et al., 2015). Due to this, the lag in initiating bioconversion was generally twice that for the untreated system. In contrast, adjusting the regression retrieved λ= 0 h in some cases indicating the absence of lag time in initiating VFA accumulation. However, such a quick start failed to achieve any significant accumulation other than for HPr in UT-BE (Table 4.3). The comparable R_{max} value and higher A_{max} indicate the advantage of the pretreated system for HAc accumulation over the untreated system. Due to the high initial VFA content and absence of consumption, the fermentation of BE had a stronger HAc accumulation, i.e., higher A_{max} compared to fermentation of RME. However, PT-RME had superior HBu accumulation compared to UT-RME and fermentation of BE indicated by higher R_{max} and A_{max} values. The cumulative HPr accumulation had lower R_{max} and A_{max} values for pretreated system compared to untreated system (Fig. A1.1a,b). HPr accumulation got effectively prevented in PT-RME than PT-BE indicated by the smaller values for biokinetic constants. For HVa accumulation, the R_{max} was similar for the acidification of RME, while PT-RME had higher A_{max} compared to UT-RME due to the possible chain elongation of HPr produced (Fig. A1.1c). This influence of pretreated system over even-numbered VFAs dilute the advantage of untreated system over TVFA accumulation (Fig. A1.2). HVa was beyond any traceable amount for the acidification of BE with the method used. Thus, the modified Gompertz equation also indicates the superiority that pretreatment gained in enhancing even-number VFA accumulation by selectively repressing odd-numbered VFA especially during the acidification of RME. Table A1.1 (Annexure 1) shows the possible stoichiometric equations that lead to VFA production.

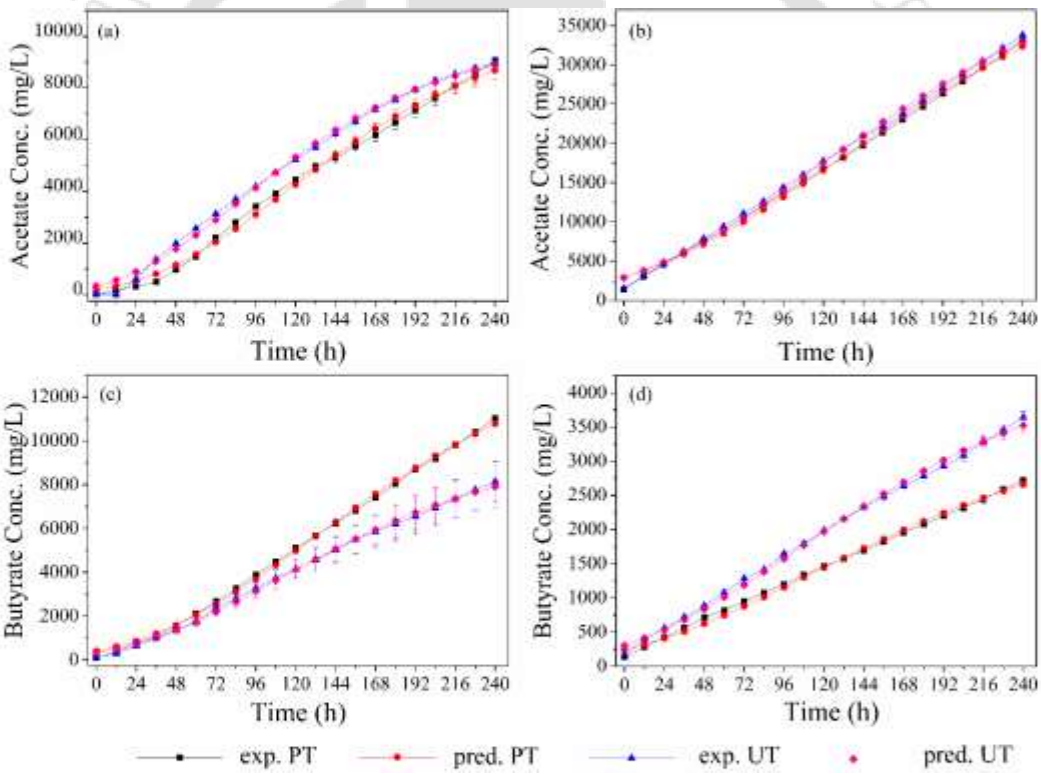


Figure 4.3: Cumulative VFA accumulation fitted to modified Gompertz equation for acetate accumulation during acidification of (a) RME and (b) BE, and for butyrate accumulation during the acidification of (c) RME and (d) BE with untreated and pretreated sludge.

4.2.6. pH assortment along the acidification process

The bioprocess monitoring of pH indicates the progress in the acidification process. In a robust acidification process, there is a decremental change in pH, and the VFA composition decides the extent of decrement. Meanwhile, pH 6.8-7.5 indicates the takeover of the system by methanogens

(Zhang et al., 2009). The pH during PT-RME remained around 6.0, with an occasional decrease at 24 h (Fig. 4.4a). This stable range in the pH for PT-RME is due to the constant TVFA production and a balance in the HAc and HBu content, as the pH is dependent on the fatty acid accumulated due to the respective pKa values HAc>HPr>HBu>HVa (Fig. 4.1b). The chain elongation of HAc at stable acidic pH due to the availability of reducing equivalents can increase HBu production. The inhibition of biomethanation is evident from the pH profile of PT-RME because of which acidogens were able to function normally. pH profile during UT-RME started in the acidic region with a slow transition into alkaline pH from 152 h. The TVFA profile (Fig. 4.1a) and COD distribution (Fig. 4.2a) also indicate the predominance of VFA consumption. The pH adjustment strategy employed in the study alone was not able to prevent VFA consumption into biogas. pH stability, $\Delta pH \leq 0.2$, depicts the ability of PT-RME to sustain acidogenesis by inhibiting methanogens compared to the eventual domination of acid consumers in UT-RME.

The pH profile for acidification of BE with pretreated and untreated systems remained within the acidic region, i.e., between 6.0-5.9 (Fig. 4.4b). The similar performance of the pretreated and untreated system brought the pH in the range. Acidogens in BAS behaved similarly in both cases producing acids without any consumption (Fig. 4.1c,d). $\Delta pH \leq 0.04$ also cements this notion that both system's acid production pattern was more or less similar and that stable VFA profile played a significant role in narrowing the pH range. The high amount of HAc accumulated lowered pH, below to that of PT-RME. A simple pH adjustment would be sufficient to direct BAS towards bioconversion and accumulation of VFA from BE.

The medium strength also might have played a role in restricting HAc production to a limit that led to a narrow and suitable pH range during the bioconversion of RME and BE. Lutpi et al. (Lutpi et al., 2016) also observed such an effect during thermophilic hydrogen fermentation of sucrose. The CO_2 evolved, and ammonia generated from the degradation of protein and other macromolecules forms bicarbonates. These bicarbonates could facilitate the buffering capacity of the acidogenic systems (Dahiya et al., 2015). Table 4.5 depicts the advantage of the present acidogenic system in elevating the even to odd ratio compared to other reported studies. The HAc content enhancing of pretreatment is evident in Table 4.5. At the same time, Table 4.5 could draw a correlation between sludge origin and heat treatment. The sludge sourced from digester treating solid wastes produces better TVFA concentration and DA under thermal pretreatment.

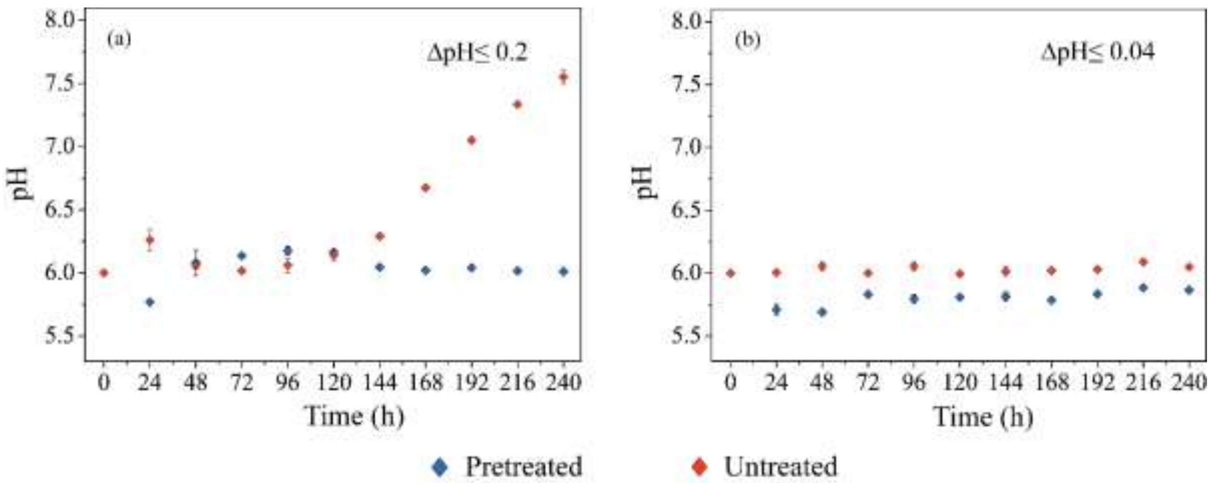


Figure 4.4: pH assortment along the retention time for the: (a) acidification of RME, (b) acidification of BE using the pretreated and untreated system

Table 4.5: A comparison between the impact of different pretreatment systems on VFA accumulation.

Substrate	Pretreatment (operation)	Anaerobic sludge origin*	TVFA Conc. (g/L)	DA (%)	VFA profile#, %w/w	Even to odd ratio	Reference
Potato and pumpkin waste	Thermal (105 °C, 4 h)	Bufallo manure +cheese whey +excess sludge	220.6[d]	-	89/3.1/7.3/0.2-0/0.6/0	24.8	(Ghimire et al., 2015)
Food waste	Acid (pH 3.0, 24 h)	Complex wastewater	11.1	37	60/10/29/1	8.1	(Sarkar et al., 2016)
Cheese whey	Thermal (100 °C, 2 h)	-	-	0.6[b]	58/19/13/6-0/0/4[a]	3.1[a]	(Colombo et al., 2016)
Primary+Excess sludge	Thermal (110 °C, 1 h)	Municipal wastewater	6.63[a]	50	28/16/30/26[a]	1.3[a]	(Zhang et al., 2019)
Food waste	Thermal (100 °C . 0.5 h)	Municipal and industrial biowaste	8.2	43[c]	66/5/20/4	9.5	(Tampio et al., 2019)
Rice mill effluent	**Thermal-acidic (100 °C, 2 h and pH 3.0, 24 h)**	Cow dung and duckweed	2.44	86	58/6.2/29/6.8	6.7	Chapter 3
		Brewery effluent	**1.35**	**55**	**54/2/41/3**	**20.97**	**This study**

*-substrate treated by inoculum at the origin, #: HAc/HPr/HBu/HVa-iHBu/iHVa/HLa, DA: Degree of acidification, a-on COD basis, b-VFA yield, mmole OA-C /mmole SS-C, c-g/g VS_{fed}, d- mmol/L, Even to odd ratio (mg/mg)

4.3. Conclusion

Regulating the VFA composition is feasible by selecting the right combination of inoculum sludge, pretreatment method, and type of waste as feedstock. Heat-acid shock efficiently inhibits methanogens, thereby enhancing VFA accumulation. At the same time, the pretreatment condition is highly dependent on the origin or type of inoculum. Untreated BAS had the upper hand in TVFA production as to pretreated sludge, probably due to the effect of pretreatment conditions. Hence, optimizing the pretreatment temperature and repeated-batch enrichment is necessary prior to the investigations. Meanwhile, the pretreated system enhanced even-numbered VFA production by suppressing odd-number VFA production with a higher yield of HAc. The production and accumulation kinetics supports even-numbered VFA accumulation by pretreated BAS. Similarly, bioconversion of RME and BE with pretreated BAS ensured better COD and pH stability leading to a system independent of retention time and pH control. Brewery anaerobic sludge was able to convert the fermentable portion of RME and BE into TVFA similarly. But pretreated BAS produced RME fermentate with desirable VFA composition possessing higher yield for HAc, HBu, and even to odd ratio with appropriate DA necessary to effect PHA composition and property.

References

Agler, M.T., Wrenn, B.A., Zinder, S.H., Angenent, L.T., 2011. Waste to bioproduct conversion with undefined mixed cultures: The carboxylate platform. Trends Biotechnol. 29, 70–78.

Arcos-Hernández, M. V, Laycock, B., Donose, B.C., Pratt, S., Halley, P., Al-luaibi, S., Werker, A., Lant, P.A., 2013. Physicochemical and mechanical properties of mixed culture polyhydroxyalkanoate (PHBV). Eur. Polym. J. 49, 904–913.

Atasoy, M., Eyice, O., Schnürer, A., Cetecioglu, Z., 2019. Volatile fatty acids production via mixed culture fermentation: Revealing the link between pH, inoculum type and bacterial composition. Bioresour. Technol. 292, 121889.

Baghchehsaraee, B., Nakhla, G., Karamanev, D., Margaritis, A., Reid, G., 2008. The effect of heat pretreatment temperature on fermentative hydrogen production using mixed cultures. Int. J. Hydrogen Energy 33, 4064–4073.

Chang, S., Li, J.Z., Liu, F., 2011. Evaluation of different pretreatment methods for preparing hydrogen-producing seed inocula from waste activated sludge. Renew. Energy 36, 1517–1522.

Colombo, B., Tommy, P.S., Maria, R., Barbara, S., Fabrizio, A., 2016. Polyhydroxyalkanoates (PHAs) production from fermented cheese whey by using a mixed microbial culture. Bioresour. Technol. 218, 692–699.

Dahiya, S., Sarkar, O., Swamy, Y.V., Venkata Mohan, S., 2015. Acidogenic fermentation of food waste for volatile fatty acid production with cogeneration of biohydrogen. Bioresour. Technol. 182, 103–113.

Ghimire, A., Frunzo, L., Pontoni, L., D'Antonio, G., Lens, P.N.L., Esposito, G., Pirozzi, F., 2015. Dark fermentation of complex waste biomass for biohydrogen production by pretreated thermophilic anaerobic digestate. J. Environ. Manage. 152, 43–48.

Hao, J., Wang, X., Wang, H., 2017. Overall process of using a valerate-dominant sludge hydrolysate to produce high-quality polyhydroxyalkanoates (PHA) in a mixed culture. Sci. Rep. 7, 1–11.

Korkakaki, E., Mulders, M., Veeken, A., Rozendal, R., van Loosdrecht, M.C.M., Kleerebezem, R., 2016. PHA production from the organic fraction of municipal solid waste (OFMSW): Overcoming the inhibitory matrix. Water Res. 96, 74–83.

Lutpi, N.A., Md Jahim, J., Mumtaz, T., Harun, S., Abdul, P.M., 2016. Batch and continuous thermophilic hydrogen fermentation of sucrose using anaerobic sludge from palm oil mill effluent via immobilisation technique. Process Biochem. 51, 297–307.

Magrini, F.E., de Almeida, G.M., da Maia Soares, D., Fuentes, L., Ecthebehere, C., Beal, L.L., da Silveira, M.M., Paesi, S., 2020. Effect of different heat treatments of inoculum on the production of hydrogen and volatile fatty acids by dark fermentation of sugarcane vinasse. Biomass Convers. Biorefinery 1–14.

Morgan-Sagastume, F., Hjort, M., Cirne, D., Gérardin, F., Lacroix, S., Gaval, G., Karabegovic, L., Alexandersson, T., Johansson, P., Karlsson, A., Bengtsson, S., Arcos-Hernández, M.V. Magnussona, P., Werker, A., 2015. Integrated production of polyhydroxyalkanoates (PHAs) with municipal wastewater and sludge treatment at pilot scale. Bioresour. Technol. 181, 78–89.

Mu, Y., Wang, G., Yu, H.Q., 2006. Kinetic modeling of batch hydrogen production process by mixed anaerobic cultures. Bioresour. Technol. 97, 1302–1307.

Ravindran, A., Adav, S., Yang, S.S., 2010. Effect of heat pre-treatment temperature on isolation of hydrogen producing functional consortium from soil. Renew. Energy 35, 2649–2655.

Saady, N.M.C., 2013. Homoacetogenesis during hydrogen production by mixed cultures dark fermentation: unsolved challenge. Internaltional J. Hydrog. Energy 8, 13172–13191.

Sarkar, O., Kumar, A.N., Dahiya, S., Krishna, K.V., Yeruva, D.K., Mohan, S.V., 2016. Regulation of acidogenic metabolism towards enhanced short chain fatty acid biosynthesis from waste : metagenomic pro fi ling. RSC Adv. 6, 18641–18653.

Tampio, E.A., Blasco, L., Vainio, M.M., Kahala, M.M., Rasi, S.E., 2019. Volatile fatty acids (VFAs) and methane from food waste and cow slurry: Comparison of biogas and VFA fermentation processes. GCB Bioenergy 11, 72–84.

Valentino, F., Morgan-Sagastume, F., Campanari, S., Villano, M., Werker, A., Majone, M., 2016. Carbon recovery from wastewater through bioconversion into biodegradable polymers. N. Biotechnol. 37, 9–23.

Zhang, D., Jiang, H., Chang, J., Sun, J., Tu, W., Wang, H., 2019. Effect of thermal hydrolysis pretreatment on volatile fatty acids production in sludge acidification and subsequent polyhydroxyalkanoates production. Bioresour. Technol. 279, 92–100.

Zhang, M., Wu, H., Chen, H., 2014. Coupling of polyhydroxyalkanoate production with volatile fatty acid from food wastes and excess sludge. Process Saf. Environ. Prot. 92, 171–178.

Zhang, P., Chen, Y., Zhou, Q., 2009. Waste activated sludge hydrolysis and short-chain fatty acids accumulation under mesophilic and thermophilic conditions: Effect of pH. Water Res. 43, 3735–3742.

Annexure 1

Table A1.1: Acidogenic bioreaction driving the fermentation system [(Saady, 2013: Agler et al., 2011)]

VFA	Stoichiometric Equation	ΔG° (kJ)
Acetate	$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2$	-206.0
Acetate	$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$	-104.0
Propionate	$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O$	-279.4
Propionate	$CH_3CHOHCOOH + H_2 \rightarrow CH_3CH_2COOH + H_2O$	-86.63[a]
Butyrate	$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2H_2 + 2CO_2$	-254.0
Butyrate	$2CH_3CHOHCOOH + 2H_2O \rightarrow CH_3CH_2CH_2COOH + 2H_2 + H^+ + 2HCO_3^-$	-56.3
Butyrate	$2CH_3COOH + 2H_2 + H^+ \rightarrow CH_3CH_2CH_2COOH + 2H_2O$	-47.55[a]
Butyrate	$CH_3COOH + CH_3OH \rightarrow CH_3CH_2CH_2COOH + 2H_2O$	-201.7[a]
Valerate	$CH_3CH_2COOH + 6H_2 + 2CO_2 \rightarrow CH_3CH_2CH_2CH_2COOH + 4H_2O$	-143.3

a: kJ/mol

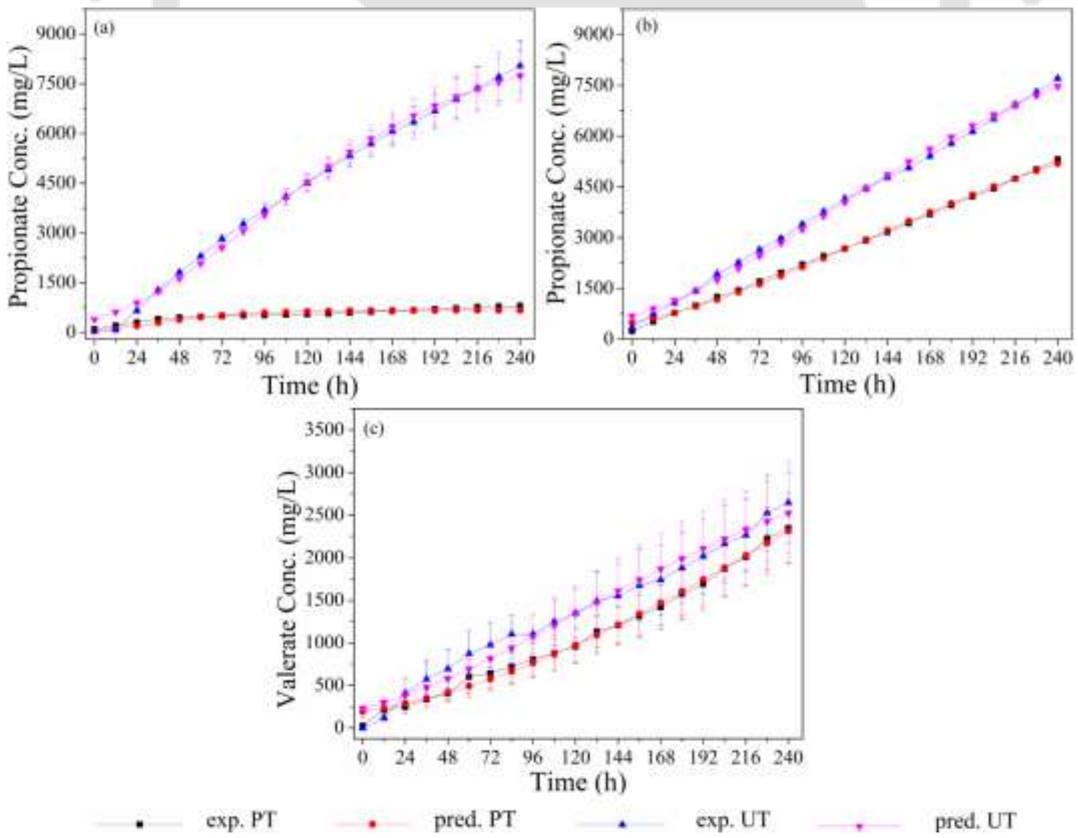


Figure A1.1: Model fitting for the cumulative accumulation of propionate, (a) RME and (b) BE and valerate, (c) RME, with untreated and pretreated sludge.

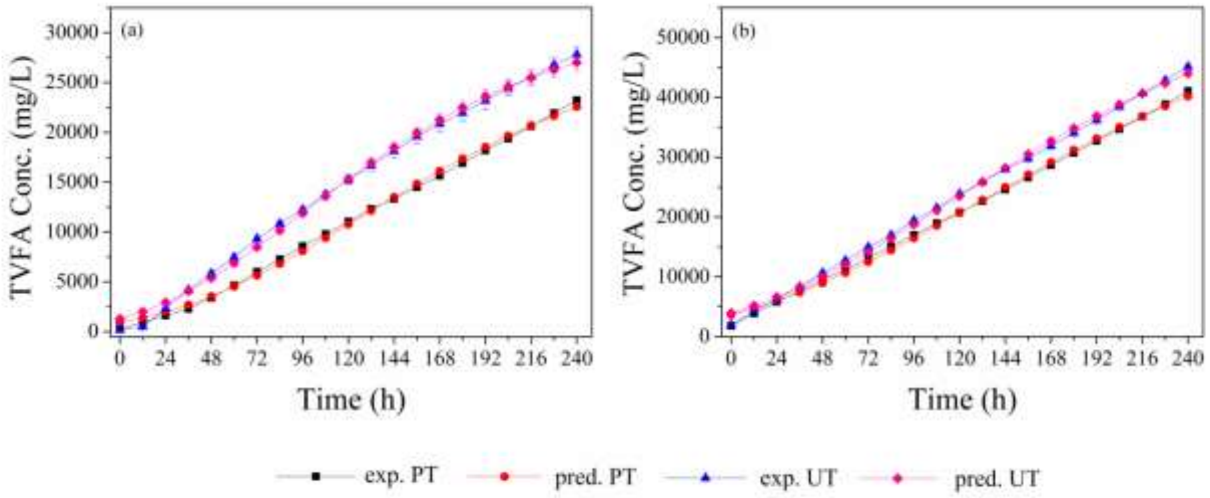
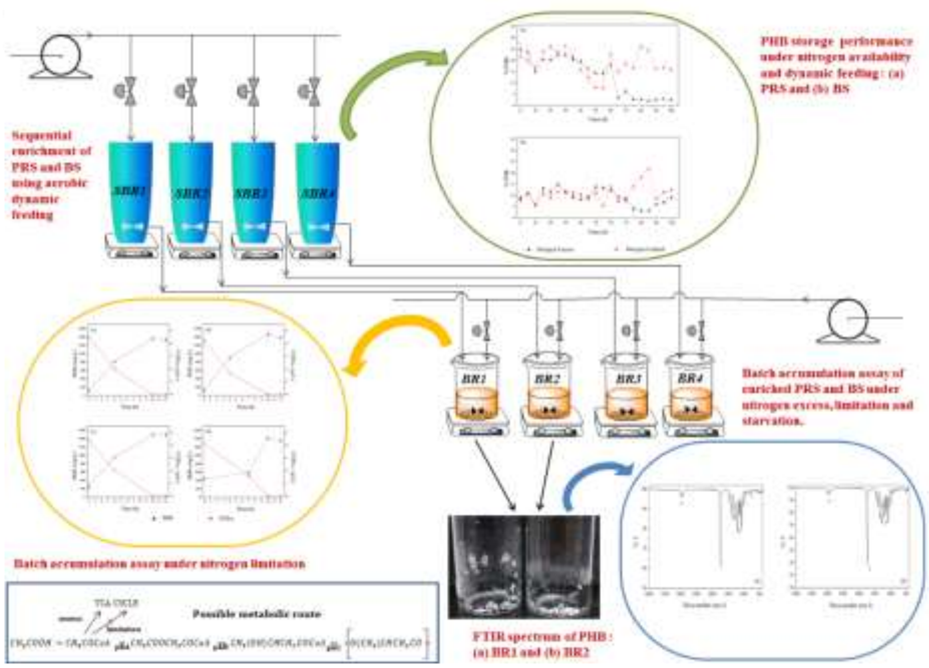


Figure A1.2: Model fitting for the cumulative accumulation of total volatile fatty acid, (a) RME and (b) BE with untreated and pretreated sludge.

Chapter 5

Effect of activated sludge origin and nitrogen availability on the bioconversion of volatile fatty acid into polyhydroxybutyrate by aerobic dynamic feeding strategy



5.1. Background and research gap

Plastics are now understood to be an unavoidable commodity in day-to-day life. Leo Hendrik Baekeland's invention of the first synthetic polymer 'Bakelite" was a stride towards reducing the impact of other such commodities as (paper, cloth items) on the environment (BBC, 2019; Feldman, 2008). However, as with everything we human touches, plastic went on to be one of the essential items of everyday life going uncheck of its misuse/overuse. Over the century, plastic pollution became one of the greatest menace the human race had to face. Plastic became so deep-rooted that a ban on its total use could be a catastrophe to the world economy that could hit the developing nations very hard. Hence, the search for an alternate brought the scientific community to one of the closest naturally occurring biodegradable polymer polyhydroxyalkanoates (PHA). The physicochemical properties were similar to that of conventional plastics depending on its monomeric composition that PHA became one of the hot topics among the research fraternity as an option to reduce (reduce, reuse, and recycle) the use of conventional plastics (Albuquerque and Malafaia, 2018),(Sudesh et al., 2000).

PHAs are condensed polymers of hydroxylated fatty acids found inside cytoplasm as lipophilic inclusions. Poly-(3)-hydroxybutyrate (PHB) is one of the PHAs that have been the center point of research. The copolymers with different monomers have also proven their varied application. But the cost of PHA-based bioplastics (per Kg) is more than twice that of conventional plastics, which had stalled the large scale use of PHA-based plastics(Valentino et al., 2016). Improving the property and reducing the operational cost of PHA had been the thrust area to improve the competitiveness of bioplastic. For the cost of PHA, the investigations concentrated on sources of carbon and synthesizing culture to influence the cost of production. In the case of PHA synthesizing culture, activated sludge was a well-known source for PHA accumulators, which was the least explored. It is also the ideal microbial source to utilize renewable carbon sources like wastewater as activated sludge is non-axenic that could survive and grow under various adverse conditions (Sabliy et al., 2019). Moreover, excess activated sludge had to be wasted in effluent treatment plants making this waste activated sludge economically and operationally the ideal source of culture. However, crude activated sludge has limited PHA storage capabilities to convert organic content to the stored polymer (Sakai et al., 2015).

Many schemes to enrich the sludge with PHA accumulators employed some form of selective pressure. Aerobic dynamic feeding (ADF) is among the widely explored options that impose a feast/famine (f/f) cycle. ADF forces the microbial mix culture (MMC) in the activated sludge to store a part of the carbon as PHA during the feast period to increase their survival ability during the long famine period. The cycle gets repeated for an extended period until the sludge gets dominated by PHA storing MMC (Serafim et al., 2008). Another route was by taking into account the relationship between volatile fatty acids (VFAs)and PHA. VFAs impart a substrate level selective pressure to increase PHA storage. In this way, renewable carbon sources can be used for PHA production from activated sludge by increasing the VFA content through acidogenic fermentation. Many studies employed the combination of ADF-VFA to influence storage capacity by adjusting different parameters like pH, temperature, feeding pattern, etc.(Valentino et al., 2016). Yet, the physical nature of the sludge itself is something least explored along with already available information. Initial biomass concentration in enrichment reactors had no significant influence on the PHA storage capacity of the sludge at a fixed sludge retention time (SRT). Even though choosing an optimal seeding concentration is necessary for setting other physical parameters (Chen et al., 2017). Sakai et al. (Sakai et al., 2015) had observed that the sludge origin plays a role when the sludge is directly utilized for PHA accumulation as they collected sludge from different municipal wastewater treatment plants that were running under different biological treatment process. At the same time, activated sludge procured from non-municipal sewage effluent treatment plants (ETP) has also shown equal capability for PHA production like sludge from food processing industrial ETP (Suresh Kumar et al., 2004), brewery ETP (Wen et al., 2010), etc. However, a comparison of the PHA production capacity of the activated sludge derived from an entirely different ETP treating different effluent and their accumulation trend under different operational conditions is yet to understand.

The most primary factor that could decide the direction of carbon flux towards growth or storage is nutrient availability. Many researchers studied the impact of nutrient availability,i.e., nitrogen and phosphorus, on PHA accumulation. Early research had shown that altering the C:N ratio to limiting conditions could enhance the PHA accumulating capacity of activated sludge (Suresh Kumar et al., 2004). Later studies implemented a combined culture enrichment and altering nutrient availability to improve polymer production. Wen et al. (2010) found that nitrogen imitation has improved PHA storage by about 59 % compared to phosphorus limitation during the acclimatization phase. At the same time, the sludge retention time of the system also influences the effect of excess or limiting the C/N ratio on culture enrichment (Johnson et al., 2010). However, most studies tuned the nutrient availability during PHA accumulation to maximize the storage of enriched MMCs. Valentino et al. (Valentino et al., 2015) proposed an optimal range of combined nitrogen and phosphorus limitation to improve the productivity over nutrient starvation during enriched sludge. On the contrary, a recent work reported nitrogen limitation as an effective route to maximize PHA production with enriched sludge compared to phosphorus and combined nitrogen-phosphorus limitation (Montiel-jarillo et al., 2017). All these studies point out the positive impact of altering nutrient availability on polymer production. Still, the impact of nutrient conditions during enrichment and their effect during accumulation under the different nutrient regimes and operational conditions is the least investigated area. Basak et al. (Basak et al., 2011) had reported the culture enriched under nitrogen deficient conditions to have a better PHA production when accumulated under nitrogen limitation.

The study explored the physical nature of activated sludge to impact biopolymer production. The study intends to quantify the effects on PHA production with respect to PHB, which is the major type of PHA synthesize and investigated. The experiments used two different activated sludge as inoculum collected from a similar unit process from two different effluent treatment plants treating extremely different categories of effluents. These sludges were then submitted to ADF enrichment to observe the influence of ecological selective pressure induced by feast/famine on the PHB accumulating potential (PAP) of inoculum. Also, an additional metabolic selective pressure was provided in the form of nitrogen (ammoniacal nitrogen) availability to determine the combined effect on the seed culture. Finally, the PHB accumulation of each of the enriched cultures was analyzed under different nitrogen availability to understand the interaction between enrichment and accumulation condition. These interactions were expounded by investigating physicochemical characteristics of the accumulated polymer followed by linking these factors to the ecology of activated sludge.

5.2. Results and discussion

5.2.1 Inoculum characterization

Petroleum refinery sludge (PRS) was collected from a petroleum refinery in Assam, India. PRS had a high amount of volatile suspended solids (VSS), 63 % of total suspended solids (TSS). Brewery sludge (BS) collected from a nearby brewery possessed a large amount of readily biodegradable carbon comprising VFAs but with a lower VSS (32 % TSS). The initial acclimatization period augmented the sludges with higher VSS due to biomass growth with acetate consumption. Both sludges had low COD, nitrogen, and phosphorus content (dissolved) contents (Table 5.1). The acclimatized sludge samples were used as inoculum for the enrichment reactors.

5.2.2. SBR performance and its effect on the PHB accumulating potential of the sludge

The primary objective of the study is the improvement of PAP of the inoculum sludge to achieve higher PHB accumulation. The primary factor that promotes PHB production is the carbon source, sodium acetate (NaAc).The presence of excess acetate increases acetyl-CoA through the β-oxidation pathway, thereby reducing the CoASH (Coenzyme A) level and uplifting the inhibition of the PHB synthesis pathway leading to improved PHB storage (Vallari et al., 1987; Tan et al., 2014). The preference of acetate as the carbon source over other uncomplex sources is a well-established fact (Cui et al., 2016). Moreover, NaAc aids in restricting the pH of the medium towards neutral.

Table 5.1: Characteristics of PRS and BS inoculums.

Parameter	PRS	BS
TSS (g/L)	24.8±0.2	71.1±2.1
VSS (g/L)	15.7±0.1	22.9±0.5
COD (g/L)	0.45±0.04	0.53±0.04
N-NH_4 (mg/L)	3.5±0.03	82.5±5.3
P-PO_4 (mg/L)	0.018±0.01	1.72±0.07

±SD, PRS: Petroleum refinery sludge, BS: Brewery sludge

Another route for influencing the storage capacity is by exposing the MMC towards recurrent feast/famine phases. The available carbon gets consumed by the end feast phase leading to a carbon starved famine phase. Thereby, a part of the carbon gets stored in a storage form to survive during the carbon starved period. This mechanism is highly dependent on the effectiveness of the ecological selective pressure implemented by feast/famine phases, shown in Fig. 5.1c,5.1d. The feast/famine condition and acetate as carbon source, in combination, influence the extent of PHB stored at the end of the feast phase. An effective ecological selective pressure imposed due to the dynamic feeding can increment the PHB stored over time (Fig. 5.1a,5.1b), which can be maximized by batch accumulation. To exert stronger selective pressure, Chen et al. (Chen et al., 2016) modified the process by inducing a carbon starved period by incorporating an additional settling, discharge, and feeding at the end of the feast period resulting in 28.3 % PHA by 30 days. The formulation of parameters in the aerobic dynamic feeding mode of SBR operation was such that the carbon source added gets consumed quickly, leaving behind a longer period without carbon. Better the feast/famine selective pressure, desirable will be the PAP of the sludge. For this purpose, the study explored a cycle length of 24 h with a fixed SRT. A prolonged cycle length was said to impose better pressure over the sludge that enables PHB accumulators to outcompete non-accumulators (Jiang et al., 2010). In the initial run, proper growth was affected in SBR2 due to higher VSS concentration causing a disparity between airflow rate and oxygen uptake. After the sludge wasting was initiated, the f/f for SBR1/SBR2 showed a narrow range of 0.2-0.4, depicting a proper dynamic selective pressure on the sludge (Fig 5.1c). Adequate biomass growth propelled by copious nitrogen and carbon resulted in a higher substrate

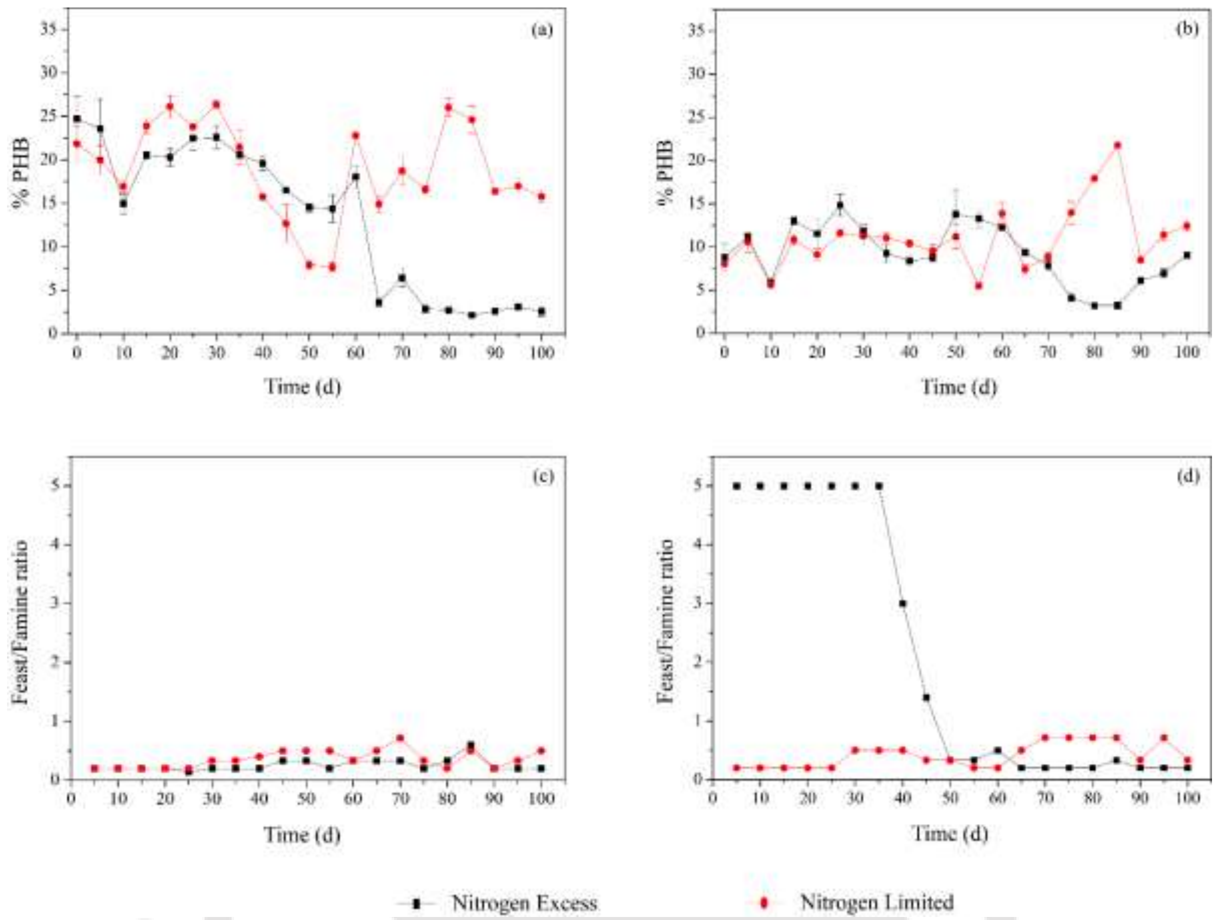


Figure 5.1: PHB storage performance in SBRs inoculated with (a) PRS and (b) BS and the associated feast/famine ratio profile of SBRs inoculated with (c) PRS and (d) BS during the enrichment process.

consumption rate (Table 5.2), thereby having a shorter feast and longer famine phases. However, f/f had a broad range between 0.2-0.7 for SBRs under nitrogen limited conditions (Fig 5.1d). This is primarily due to a growth restriction imposed by inadequate nitrogen supply that tends to slow the growth and divert the carbon mainly towards storage and maintenance. The specific substrate consumption rate in the limiting enrichment culture prevented f/f from slipping further to lower value. Nonetheless, maintaining the f/f ratio in SBR3/SBR4 was cumbersome due to the growth limitation.

So it is evident that when nitrogen is in excess, a lower f/f is obtained compared to when nitrogen is limited (Fig. 5.1c,5.1d). Better selective pressure sums up better PHB storage. However, it was interesting to notice that SBR3and SBR4 had a narrow and superior PHB storage distribution compared to SBR1and SBR2 (Fig. 5.1a,5.1b). This phenomenon is similar to the higher PHA storage (14.7 %) observed in the nitrogen limited SBR compared to nitrogen excess SBR (10.6 %) by Basak et al. (Basak et al., 2011). The difference between PHB storage for SBR1 and SBR3 was nearly double from the start of nitrogen limitation (60 d) for SBR3. The effect of the limitation kicked in only by 70th days for SBR4 with a spike of 20 %,

Table 5.2: Kinetic and stoichiometric parameters for the enrichment of the activated sludge under different nitrogen availability conditions.

Regime	SBR1	SBR2	SBR3	SBR4
Seed culture	PRS	BS	PRS	BS
Nutrient availability	100:7:1*	100:7:1*	100:1:1†	100:1:1†
f/f	0.2	0.2	0.5	0.3
% PHB	7.8±0.05	14.4±0.04	14.4±0.04	13.4±0.02
$Y_{P/S}$	0.21±0.07	0.27±0.06	0.095±0.04	0.078±0.07
$Y_{X/S}$	0.73±0.01	0.72±0.01	0.39±0.01	0.18±0.04
q_p	0.017±0.003	0.012±0.04	0.006±0.06	0.01±0.14
-qs	0.12±0.05	0.11±0.02	0.074±0.02	0.10±0.04
Productivity	10.3±0.06	13.4±0.08	4.7±0.16	4.3±0.1
r_p	119.9±0.07	136.6±0.02	34.4±0.05	34.2±0.08
$-r_p$	26.5±0.04	76.2±0.04	34.6±0.14	27.5±0.02
ΔY	0.94±0.03	0.99±0.02	0.49±0.02	0.26±0.01
m_s	0.33±0.09	0.24±0.06	0.27±0.01	0.96±0.1
m_p	0.019±0.16	0.028±0.09	0.022±0.12	0.10±0.2
$Y_{P/S}^{Pred}$	0.16±0.03	0.22±0.07	0.04±0.04	0.01±0.06

±% SD, * nitrogen excess, †nitrogen limited, f/f:h/h, q_p: $mgCOD_{PHB}$ /mg COD_X h, q_s: mg COD_{Ac} / $mgCOD_X$ h, $Y_{P/S}$: mg COD_{PHB}/$mgCOD_S$, $Y_{X/S}$: $mgCOD_{PHB}$/$mgCOD_{Ac}$, Productivity: $mgCOD_{PHB}$/Lh, r_{PHB}/-r_{PHB}: $mgCOD_{PHB}$/Lh, m_s/m_p: mgCOD/mgCOD h

subsequently stabilizing around 14 % (during the assessment period). The average difference between the PHB storage of SBR2 and SBR4 was ≈ 10 %. The higher PHB storage of SBR3 and SBR4 can be correlated to be the cumulative impact of growth imbalance. This imbalance shifts carbon flux to other non-growth-related activities. Therefore, the impact of the feast/famine phase can be considered negligible due to growth limitation with limited or absence of nitrogen for culture to aid PHB consumption during the famine phase. However, the active feast/famine cycle could put enough selective pressure by the 100th day that nitrogen excess cultures could match that of limited culture. SBR1 and SBR2 could have challenged SBR3 and SBR4 in the initial leg of the run if the washout observed from day 30 due to implementation of SRT 10 d by sludge wasting were avoided (section 5.2.3).

Similar to the observation of Johnson et al. (Johnson et al., 2010), a prolonged period of nitrogen limitation caused SBR4 to develop filamentous growth. This excess bulking might have impaired proper oxygen mass transfer leading to a highly unstable f/f ratio towards the end of reactor operation, finally resulting in the loss of its PAP. At the same time, SBR3 ran the entire run of the experiment with stable storage. Serafim et al. (Serafim et al., 2004)had observed consistent performance for nitrogen limited SBR. The microbial community in the sludge might have responded to the limiting condition contrastingly. Unstable extracellular polymer metabolism might have led to the loss of physical characteristics of sludge in SBR3 as microscopic analysis failed to find any serious filamentous growth, as observed by Chen et al.(2017). Therefore, nitrogen limitation can be a potential route to improve the PAP of activated sludge, but extended exposure to limiting conditions can cause reactor instability and loss of storage capacity. On the contrary, the nitrogen excess condition ensures stable operation and lower f/f ratio, thereby implementing a strong selective pressure leading to improved PAP when exposed to a long duration of time. So nitrogen excess enrichment has the potency to establish a long-term enrichment system with adequate PHB storage. Submitting the enriched culture to PHB accumulation could further understand this effect (section 5.2.6).

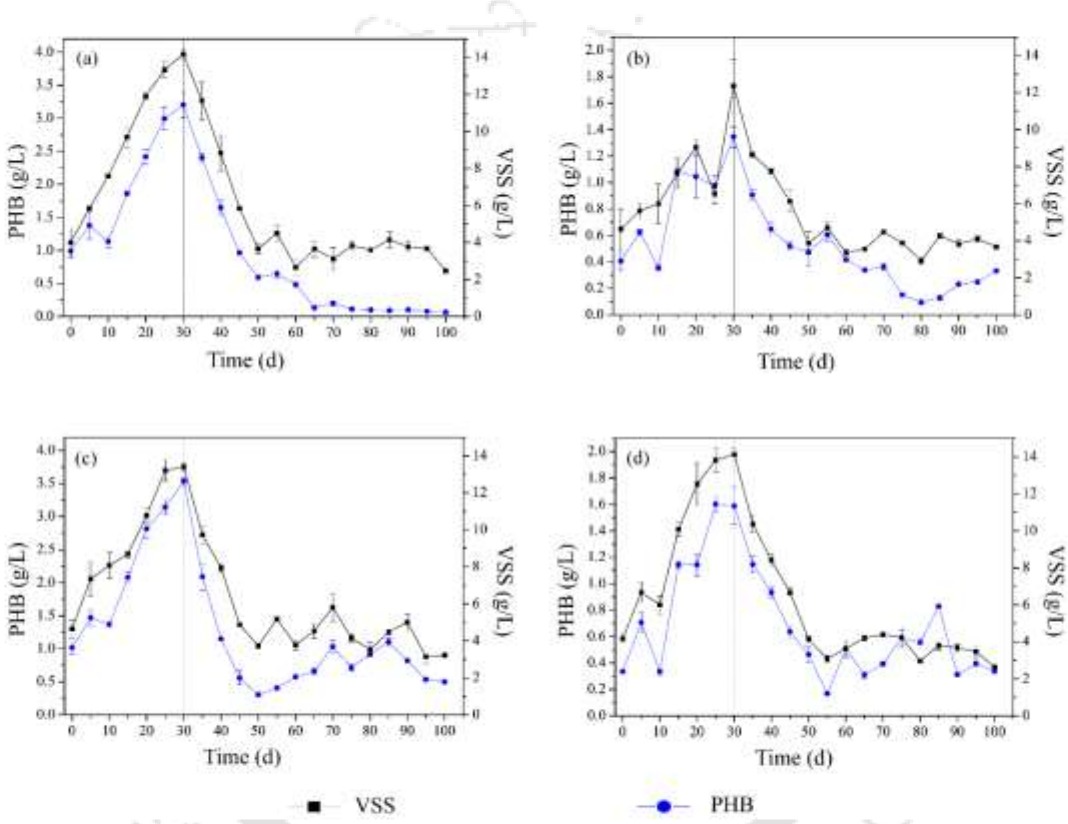


Figure 5.2: The impact of sludge wastage on PHB production and the enrichment of activated sludge in (a) SBR1, (b) SBR2, (c) SBR3, (d) SBR4

5.2.3. SRT regulation and impact on PAP of the sludge

The SRT dictates the time sludge spends in the reactor being exposed to the process conditions, thereby molding the microbial community in the desired route. The microbial community would grow and replace the population removed within the period of fixed sludge retention time, thereby assisting in proper growth and maintenance of the reactor by ensuring the required availability of oxygen, carbon, nutrient, mixing, etc. A higher SRT, longer period of exposure should allow the process parameter to influence the MMC to the maximum. However, the SRT for SBR enrichment of PHA generally ranges between 1-10 days (Valentino et al., 2016). Johnson et al. (Johnson et al., 2010) observed that a lower SRT has a longer feast time, causing a weaker limitation on the culture. So, a combination of longer cycle and lower SRT proposed improves the feast/famine enrichment of PHB accumulators due to the longer famine phase accompanied by fast growth (Johnson et al., 2009).

In the study, Eq. 6 was followed for monitoring SRT of the SBRs as laid out by Liu and Tay (Liu and Tay, 2007) (section 2.5.2). SBRs ran without sludge wasting (V_w=0) and controlling SRT until the 30th day to observe the effect of uncontrolled SRT on enrichment. SRT was in the range of 5-10 d during the start-up of the reactor. In the absence of sludge removal, the biomass kept on building up such that the VSS content of SBRs jumped from 4-4.8 g/L at 0 d to 12-14 g/L by 30 days (Fig. 5.2). The seed culture had fine settling properties (<<X_e), due to which the sludge wastage through effluent discharge reduced drastically, resulting in the scaling of SRT. Allied to this feast/famine ratio, which was around 0.2, also started to get affected for SBR2/SBR4due to the disparity between biomass growth and airflow rate (Fig. 5.1d). However, there was no significant effect of increasing biomass load on storage performance until 30 d as the PHB storage remained, on average, between 20-23 % and 5-11 % for SBRs inoculated with PRS and BS respectively (Fig. 5.2). This observation hints at the dominance of slow-growing PHB accumulators. However, sludge wasting restricts the SRT and biomass concentration in the reactors. Sludge wasting of 200mLfrom the 30 d maintains an SRT of 10 d by assuming X_e=0 as VSS in the effluent was negligible compared to that in the reactor. A plunge in PHB content by ten folds followed the initiation of sludge wasting (Fig 5.2). Sludge wasting caused a washout of biomass as VSS fell concomitantly with PHB content for all SBRs. The decline was explicit for reactors inoculated with PRS than BS, as observed by a steep reduction in PHB storage (Fig. 5.1a). Chau et al. (Chua et al., 2003) also made a similar observation, where the SRT maintained at 3 d and 10 d leads to a decrease in PHA production rate drastically till the 25th day. The washout removed the pre-existing slow-growing PHB accumulators during the sludge wasting. An SRT control implements late into reactor start-up caused the slow-growing accumulators, that might be dominant, in SBR1/SBR3 unable to keep up their growth to replace the wasted amount within ten days (Fig. 5.1a,5.2a,5.2c). However, biomass loss had a milder effect on PHB storage for SBR2/SBR4, which might be due to the presence of relatively fast-growing PHB-MMC in BS compared to PRS (Fig. 5.1b,5.2b,5.2d).

For SBR3, the effect was profound due to added loss of biomass owing to deterioration in settleability of sludge as the reactor had moved into near limiting C/N (50) (Fig. 5.1a,5.2c). A similar effect was also observed by Chen et al. (Chen et al., 2017) by 40 days when SBR was operated with fermented sugar cane wastewater at SRT of 10 d. Even though SBR3 maintained its poor settleability and storage capacity even after establishing C/N: 100, SBR4 lost its settling characteristics and storage entirely (which started deteriorating after 60 days) due to extensive filamentous growth by the 120th day. Similar to the case of SBR4, Johnson et al. (Johnson et al., 2010) observed that SBR under nutrient limitation maintained at 0.5d SRT lost its PAP within five months of operation. Thus no effluent is discharged after 80 days in SBR3 and SBR4 to prevent extensive sludge washout (when limitation became prominent). SRT was maintained only by sludge wasting (V_e=0, Eq. 1).

The biomass washout got stabilized with a 10 d SRT by the 50th day for all SBRs, indicating that the sludge got populated with faster-growing MMCs. These fast-growing MMCs under nitrogen excess regime required an extended period of enrichment to regain the storage capacity by the effect of selective pressure induced by feast/famine enrichment. In contrast, nitrogen limitation implemented in SBR3 and SBR4 restocked the new MMCs with the population having higher PAP in a limited period of run from the 60th day due to growth imbalance, as observed from Fig 5.1a,b.Thus the study implies that while the nutrient condition can alter the dominance of the microbial community, SRT dictates which community is permitted in the system depending on its growth. SRT is a crucial parameter to be optimized for a particular sludge considering the operating parameters to improve PHB accumulating potential of activated sludge.

5.2.4. Influence of the C/N ratio on PHB accumulating potential of the sludge

The optimal ratio of C:N:P for a stable activated sludge system is 100:5:1 (C/N:20)(Basak et al., 2011). Most of the studies differentiate the nitrogen excess or limited based on optimal ratios, such as C/N<20 is "nitrogen excess," while C/N>20 is "nitrogen limited". However, we observed that the ratio could vary significantly with inoculum, operational conditions, etc. A C/N ratio of 14 (±2) established a nitrogen excess condition in SBR1 and SBR2, which principally means that nitrogen was available in a higher amount even after the carbon was consumed entirely as the % $N\text{-}NH_4$ consumption till feast period was around 50-70 % (Fig. 5.3a,5.3b). In the system, the acetate concentration solely dictates the extend of feast/famine selective pressure that exerts a "carbon limiting" condition. The presence of nitrogen after the feast phase ensures that the MMC could survive during the famine phase using PHB as a carbon source. But during the famine phase, nitrogen consumption is meager as to 5-10 %, leaving a high amount of nitrogen at the end of the cycle (Fig. 5.3a,5.3b). Therefore, the necessity to narrow down the optimal C/N ratio exists so that nitrogen left at the end of the cycle would be minimal. Thereby, the nitrogen leakage to PHB accumulation could be contained, maintaining proper condition during accumulation using fermented or non-fermented effluent. For carbon limited enrichment, the restriction implemented by the shorter feast phase and extend famine phase prompt the MMC to alter the carbon flux towards PHB. This process over a prolonged period results in higher PHB storage. This trend, followed by SBR1 and SBR2, is visible from Fig. 5.3a,5.3b.

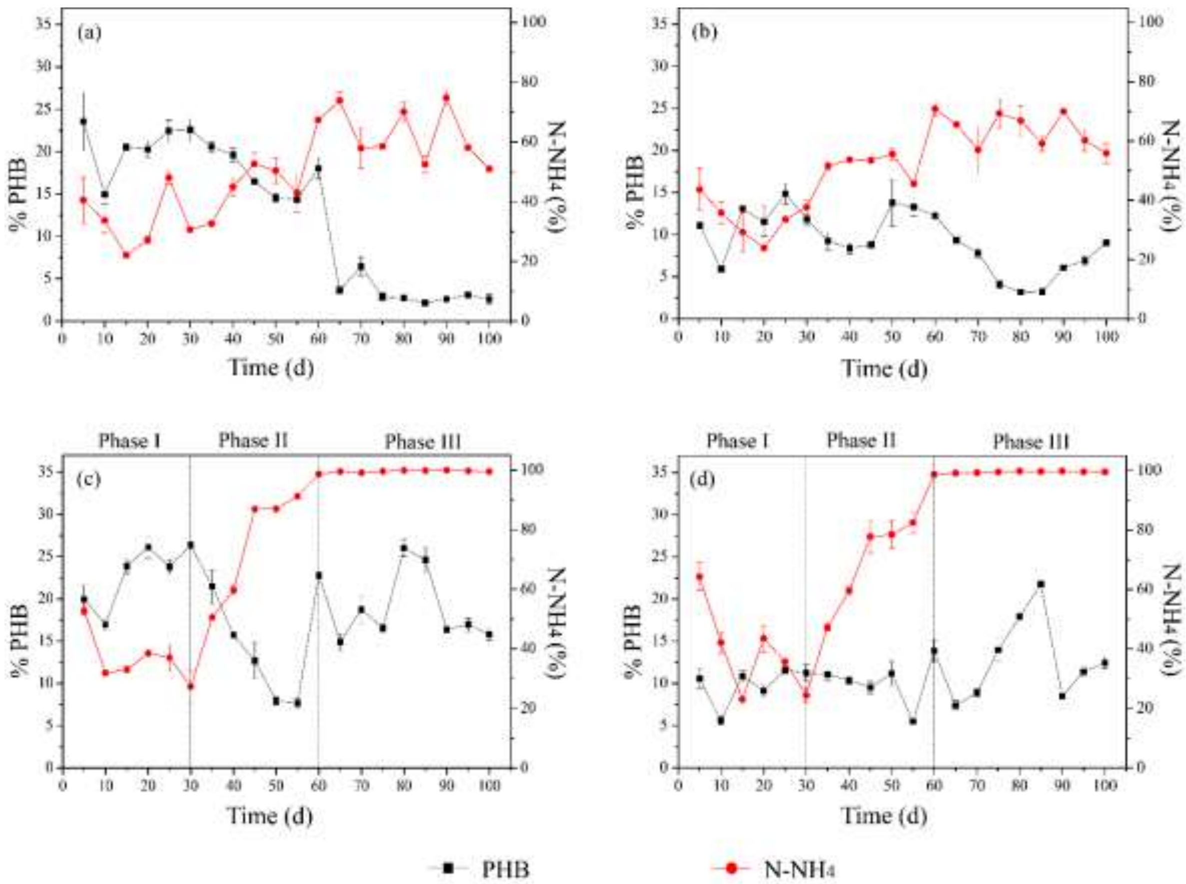


Figure 5.3: Influence of C/N ratio on PHB accumulating potential of activated sludge inoculated to (a) SBR1, (b) SBR2, (c) SBR3, (d) SBR4 during the sequential enrichment processes.

The term nitrogen limitation is also ambiguous. It can also vary from one system to another. A C/N ratio is limiting when the nitrogen gets consumed completely or exhausted before the carbon source. The real effect of limitation is visible at this equilibrium point. As observed in Fig. 5.3c,5.3d, during phase I C/N ratio of 25 was implemented that resulted in 20-40 % consumption by the end of feast reverberated by hardly any improvement in PHB storage. In phase II also, with a C/N ratio of 50, PHB storage was unable to show any acceptable increment from the initial. However, nitrogen consumption reached 80% for SBR3and SBR4 at the end of the feast phase, with the remaining amount available during the famine phase. Interestingly, the PHB storage showed a spike from the start of phase III, where the C/N ratio was 100. During phase II, the reactors underwent a kind of washout condition (mentioned in section 5.2.3), which might have diluted the limiting effect of the C/N ratio 50 without any improvement in the PHB storage. However, in phase III, the reactors were able to recover from the washout effect, thereby implementing the effect of limiting the condition imposed by C/N:100 (Fig. 5.3c,5.3d). PHB storage spikes to 26 % and 21 % for SBR3 and SBR4, respectively, during phase III, followed by reaching an equilibrium at 15 % and 12 %, respectively. In nitrogen limitation, the faster consumption of nitrogen leaves behind excess carbon. This induces a drop in protein synthesis and other essential growth-promoting molecules that slow down the TCA cycle and growth. Thereby, inhibition of the PHB synthesis pathway is uplifted and diverts more acetate into the pathway resulting in improved storage (deliberated in section 5.2.6).

The study clearly indicates that it is necessary to standardize the C/N ratio according to the reactor operating condition. The PHB storage was higher and consistent for nitrogen limited SBRs than nitrogen excess for the accessed period (Fig. 5.3). Basak et al. (Basak et al., 2011) also observed similar effects where a combination of nitrogen limitation and aerobic dynamic feeding had a positive result on PHA accumulation when enriched for 82 days. The proper limiting C/N ratio is also necessary to maximize PHB accumulation during the batch assay. However, longer exposure to complete limiting conditions could affect the stability of the SBRs and the storage capacity of the sludge. Determining the optimum excess C/N ratio delivers stable reactor operation and strong feast/famine conditions that could maintain the storage capacity for an extended period.

5.2.5. Kinetics of PHB storage during enrichment

The entire process of feast/famine enrichment, in a nutshell, is depicted in Fig. 5.4. In SBR1and SBR2, the MMC had faster growth and consumption of carbon due to the presence of NaAc and excess availability of $N\text{-}NH_4$, thereby having a shorter feast phase and extended famine phase (Fig. 5.4a,5.4b). But for SBR3 and SBR4, the availability of nitrogen gets highly restricted than carbon. Due to this, competition generates among the MMC in sludge to quickly consume the available nitrogen, increasing the substrate consumption rate. For this reason, acetyl-Coenzyme A also gets diverted to keep the consumption rate. The result is the delay in the transition from feast to famine phase (Johnson et al., 2010). Thus, nitrogen limited culture has a longer feast phase than nitrogen excess evident even though they have a comparable specific substrate consumption rate (Table 5.2). In nitrogen limitation, 'growth limitation' primarily attributes PHB storage rather than the feast/famine cycle.

In SBR1 and SBR2, a carbon limitation develops due to the faster consumption of acetate in the presence of excess nitrogen. Due to the carbon limitation, the accumulated PHB gets consumed for growth and maintenance during the extended famine phase. Nitrogen gets exhausted faster than carbon within the feast period creating a 'nitrogen limited condition in SBR3/SBR4. Therefore, PHB consumption during the famine period becomes slower due to insufficient nitrogen. The PHB consumption rate ($-r_p$) was slower till 16 h, followed by a sudden dip in PHB concentration at 18 h due to possible cell death (Fig. 5.4c,5.4d). Thus, the endogenous metabolism would have resulted in a similar $-r_p$, between limited and excess culture until the end of the cycle (Table 5.2). PHB production rate (r_p) casts the effect of nitrogen availability where r_p was higher for excess enrichment (119.9 and 136.6 mgCOD$_{PHB}$/Lh) while lower for limited enrichment (34.4 and 34.2 mgCOD$_{PHB}$/Lh). The combined effect of biomass growth and PHB accumulation catered by excess nitrogen resulted in better r_p. In contrast, insufficient nitrogen causes decelerated growth affecting cellular metabolism, eventually leading to lower storage.

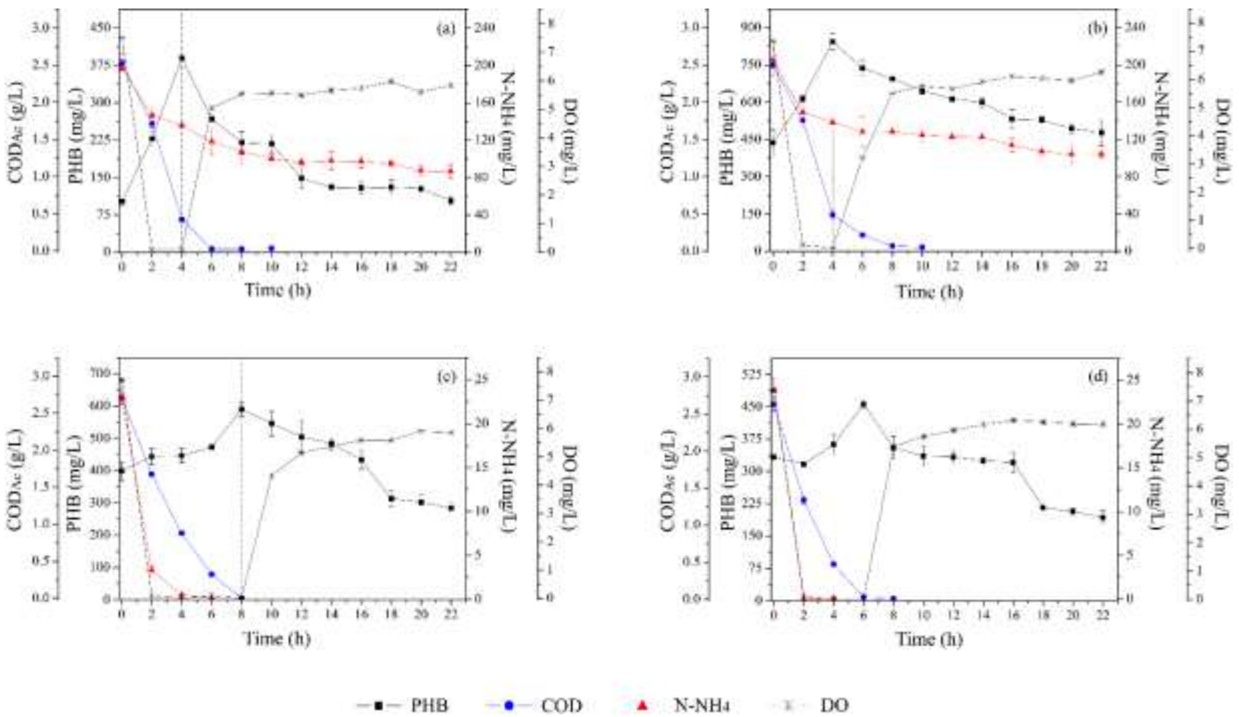


Figure 5.4: Kinetics of PHB production and it's regulation during a single cycle in the sequential enrichment of activated sludge in (a) SBR1, (b) SBR2, (c) SBR3, (d) SBR4

The PHB yield ($Y_{P/S}$) also depicts the negative impact of limiting conditions on culture enrichment. The substrate conversion into PHB was more efficient for SBR1 and SBR2 with $Y_{P/S}$ around 0.21 mgCOD$_{PHB}$/mgCOD$_S$ and 0.27 mgCOD$_{PHB}$/mgCOD$_S$ than that of SBR3and SBR4 with $Y_{P/S}$ of 0.095 mgCOD$_{PHB}$/mgCOD$_S$ and 0.078 mgCOD$_{PHB}$/mgCOD$_S$, respectively. The biomass yield ($Y_{X/S}$) indicates the nitrogen excess enrichment to be growth-oriented while nitrogen limited to be a growth-restricted regime. The insufficient nitrogen availability depleted the vital cell machinery (protein, enzyme, nucleic acid) as nitrogen is unavailable for protein synthesis, DNA replication/repair, etc. Such an extended period could weaken MMC that downturns the conversion capacity of the sludges. Also, the scarcity of nitrogen propels a competition between microbial communities to increase the nitrogen uptake rate causing faster substrate uptake. Some part of the stored PHB also gets consumed to keep the rate that might have affected the $Y_{P/S}$ (Johnson et al., 2010). Meanwhile, surplus nitrogen maintains well-functioning cell machinery, which in combination with the selective pressure, enables MMCs to divert more carbon into the storage form.

PHB storage denotes the part of the biomass occupied by the polymer on a weight basis at the end of the feast. The initiation of nitrogen limitation bulked the MMCs in SBR3/SBR4 with high PHB. However, limitations caused the weakening of cell functioning that prevented MMCs from improving the storage further (SBR3-14.4 % and SBR4-13.4 %). Also, an excessive amount of acetyl-CoA and NADH+ could have allosterically inhibited pyruvate dehydrogenase, thereby draining the cells of vital functional ability (Berg et al., 2002). The severe growth restriction caused SBR4 to lose the PHB storage altogether by 120 days. At the same time, nitrogen available in excess promoted biomass growth, slowly inflicting selective pressure. In a way, the loss from biomass washout was balanced for SBR1 (7.8 %), while the storage maximized for SBR2 to 14.4 %. However, specific PHB storage or production rate (q_P) also points out that the upper hand excess condition had over limiting conditions during enrichment. Due to the metabolically active and highly selective MMC formulated in SBR1 and SBR2, acetate got quickly converted to maximum PHB denoted by a high q_P of 0.017 $mgCOD_{PHB}/mgCOD_{X}h$ and 0.012 $mgCOD_{PHB}/mgCOD_{X}h$, respectively. But prolonged growth restriction and additional pressure imposed on MMC for faster uptake of metabolites might have attenuated the high PAP that SBR3 and SBR4 possed, culminating in slower carbon to PHB conversion denoted by lower q_P 0.006 $mgCOD_{PHB}/mgCOD_{X}h$ and 0.01 $mgCOD_{PHB}/mgCOD_{X}h$. PHB productivity is also higher for SBR1/SBR2 (Table 5.2). Therefore, a kinetically active PHB storing MMC gets enriched under extend period of ecological selective pressure compared to more or less attenuated MMC from metabolic selective pressure. Nitrogen excess is the best possible route to acquire a high PHB accumulating MMC from activated sludge with the aim of an extended period of operation.

The material balance for the enrichment under the feast period points out a near-complete recovery of carbon coming into the system for SBR1 and SBR2 indicated by the global yield of $\Delta Y \geq 0.9$ with the highest conversion to biomass followed by PHB (Table 5.2). ΔY indicates a fine balance between biomass growth and PHB storage, improving with further exposure to selective pressure. Cellular maintenance and extracellular polymeric substance production would be a possible sink for the remaining carbon. Thus, nitrogen excess enrichment reduces the wastage of carbon. However, ΔY was less than 0.5 for nitrogen limited enrichment indicating reduced efficiency of carbon utilization. The bulking of sludge could attribute to the wastage of carbon, which also caused some irregularity in SBR3/SBR4 functioning. The bulking of sludge was minimal for SBR4, while excessive filamentous growth drained the sludge in SBR4 of its PAP as time elapsed. Therefore, a growth-limited enrichment may lead to the diversion of carbon from PHB accumulation if exposed for a long period, as in the present study.

The kinetic model relates feast/famine enrichment as a function of active biomass growth. Table 5.2 gives the maintenance coefficient derived from fitting Fig. 5.4 to the kinetic model. The maintenance coefficient is comparatively lower during the feast phase except for SBR4. A large portion of influent carbon gets used up for active biomass growth during the feast phase exposing the MMCs to stronger selective pressure in SBR1/SBR2. The m_s was higher for SBR4due to the diversion of carbon from the system mostly related to filamentous growth and maintenance due to prolonged exposure to nitrogen limitation, which could reason for the lowest ΔY. This effect was milder for SBR3 as carbon wastage was less compared to SBR4 due to limited growth and lower bulking of sludge attributed to the microbial community in the MMC. The predicted conversion yield is close to that of the experimental value except for SBR4. Thus, the nitrogen excess enrichment in the present study closely follows the kinetic model. On the contrary, fitting of the famine phase fitted to the linear equation a negative slope. This indicates that the cause of the reduction in PHB was the decline in the active biomass with a very low m_p value. The cycle length of 24 h might have extended the famine phase far that after a brief period of biomass growth, the death phase occupies a significant portion of the famine phase. The close $-r_p$ values can be correlated with this effect for the enrichment reactors. Therefore, in the study, the enrichment of MMC with PHB accumulators is due to the cumulative impact of cellular maintenance, limited growth, and cell death during the famine phase in Fig. 5.4.

5.2.6. Batch accumulation to maximize PHB storage

The sequencing batch reactor is an ideal system to induce sludge into storing a significant part of the carbon in the form of PHB. But there is subsequent degradation of the granules to survive under famine condition. However, the extent of sludge coerced into storage can be appreciated under a constant carbon load without allowing advancement into the famine phase. Therefore, batch reactors have been the ideal contour to expose the enriched sludge to such a short and fixed condition to achieve a calculated goal (Fauzi et al., 2019). In addition, Fed-batch (pulse feed) and continuous reactors (continuous feed) utility to implement similar selective pressure upon enriched sludge to entice PHB accumulation were also explored (Valentino et al., 2015)(Chen et al., 2015). The excess carbon in the presence of some restrictions would drive the enriched culture to accumulate PHB maximally. Relatively higher COD_{Ac} (COD conversion value of acetate concentration) provided excess carbon in the batch assay. In the case of batch assay under nitrogen excess condition, PHB concentration increased until a point (7-8 h) with a concurrent decline in COD_{Ac} (90-95 %) followed by the initiation of PHB degradation (Fig. A2.1). In nitrogen starved accumulation, only 65 % of COD_{Ac} was consumed in BR1 and BR2 with a proportional increase in PHB production till the 10h. While in BR3, 90 % COD_{Ac} is consumed by 4 h with ≈1000 mg/L increment in PHB production followed by a saturation (Fig. A2.2c). Similar is the case of BR4, but PHB concentration kept on increasing till 12 h even though the increment was only about 100 mg/L (Fig. A2.2d). PHB concentration had an exponential increase till the 10 h with 98 % consumption of COD_{Ac} without any significant PHB consumption till 12th h for nitrogen limited batch assay (Fig. 5.5).

Table 5.3: Kinetics and stoichiometric parameter of batch accumulation of enriched sludge under different nitrogen availability conditions.

Regime	Nutrient Excess (NE)				
	% PHB	$Y_{P/S}$	q_p	q_s	Productivity
BR1	20.6±0.03	0.18±0.02	0.04±0.04	0.59±0.0	71.4±0.03
BR2	39.8±0.04	0.24±0.03	0.13±0.08	0.19±0.0	92.2±0.05
BR3	26.2±0.02	0.2±0.01	0.053±0.05	0.22±0.0	85.5±0.02
BR4	4.1±0.08	0.03±0.007	0.003±0.01	0.06±0.09	5.6±0.06
Regime	Nutrient Limited (NL)				
BR1	76.1±0.03	0.48±0.0	0.71±0.03	0.57±0.02	219.2±0.04
BR2	71.7±0.05	0.54±0.07	0.53±0.09	0.26±0.04	230.0±0.09
BR3	39.4±0.03	0.49±0.04	0.09±0.05	0.13±0.07	209.2±0.04
BR4	24.4±0.01	0.39±0.05	0.04±0.02	0.14±0.01	164.5±0.06
Regime	Nutrient starved (NS)				
BR1	40.3±0.02	0.31±0.007	0.078±0.02	0.23±0.02	83.4±0.01
BR2	36.6±0.06	0.5±0.09	0.036±0.09	0.37±0.05	110.8±0.09
BR3	58.3±0.04	0.43±0.03	0.19±0.08	0.13±0.005	161.1±0.03
BR4	7.27±0.03	0.05±0.002	0.003±0.0	0.067±0.0	14.9±0.005

±% SD: q_p: $mgCOD_{PHB}$ /mg COD x h, q_s: mg COD_{Ac} / mgCOD x h, $Y_{P/S}$: mg COD_{PHB}/ $mgCOD_S$, Productivity: $mgCOD_{PHB}$/Lh

Table 5.3 depicts the effect of enrichment. BR1 and BR2 under nitrogen limitation had the highest PHB accumulation, 76.1 % and 71.7 %, respectively, compared to all other batch assays with the highest yield coefficient as well. Nitrogen starvation had comparatively reduced storage and yield compared to nitrogen limitation. Nitrogen excess condition had the least output (highest for BR2, 39.8 %) PHB storage and yield. However, the variation in the yield coefficient between BR1, BR2, BR3, and BR4 within the nitrogen excess and starvation regime was minimal. The q_p for BR1and BR2 under nitrogen limitation was much faster (0.71 and 0.53 $mgCOD_{PHB}/mgCOD_X h$), followed by nitrogen starvation and nitrogen excess. Similar was the trend for volumetric productivity, with BR1 and BR2 having better productivity of 219 and 230 $mgCOD_{PHB}$/Lh. Meanwhile, BR4 gave the least PHB accumulation performance in all the three nitrogen regimes

(Table 5.3). From these observations, we can conclude that nitrogen limitation is the most desirable scheme for maximizing the PHB content for a short retention time. In a batch assay, the faster nitrogen uptake (as seen in Fig. 5.4c,5.4d) leads to an extended period of nitrogen deficiency to a starved period in the presence of excess carbon. Due to these essential growth-related proteins, DNA/RNA synthesis gets stalled, which negatively affects the TCA cycle. CoASH generated from the TCA cycle inhibits 3-ketothiolase (PhA) in the PHA synthesis pathway during optimum growth, thereby blocking PHB production. Due to imbalance growth induced by nitrogen limitation, $NADH^+$ increase, and CoASH goes to a non-inhibitory level, thus releasing the lock in the PHA synthesis pathway leading to increased productivity (Tan et al., 2014). An increase in $NADH^+$ also aids in PHB synthesis by inhibiting citrate synthase activity. Besides, the increase in acetyl-CoA due to the limiting C/N ratio regulates acetyl phosphate synthesis by phosphotransacetylase that post-translationally activates PHB synthase (Kessler and Witholt, 2001).

The imbalance of growth lacks in nitrogen starvation due to which the PHB storage was quite less than nitrogen limitation. Except for BR3, q_s for nitrogen starvation were slower than nitrogen excess and limitation, indicating poor storage and growth. Valentino et al. (2015) had proposed that nutrient limitation (between starvation and excess) possesses a better opportunity to augment the PHA accumulation of enriched mix culture. Also, microbial growth becomes a preferred route in nitrogen excess accumulation leading to reduced PHB storage and consumption of stored PHB after depletion of external carbon sources. The degradation of stored polymer is comparatively lesser in nitrogen limited, followed by starved conditions (Fig. 5.5,A2.3). However, the death phase can initiate loss of stored PHB beyond the peak retention time.

From Table 5.3, it is evident that sludge enriched in nitrogen excess SBRs (SBR1/SBR2) were able to accumulate PHB better and faster as compared to MMC enriched in nitrogen limited conditions (SBR3/SBR4). A reason for this leverage would be that the excess nitrogen ensured the presence of all the essential protein, enzyme, and nucleic acid reserves before going into the famine phase due to a well-functioning metabolic system. In this way, an extended feast/famine cycle developed an enriched MMC that was actively growing with dominant PHB accumulators. Furthermore, during batch accumulation, this culture diverted the excess carbon towards PHB effectively with the implementation of growth limitation.

For MMC enriched under limited conditions, extensive exposure to the imbalance growth could have limited PHB production. Furthermore, restriction of protein, enzyme, and other factors synthesis due to the unavailability of nitrogen might have weakened MMCs after extended exposure. This metabolic starvation could initiate catabolism of PHB synthesis related enzymes, other essential and non-essential proteins into the growth-related cycle. This could have drained the cell machinery leading to loss of PHB storage altogether under extended limitation during the batch assay. Meanwhile, the robustness of excess condition could have led MMC from SBR1 and SBR2 to outperform MMC from SBR3 and SBR4 when exposed to further nitrogen limitation during the batch assay. Due to this, the acetate was quickly assimilated by the former (>>q_s) than the latter. Besides that, the culture enriched under nitrogen excess condition possesses maximal volumetric and kinetic accumulation capacity even though close values of $Y_{P/S}$ point out to the similar conversion of acetate to PHB for nitrogen limited batch accumulation.

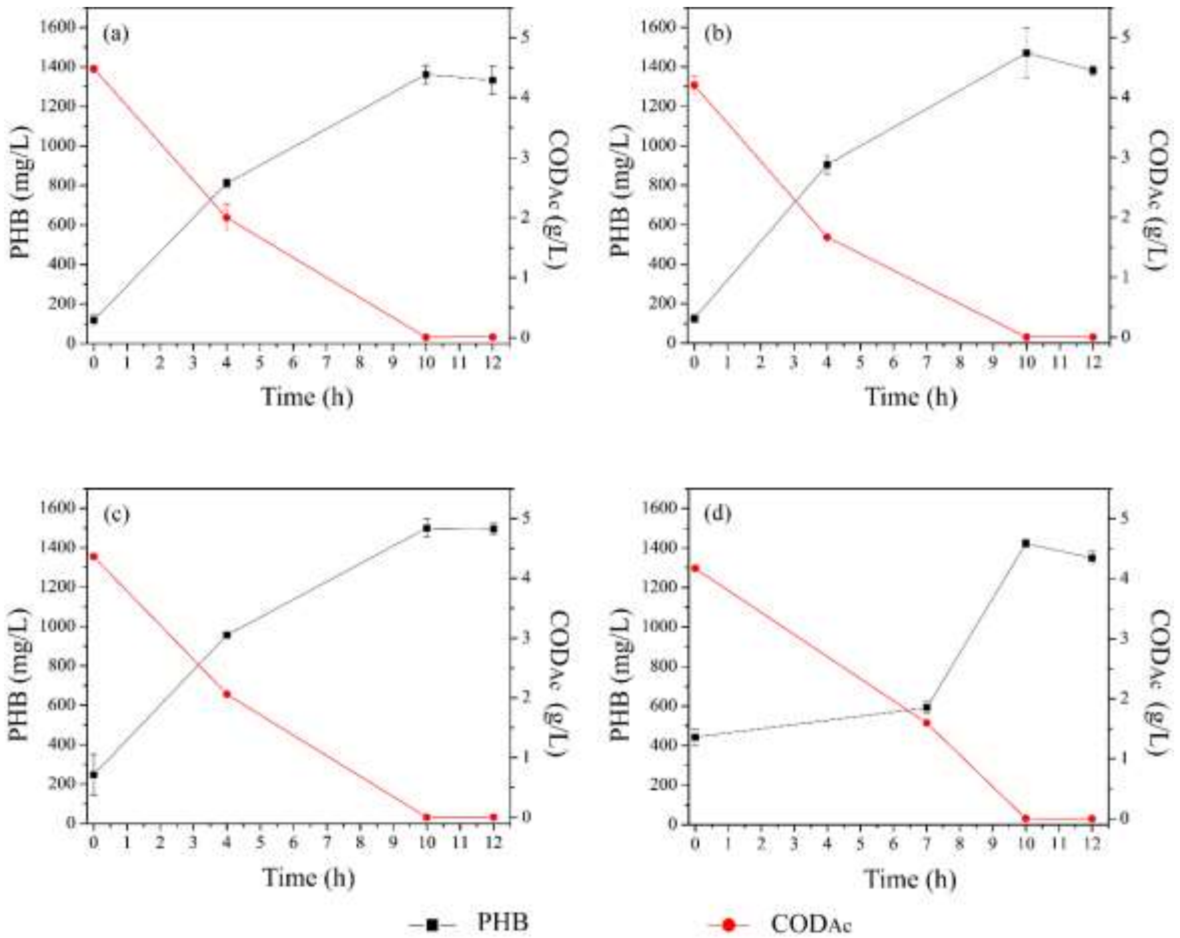


Figure 5.5: PHB accumulation and substrate consumption profile of enriched sludge in (a) BR1,(b) BR2, (c) BR3,(d) BR4 during batch accumulation under nitrogen limited condition.

The PHB accumulation by the culture inoculated from SBR4 showed the least performance. The probable reason, as laid down in other sections, would be due to the excess filamentous growth affecting the proper mass transfer of carbon, nutrient, oxygen, etc. This might be the reason that cell growth, as well as accumulation, got retarded with a lower q_s and $Y_{P/S}$ value for batch assay under nitrogen excess and starvation (Table 5.3). Thus culture enrichment under nitrogen limitation has a high chance of failure without proper process control, as stress condition is known to induce filamentous growth (Metcalf and Eddy, 2003). Yet Wen et al. (2012) had reported a bulking sludge enriched under nitrogen excess condition to provide about 53 % PHB accumulation under nitrogen starvation, thereby pointing out the applicability of nitrogen excess condition during enrichment over nitrogen limitation. Thus from this study, it is clear that sludge enriched under nitrogen excess condition becomes well acclimatized to convert maximum available carbon into PHB utilizing a balanced restriction imposed by nitrogen limitation during the batch assay.

5.2.7. Microbial community structure of the enriched activated sludge

One sample each of petroleum refinery sludge (sample 1) and brewery sludge (sample 2) enriched under nitrogen excess condition with maximized PHB accumulation under nitrogen

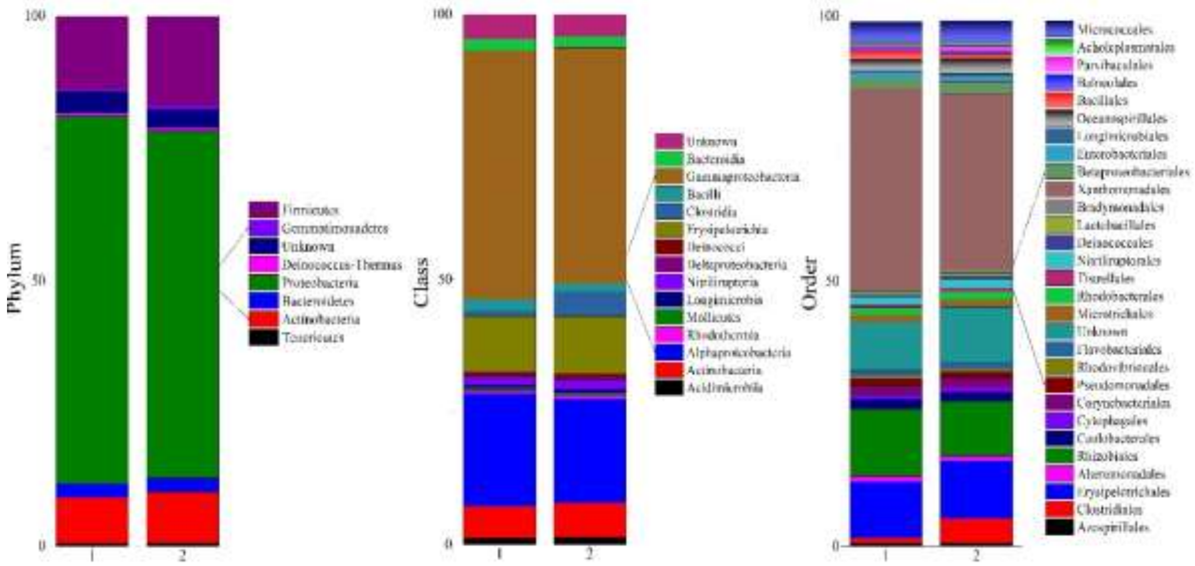


Figure 5.6: Phylum, class and order level of taxonomic distribution of the microbial community from PRS (sample 1) and BS (sample 2) enriched under nitrogen excess condition and PHB accumulation maximized under nitrogen limitation based on relative abundance.

limited condition were collected. Samples 1 and 2 were subjected to 16S rRNA metagenomic sequencing to identify the dominant microbial community performing the accumulation that revealed a high similarity in the dominant operational taxonomic unit (OTU) between samples 1 and 2. Phylum *Proteobacteria* contributed 82.2 % and 74.4 % of the sequenced genome from samples 1 and 2, respectively. The inoculum got enriched at the class level by *Gammaproteobacteria*, *Alphaproteobacteria* and *Erysipelotrichia* 61.8%, 12.3 %, 15.1 %, for sample1 and 46.4 %, 35.3 %, 13.2 %, for sample 2 respectively. At the order level, PHB production in samples (1 and 2) was tuned significantly by *Xanthomonadales* (43.57% and 59.4 %), *Rhizobiales* (32.7 % and 8.0 %), and *Erysipelotrichales* (13.2 % and 15.1 %). The microbial population shifted a bit at the family level. The percentage of *Erysipelotrichaceae* remained similar (13.2 % and 15.1%)for both samples. *Xanthomonadaceae* (38.4 %) and *Rhizobiaceae* (32.34 %) dominated for sample 1, while *Rhodanobacteraceae* (54.6 %) and *Rhizobiaceae* (7.8 %) were the major microbial family in sample 2. The microbial community distribution follows a similar pattern as described in other studies that investigated PHA production through feast/famine enrichment (Huang et al., 2018),(Sruamsiri et al., 2020). Thus, the MMCs from sludge originated

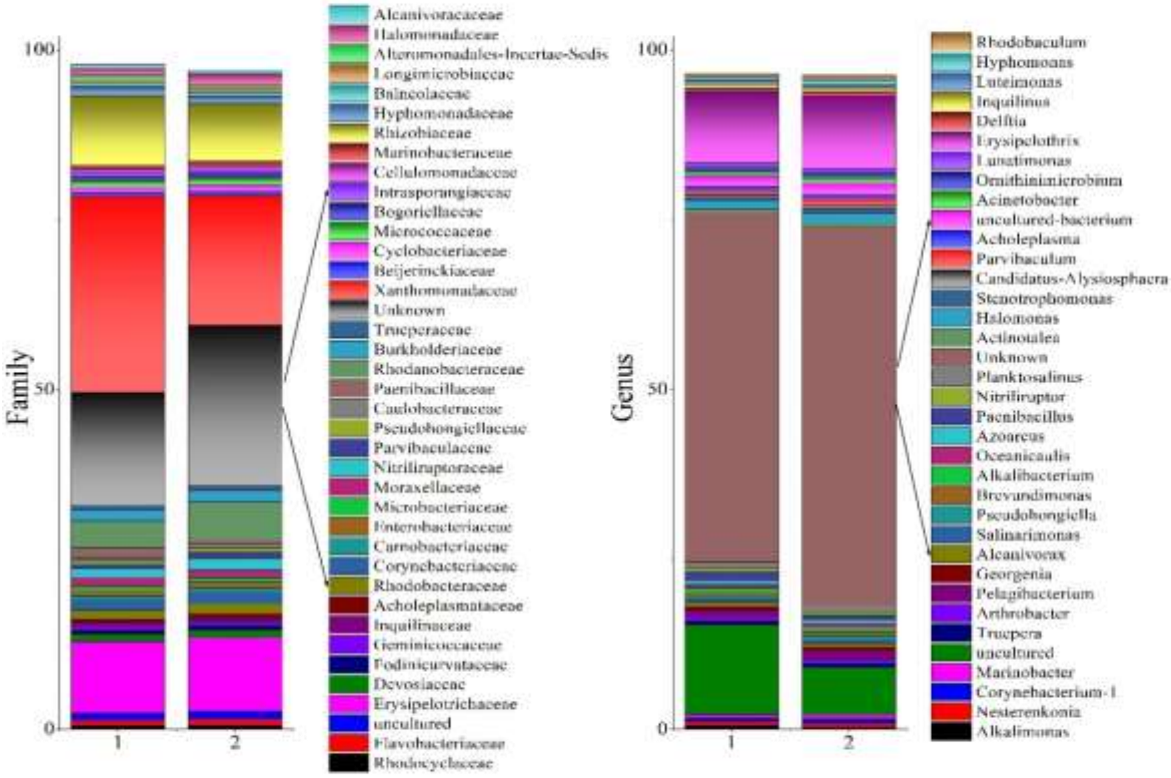


Figure 5.7: The family and genus level taxonomic distribution of the microbial community from PRS (sample 1) and BS (sample 2) enriched under nitrogen excess condition and PHB accumulation maximized under nitrogen limitationbased on relative abundance.

from entirely different environments strongly converged under feast/famine enrichment such that nitrogen limitation during batch accumulation did not produce any considerable difference in the PHB storage of MMC enriched under nitrogen excess. Fig. 5.6,5.7gives the detailed distribution of different microbial communities identified in sample 1 and sample 2 according to the OTU % (OTU with relative distribution ≤0.2 % were filtered out).

5.2.8. Physiochemical characterization of the PHB synthesized

Polymer accumulated in the batch accumulation was subjected to different characterization methods to confirm PHA production and determine the composition, thermal properties, crystallinity, and molecular weight.

Functional group Identification by Fourier-transform infrared spectroscopy (FTIR): Functional groups are the signature of a particular compound that enables the deciphering of the structure, thereby identifying the material. Fig. A2.3 depicts the associated peaks of the produced compound represented here as a range between the value for BR1 and BR2, respectively. The bands at 1722-

1724 cm^{-1} indicate the presence of C=O group (1), bands near 1452-1454 cm^{-1} depict the asymmetric bending of the C-H group in the $–CH_2$ or $–CH_3$ group (2) while symmetric bending of C-H group in $–CH_3$is banded at 1378-1380 cm^{-1} (3). The bands between 1056-1290 cm^{-1} correspond to stretching of C-O bond (4), and the bands suggest the presence of $–CH_3$ at 2977-2984 cm^{-1} and 2935-2937 cm^{-1} (5). These FTIR bands confirm PHB to be the produced compound(Ramezani et al., 2015; Reddy et al., 2015).

Molecular arrangement by powder X-ray diffraction spectroscopy (PXRD): The pattern of arrangement of molecules in solid manifests in its X-ray diffraction spectrum, identifying it to be crystalline/amorphous, thereby understanding the suitability of solids to a particular application. Here FTIR and NMR have given ample evidence for the precipitate to be composed of homopolymer, PHB. The PXRD spectrum also points out in the same direction (Fig. A2.4). The peak 2ϴ value obtained from the precipitate, 13, 17, 20, 22, 25, 27, 32, 44 deg., match to that of PHB as reported in available pieces of literature (Reddy et al., 2015), (Kiran et al., 2014), (Saratale and Oh, 2015). The sharp peaks at 13 deg. and 17 deg. can be identified as an orthorhombic unit cell, while weaker reflection at 20 deg. and 22 deg. corresponds to the reflections of α and β PHB, respectively (Mottin et al., 2016). The crystallinity index (CI) of the extracted polymer measured to be about 50 % (Table 5.4), i.e., the extracted PHB is semicrystalline, similar to that reported by Albuquerque et al. (Albuquerque et al., 2011). The higher temperature of extraction (100 °C) for polymer recovery in the study might also have aided in the semicrystalline nature of the polymer (Qin et al., 2020). The average crystalline size (L) obtained for the extracted polymer was about 10.4 nm, which is smaller than the earlier reported size derived from microalgae (Kavitha et al., 2016).

Deduction of chemical structure using nuclear magnetic resonance (NMR): The purified polymer was dissolved in $CDCl_3$ and analyzed under nuclear magnetic resonance for 1H and 13C for confirming PHB production, deducing the chemical structure, and predicting the primary sequence of the polymer chain. The respective peaks are labeled based on the chemical shift, the area under the curve, and chemical bonding between molecules, as shown in the chemical structure in Fig. A2.5, A2.6. The $CDCl_3$ and water present in $CDCl_3$ account for chemical shifts at 7.2 ppm and 1.6 ppm, respectively. Fig. A2.5 shows the NMR spectra of material extracted from BR1, while Fig. A2.6 is of BR2. 1H spectrum is a clear indication of PHB. The peaks in the chemical shifts are assigned to protons on -CH (5.3 ppm), $-CH_2$ (2.6, 2.5 ppm), and $–CH_3$ (1.3 ppm). The area under the curve for a chemical shift at 1.3 ppm is 3 times that of 5.3 ppm that denotes the methyl group. The chemical shift at 2.6 and 2.5 ppm (area similar to 5.3 ppm) depicts protons attached to $-CH_2$appearing as couplets due to bonding between –CH and $–CH_3$. Similar is the case for a chemical shift at 5.3 ppm (Fig. A2.5, A2.6). This helps to draw the structure of the compound extracted, as shown in the figures keeping in mind that the flanking protons in the monomer get condensed to form the polymer.

NMR spectrum gives insight into the carbon backbone of the compound, which is a tag for that compound. The higher the electro-negativity, the higher is the resonant frequency shift (chemical shift) in NMR. In that perspective, the lowest chemical shift of 19.8 ppm denotes carbon in $–CH_3$ while that at 169.2 ppm denotes carbon in –C=O. The intervening chemical shift at 57.6 ppm and 60.7 ppm indicate carbon on $–CH_2$and –CH, respectively. Therefore, considering the arrangement of carbon backbone according to the order of chemical shift, the structure of the compound can be formulated as given in Fig. A2.5,A2.6. Compared with existing literature, we can confirm the produced compound as a homopolymer, polyhydroxybutyrate, and can expect to have a reasonable degree of purity according to NMR spectra (Reddy et al., 2015).

Table 5.4: Characteristics of PHB synthesized from the batch accumulation under nitrogen limitation

Reactor	Inoculum	M_w (gmol^{-1} x10^5)	M_n (gmol^{-1} x10^5)	PDI	T_m (°C)	T_d onset (°C)	T_d end (°C)	DP_w (x 10^3)	L (nm)	CI (%)
BR1	SBR1	4.3	2.8	1.5	168	258	289	4.13	10.43	50.4
BR2	SBR2	4.3	2.5	1.7	165	268	293	4.13	10.45	53.3

$T_{d\ onset}$: Onset of degradation, $T_{d\ end}$: peaks of thermal degradation

Determination of the thermal properties by thermo-gravimetric analysis (TGA): The melting (T_m) and decomposition temperature (T_d) determined here portray the thermal stability of the polymer to be molded and cast according to application. From Fig. A2.7, it is evident that the polymer extracted was pure PHB rather than a copolymer as the TGA curve is devoid of any intervening curves. Thereby, comparing the respective regions between TGA and differential scanning calorimetry (DSC) curves can determine the changes in phase for the precipitate. The region where the DSC curve dips to the maximum with about 99 % weight loss indicating a phase change gives T_d. The onset of T_d for BR1 was from about 258 °C till 99 % got decomposed at 289 °C. Similarly, for BR2, T_d was onset about 268 °C till complete decomposition at 293 °C. Meanwhile, the TGA curve shows a 1% weight loss during the initial dip in the DSC curve depicting a phase change due to the melting of the polymer. Hence the T_m of BR1 and BR2 are approximately168 °C and 165 °C, respectively. Similar results were observed by Arcos-Hernández et al. (2013) when the feeding sequence resulted in a copolymer with more than 70 % 3HB. Meanwhile, the similar inflammability and combustion heat release rate with temperature observed from Fig. A2.7 indicates the similar exothermic properties of polymer extracted from both the sludges (Zhang et al., 2019). T_d and T_m observed here also nears to that of T_d of pure PHB, which is around 274-310 °C and T_m of 178 °C (Reddy et al., 2015).

Molecular weight estimation by gel permeation chromatography: The number-average molecular weight (M_n) depends on the molecular weight of individual polymer chains. At the same time, the weight-average molecular weight (M_w) takes care of the molecular size, i.e., the weight of each chain gets accounted for in addition to the number of polymer chains. The polydispersity index (PDI) gives the range of molecular weight of the polymer, while the weight average degree of polymerization (DP_w) provides the number of monomer units in the polymer (Shrivastava, 2018). In the study, the precipitate confirmed to be PHB by other qualitative analyses has M_w in the range of 4.3 x 10^5 g/mol and M_n between 2.5-2.8 x 10^5 g/mol (Table 5.4). Thus the PHB produced from both the sludge has a comparatively higher molecular weight as reported by other researchers and is predicted to have wide applicability (Arcos-Hernández et al., 2013). Also, a lower PDI of 1.5-1.7 indicates the size of the different chains in the polymer from both reactors has a narrow range, that individual chain doesn't vary from each other drastically in size. A narrow PDI is desirable for mechanical applications. A high of DP_w 4130 for PHB extracted from both the sludge depicts a large number of monomeric units or longer chain length. A high DP_w also points towards its utility in processes where better mechanical properties are the demand.

5.2.9. Response of inoculum origin on PHB production through altering nitrogen availability

In an overall assessment, the enrichment process through dynamic feeding and nitrogen excessiveness had resulted in the copious amount of PHB under nitrogen limited batch accumulation. Recently, uncoupled carbon and nitrogen feeding strategy was formulated and amalgamated with aerobic dynamic feeding to increase the efficiency of nitrogen excess enrichment to impact and maximize PHA accumulation (Silva et al., 2017). Table 5.3 points out that the influence of sludge origin on PHB accumulation to be insignificant. Acclimatization of different inoculum for an extended period under the same operational condition influenced the MMCs. The convergence of the microbial community and similar physiochemical properties also points in the same direction. PRS had the upper hand over the BS during the initiation of the SBRs with 24.7 % and 8.8 % PHB storage, respectively (Fig. 5.1a,5.1b). However, SRT of 10 days diminished this capacity of PRS while BS stayed put with minor loss. PRS was able to make up for the loss with long-term enrichment, effecting its influence in the batch assay. This independence of PHB production from inoculum origin is more evident in nitrogen excess enrichment. The physical or ecological selective pressure stabilizes any critical community flux that appeared during the enrichment (Huang et al., 2018). While in nitrogen limited enrichment, the growth imbalance could have asserted a metabolic selective pressure that spiked the storage of accumulators, also forcing the non-accumulators into storing carbon very quickly. These altered non-accumulators would have perished and the accumulators impaired after a long period of extreme stress imparted by insufficient nitrogen, the pressure-induced by feast/famine cycle, and other physical parameters before impacting on PHB accumulation in the batch assay. Thus, the conditions in which MMC got enriched weighed than the condition in which inoculum sludge had survived. Another similar study utilized sludge from different secondary biological treatment units without any prior acclimatization or enrichment that, as expected, showed the dependence of origin on PHA production (Sakai et al., 2015). Janarthanan et al. (Janarthanan et al., 2016) had also forwarded that sequential enrichment of sludge under dynamic feeding to be a robust process and that the PHA accumulation profile to be consistent despite the shifts in microbial community fluxes throughout enrichment. Therefore, the long-term feast/famine enrichment in the presence of excess nitrogen and a short-term accumulation under growth limiting condition leads to improved PHB production irrespective of the origin of activated sludge. A comparative summary of the ADF enrichment of activated sludge is given in Table 5.5.

5.2.10. Prospective application of extracted PHB

The characterization of the precipitate concludes that the accumulated product is a homo-polymer of PHB. The polymer recovery protocol used in the study purified the extract to an acceptable degree (appendix 2). The properties listed in Table 5.4 point towards the broad scope of its applicability. The extracted PHB is semicrystalline in nature with a smaller crystalline structure pointing towards a well-ordered molecular structure that could impart desirable mechanical properties. The T_m of the polymer depicts the ease of melting the polymer and casting into the required size according to use without degradation, $T_d \approx 2 \times T_m$. The higher M_w and narrow M_w distribution (PDI 1.5-1.7) of the polymer guarantee better mechanical properties like Tensile strength, Elongation to break, toughness, weatherability, and chemical resistance. The entanglement of these long chains (DP_w) makes the polymer highly viscous that prevents the flexible chains from moving relative to each other when a substantial external force is applied (Shrivastava, 2018). These properties make the extracted polymer an attractive competitor for the conventional plastics in the areas that require satisfactory mechanical properties like reinforcement, automobile parts, scaffolds for tissue engineering, consumer and household goods, etc. The hydroxyl group (C-OH) is an electron donor group (Liu et al., 2020). Its absence due to monomer linkage and hydrocarbon backbone gives the polymer an insulating property. Also, it possible to draw thin sheets from extracted PHB without losing the strength for food packaging applications due to these properties. The various blends of PHB, like PHB-Chitosan, have shown improved flexibility, tensile strength, thermal stability, and swelling capacity in comparison to the crude polymer converting the biodegradable polymer as an apt substitute for conventional polymer (Albuquerque and Malafaia, 2018). The copolymer of PHB, like P(3HB-3HV), also finds application in food packaging due to excellent gas barrier property (Anjum et al., 2016). These advancements can convert activated sludge derived PHB as an acceptable solution for mitigating the current environmental issues posed by plastics.

Table 5.5: Comparative summary of PHB production performance from volatile fatty acids using feast-famine enriched activated sludge

Substrate	Regime	Organic content (g/L, COD or C)	Temperature (°C) /pH	PHB storage[#] (%)	PHB yield [#] (g/g, COD or C)	Reference
Sodium acetate	Fed batch	60[b]	20/7.0	70	-	(Johnson et al., 2010)
Sodium acetate	Fed Batch	30 [b]	30	64.7	-	(Cui et al., 2016)
Butyrate	Batch	1 (g/L)	28/n.c	62.9	0.93[a]	(Kourmentza and Kornaros, 2016)
Acetate	Batch	1.2	22/n.c	51	0.78 [a]	(Montiel-jarillo et al., 2017)
Sodium acetate	Fed batch	10-30[b]	30/7.0	53 85	0.5 [a] 0.61[a]	(Marang et al., 2018)*
Acetate	Fed batch	60	n.c	49.9	0.7 [a]	(Mannina et al., 2019)
Sodium acetate	Batch	1	28/n.c	43.8	0.48 [a]	(Inoue et al., 2019)
Acetate	Batch	5	20–25/n.c	66.4[c]	0.54	(Ahmadi et al., 2020)
Acetate+ Butyrate	Sequential batch	4.3+10.9	37/7.0	2.6 (g/L)	0.36 (g/g)	(Naresh Kumar et al., 2021)
Acetate+ Propionate	Fed-batch	25-20[b]	n.a/7.5	42	0.51[a]	(Cruz et al., 2022)
Sodium acetate	**Batch**	**4.5**	**n.c**	**76.1 71.7**	**0.48 0.54**	**This study**

*: continuous and semi-continuous fed enrichment, #:: Some values are approximated from data given in the respective research articles a: C mol/C mol, b: mmol/L, c: gPHB/gTSS, nitrogen and phosphorus source are not added creating limited or starved condition, fed-batch: organic content given is for each pulse

5.3. Conclusion

So, it can be surmised that an extended period of sequential enrichment under a feast/famine cycle could upgrade the PHB storage capacity of the activated sludge under appropriate nitrogen excess and nitrogen limiting condition. Nitrogen limited batch condition maximized the diversion of excess carbon into PHB accumulated against nitrogen excessive or starvation environment. However, the MMC enriched under nitrogen excess condition attained maximal PHB accumulation compared to nitrogen limitation with dynamic feeding. So nitrogen excess enrichment can improvise the PHB accumulating potential of the activated sludge under aerobic dynamic feeding. Meanwhile, the effect of sludge origin was negligible on the PHB accumulating potential of the MMC enriched under ADF. The long-term selective pressure imposed by the feast/famine enrichment under excess nitrogen availability directed similar microbial communities to adapt and dominate. The PHB produced from enriched PRS and BS sludge had similar physiochemical properties. The PHB produced was semicrystalline with high molecular weight and suitable thermal properties. These characteristics of PHB produced points towards the broad applicability with a clear potential to balance with conventional petroleum-derived polymers.

References

Ahmadi, F., Zinatizadeh, A.A., Asadi, A., 2020. The effect of different operational strategies on polyhydroxyalkanoates (PHAs) production at short-term biomass enrichment. J. Environ. Chem. Eng. 8, 103531.

Albuquerque, M.G.E., Martino, V., Pollet, E., Avérous, L., Reis, M.A.M., 2011. Mixed culture polyhydroxyalkanoate (PHA) production from volatile fatty acid (VFA)-rich streams: Effect of substrate composition and feeding regime on PHA productivity, composition and properties. J. Biotechnol. 151, 66–76.

Albuquerque, P.B.S., Malafaia, C.B., 2018. Perspectives on the production, structural characteristics and potential applications of bioplastics derived from polyhydroxyalkanoates. Int. J. Biol. Macromol. 107, 615–625.

Anjum, A., Zuber, M., Zia, K.M., Noreen, A., Anjum, M.N., Tabasum, S., 2016. Microbial production of polyhydroxyalkanoates (PHAs) and its copolymers: A review of recent advancements. Int. J. Biol. Macromol. 89, 161–174.

Arcos-Hernández, M. V, Laycock, B., Donose, B.C., Pratt, S., Halley, P., Al-luaibi, S., Werker, A., Lant, P.A., 2013. Physicochemical and mechanical properties of mixed culture polyhydroxyalkanoate (PHBV). Eur. Polym. J. 49, 904–913.

Basak, B., Ince, O., Artan, N., Yagci, N., Ince, B.K., 2011. Effect of nitrogen limitation on enrichment of activated sludge for PHA production. Bioprocess Biosyst. Eng. 34, 1007–1016.

BBC, 2019. How plastic bags were supposed to help the planet [WWW Document]. URL https://www.youtube.com/user/bbcnews/featured (accessed 4.5.20).

Berg, J.M., Tymoczko, J.L., Stryer, L., 2002. Section 17.2 Entry to the Citric Acid Cycle and Metabolism Through It Are Controlled, in: Freeman, W.H. (Ed.), Biochemistry. New York.

Chen, Z., Guo, Z., Wen, Q., Huang, L., Bakke, R., Du, M., 2016. Modeling polyhydroxyalkanoate (PHA) production in a newly developed aerobic dynamic discharge (ADD) culture enrichment process. Chem. Eng. J. 298, 36–43.

Chen, Z., Huang, L., Wen, Q., Guo, Z., 2015. Efficient polyhydroxyalkanoate (PHA) accumulation by a new continuous feeding mode in three-stage mixed microbial culture (MMC) PHA production process. J. Biotechnol. 209, 68–75.

Chen, Z., Huang, L., Wen, Q., Zhang, H., Guo, Z., 2017. Effects of sludge retention time, carbon and initial biomass concentrations on selection process : From activated sludge to polyhydroxyalkanoate accumulating cultures. J. Environ. Sci. 2, 76–84.

Chua, A.S.M., Takabatake, H., Satoh, H., Mino, T., 2003. Production of polyhydroxyalkanoates (PHA) by activated sludge treating municipal wastewater: Effect of pH, sludge retention time (SRT), and acetate concentration in influent. Water Res. 37, 3602–3611.

Cruz, R.A.P., Oehmen, A., Reis, M.A.M., 2022. The impact of biomass withdrawal strategy on the biomass selection and polyhydroxyalkanoates accumulation of mixed microbial cultures.

N. Biotechnol. 66, 8–15.

Cui, Y., Zhang, H., Lu, P., Peng, Y., 2016. Effects of carbon sources on the enrichment of halophilic polyhydroxyalkanoate-storing mixed microbial culture in an aerobic dynamic feeding process. Sci. Rep. 6, 30766.

Fauzi, A.H.M., Chua, A.S.M., Yoon, L.W., Nittami, T., Yeoh, H.K., 2019. Enrichment of PHA-accumulators for sustainable PHA production from crude glycerol. Process Saf. Environ. Prot. 122, 200–208.

Feldman, D., 2008. Polymer history. Des. Monomers Polym. 11, 1–15.

Huang, L., Chen, Z., Wen, Q., Zhao, L., Lee, D.J., Yang, L., Wang, Y., 2018. Insights into Feast-Famine polyhydroxyalkanoate (PHA)-producer selection: Microbial community succession, relationships with system function and underlying driving forces. Water Res. 131, 167–176.

Inoue, D., Fukuyama, A., Ren, Y., Ike, M., 2019. Rapid enrichment of polyhydroxyalkanoate-accumulating bacteria by the aerobic dynamic discharge process: Enrichment effectiveness, polyhydroxyalkanoate accumulation ability, and bacterial community characteristics in comparison with the aerobic dynamic fe. Bioresour. Technol. Reports 7, 100276.

Janarthanan, O.M., Laycock, B., Montano-herrera, L., Lu, Y., Arcos-hernandez, M. V, Werker, A., Pratt, S., 2016. Fluxes in PHA-storing microbial communities during enrichment and biopolymer accumulation processes. N. Biotechnol. 33, 61–72.

Jiang, Y., Marang, L., Kleerebezem, R., Muyzer, G., van Loosdrecht, M.C.M., 2010. Effect of temperature and cycle length on microbial competition in PHB-producing sequencing batch reactor. Int. Soc. Microb. Ecol. 5, 896–907.

Johnson, K., Jiang, Y., Kleerebezem, R., Muyzer, G., van Loosdrecht, M.C.M., 2009. Enrichment of a mixed bacterial culture with a high polyhydroxyalkanoate storage capacity. Biomacromolecules 10, 670–676.

Johnson, K., Kleerebezem, R., van Loosdrecht, M.C.M., 2010. Influence of the C/N ratio on the performance of polyhydroxybutyrate (PHB) producing sequencing batch reactors at short SRTs. Water Res. 44, 2141–2152.

Kavitha, G., Kurinjimalar, C., Sivakumar, K., Palani, P., Rengasamy, R., 2016. Biosynthesis, purification and characterization of polyhydroxybutyrate from Botryococcus braunii kütz. Int. J. Biol. Macromol. 89, 700–706.

Kessler, B., Witholt, B., 2001. Factors involved in the regulatory network of polyhydroxyalkanoate metabolism. J. Biotechnol. 86, 97–104.

Kiran, G.S., Lipton, A.N., Priyadharshini, S., Anitha, K., Suárez, L.E.C., Arasu, M. V., Choi, K.C., Selvin, J., Al-Dhabi, N.A., 2014. Antiadhesive activity of poly-hydroxy butyrate biopolymer from a marine Brevibacterium casei MSI04 against shrimp pathogenic vibrios. Microb. Cell Fact. 13, 1–12.

Kourmentza, C., Kornaros, M., 2016. Biotransformation of volatile fatty acids to polyhydroxyalkanoates by employing mixed microbial consortia : The effect of pH and carbon source. Bioresour. Technol. 222, 388–398.

Liu, S., Lai, C., Li, B., Zhang, C., Zhang, M., Huang, D., Qin, L., Yi, H., Liu, X., Huang, F., Zhou, X., Chen, L., 2020. Role of radical and non-radical pathway in activating persulfate for degradation of p-nitrophenol by sulfur-doped ordered mesoporous carbon. Chem. Eng. J. 384, 123304.

Liu, Y.Q., Tay, J.H., 2007. Influence of cycle time on kinetic behaviors of steady-state aerobic granules in sequencing batch reactors. Enzyme Microb. Technol. 41, 516–522.

Mannina, G., Presti, D., Montiel-Jarillo, G., Suárez-Ojeda, M.E., 2019. Bioplastic recovery from wastewater: A new protocol for polyhydroxyalkanoates (PHA) extraction from mixed microbial cultures. Bioresour. Technol. 282, 361–369.

Marang, L., van Loosdrecht, M.C.M., Kleerebezem, R., 2018. Enrichment of PHA-producing bacteria under continuous substrate supply. N. Biotechnol. 41, 55–61.

Metcalf, Eddy, 2003. Wastewater engineering : treatment and resource recovery. McGraw-Hill Higher Education, New York.

Montiel-jarillo, G., Carrera, J., Suárez-ojeda, M.E., 2017. Enrichment of a mixed microbial culture for polyhydroxyalkanoates production : Effect of pH and N and P concentrations. Sci. Total Environ. 583, 300–307.

Mottin, A.C., Ayres, E., Orefice, R.L., Câmara, J.J.D., 2016. What changes in poly(3-hydroxybutyrate) (PHB) when processed as electrospun nanofibers or thermo-compression molded film? Mater. Res. 19, 57–66.

Naresh Kumar, A., Kim, G.-B., Muhorakeye, A., Varjani, S., Kim, S.H., 2021. Biopolymer production using volatile fatty acids as resource: Effect of feast-famine strategy and lignin reinforcement. Bioresour. Technol. 326, 124736.

Qin, F., Peng, Y., Song, G., Fang, Q., Wang, R., Zhang, C., Zeng, G., Huang, D., Lai, C., Zhou, Y., Tan, X., Cheng, M., Liu, S., 2020. Degradation of sulfamethazine by biochar-supported bimetallic oxide/persulfate system in natural water: Performance and reaction mechanism. J. Hazard. Mater. 398, 122816.

Ramezani, M., Amoozegar, M.A., Ventosa, A., 2015. Screening and comparative assay of poly-hydroxyalkanoates produced by bacteria isolated from the Gavkhooni Wetland in Iran and evaluation of poly-β-hydroxybutyrate production by halotolerant bacterium Oceanimonas sp. GK1. Ann. Microbiol. 65, 517–526.

Reddy, M. V, Mawatari, Y., Yajima, Y., Seki, C., Hoshino, T., Chang, Y., 2015. Poly-3-hydroxybutyrate (PHB) production from alkylphenols , mono and poly-aromatic hydrocarbons using Bacillus sp . CYR1 : A new strategy for wealth from waste. Bioresour. Technol. 192, 711–717.

Sabliy, L., Kuzminskiy, Y., Zhukova, V., Kozar, M., Sobczuk, H., 2019. New Approaches in Biological Wastewater Treatment Aimed at Removal of Organic Matter and Nutrients. Ecol. Chem. Eng. S 26, 331–343.

Sakai, K., Miyake, S., Iwama, K., Inoue, D., Soda, S., Ike, M., 2015. Polyhydroxyalkanoate (PHA) accumulation potential and PHA-accumulating microbial communities in various activated sludge processes of municipal wastewater treatment plants. J. Appl. Microbiol. 118, 255–266.

Saratale, G.D., Oh, M.K., 2015. Characterization of poly-3-hydroxybutyrate (PHB) produced from Ralstonia eutropha using an alkali-pretreated biomass feedstock. Int. J. Biol. Macromol. 80, 627–635.

Serafim, L.S., Lemos, P.C., Albuquerque, M.G.E., Reis, M.A.M., 2008. Strategies for PHA production by mixed cultures and renewable waste materials. Appl. Microbiol. Biotechnol. 81, 615–628.

Serafim, L.S., Lemos, P.C., Oliveira, R., Reis, M.A.M., 2004. Optimization of polyhydroxybutyrate production by mixed cultures submitted to aerobic dynamic feeding conditions. Biotechnol. Bioeng. 87, 145–160.

Shrivastava, A., 2018. 2. Polymerization, in: Andrew, W. (Ed.), Introduction to Plastics Engineering. Elsevier, Cambridge, pp. 17–48.

Silva, F., Campanari, S., Matteo, S., Valentino, F., Majone, M., Villano, M., 2017. Impact of nitrogen feeding regulation on polyhydroxyalkanoates production by mixed microbial cultures. N. Biotechnol. 37, 90–98.

Sruamsiri, D., Thayanukul, P., Suwannasilp, B.B., 2020. In situ identification of polyhydroxyalkanoate (PHA)-accumulating microorganisms in mixed microbial cultures under feast/famine conditions. Sci. Rep. 10, 1–10.

Sudesh, K., Abe, H., Doi, Y., 2000. Synthesis, structure and properties of polyhydroxyalkanoates: Biological polyesters. Prog. Polym. Sci. 25, 1503–1555.

Suresh Kumar, M., Mudliar, S.N., Reddy, K.M.K., Chakrabarti, T., 2004. Production of biodegradable plastics from activated sludge generated from a food processing industrial wastewater treatment plant. Bioresour. Technol. 95, 327–330.

Tan, G.-Y., Chen, C.-L., Li, L., Ge, L., Wang, L., Razaad, I., Li, Y., Zhao, L., Mo, Y., Wang, J.-Y., 2014. Start a Research on Biopolymer Polyhydroxyalkanoate (PHA): A Review.

Polymers (Basel). 6, 706–754.
Valentino, F., Karabegovic, L., Majone, M., Morgan-Sagastume, F., Werker, A., 2015. Polyhydroxyalkanoate (PHA) storage within a mixed-culture biomass with simultaneous growth as a function of accumulation substrate nitrogen and phosphorus levels. Water Res. 77, 49–63.
Valentino, F., Morgan-Sagastume, F., Campanari, S., Villano, M., Werker, A., Majone, M., 2016. Carbon recovery from wastewater through bioconversion into biodegradable polymers. N. Biotechnol. 37, 9–23.
Vallari, D.S., Jackowski, S., Rock, C.O., 1987. Regulation of pantothenate kinase by coenzyme A and its thioesters. J. Biol. Chem. 262, 2468–2471.
Wen, Q., Chen, Z., Tian, T., Chen, W., 2010. Effects of phosphorus and nitrogen limitation on PHA production in activated sludge sludge. J. Environ. Sci. 22, 1602–1607.
Wen, Q., Chen, Z., Wang, C., Ren, N., 2012. Bulking sludge for PHA production: Energy saving and comparative storage capacity with well-settled sludge. J. Environ. Sci. 24, 1744–1752.
Zhang, C., Wang, W., Duan, A., Zeng, G., Huang, D., Lai, C., Tan, X., Cheng, M., Wang, R., Zhou, C., Xiong, W., Yang, Y., 2019. Adsorption behavior of engineered carbons and carbon nanomaterials for metal endocrine disruptors: Experiments and theoretical calculation. Chemosphere 222, 184–194.

Annexure 2

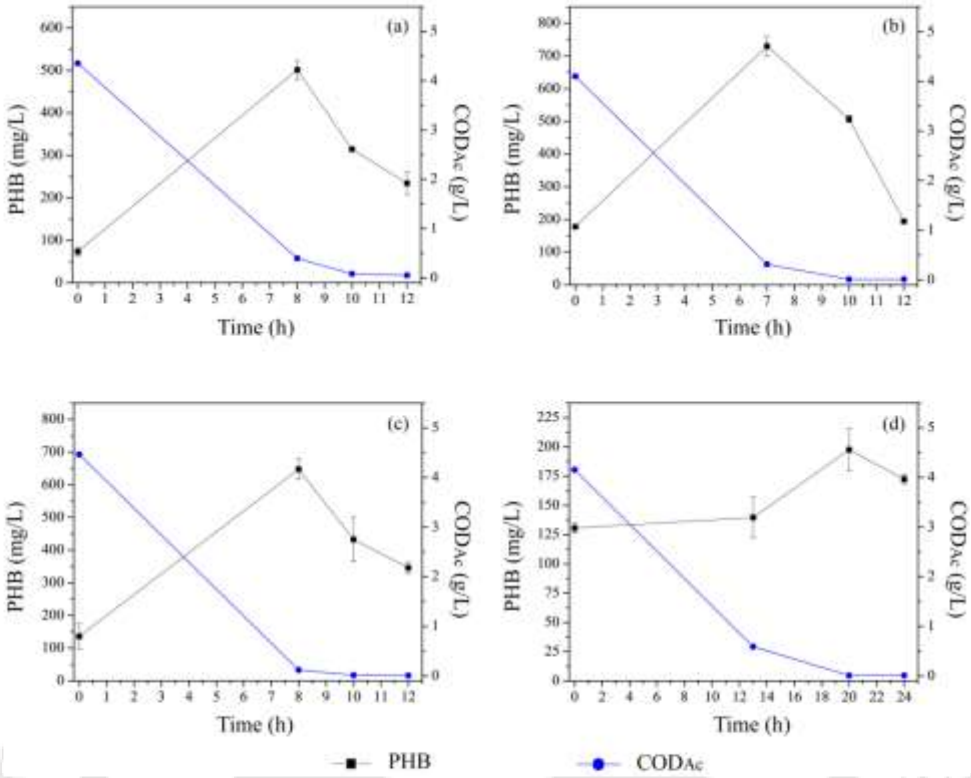


Figure A2.1: PHB accumulation during batch accumulation under nitrogen excess condition for (a) BR1, (b) BR2, (c) BR3, (d) BR4

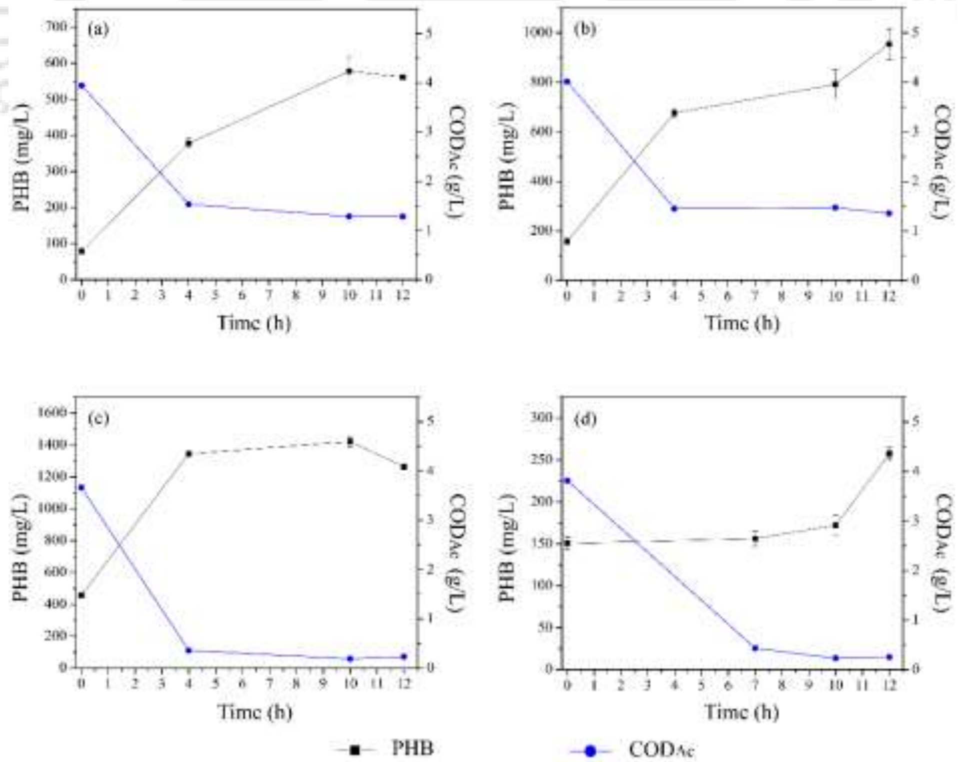


Figure A2.2: PHB accumulation during batch accumulation under nitrogen starved condition for (a) BR1, (b) BR2, (c) BR3, (d) BR4

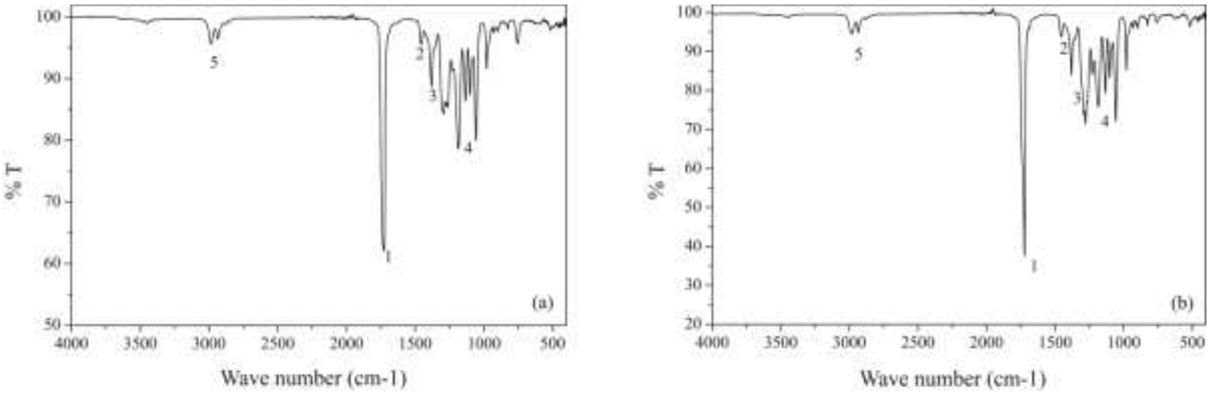


Figure A2.3: Fourier Transform Infrared Spectrum (FTIR) of PHB: (a) BR1 and(b) BR2 (b) R2

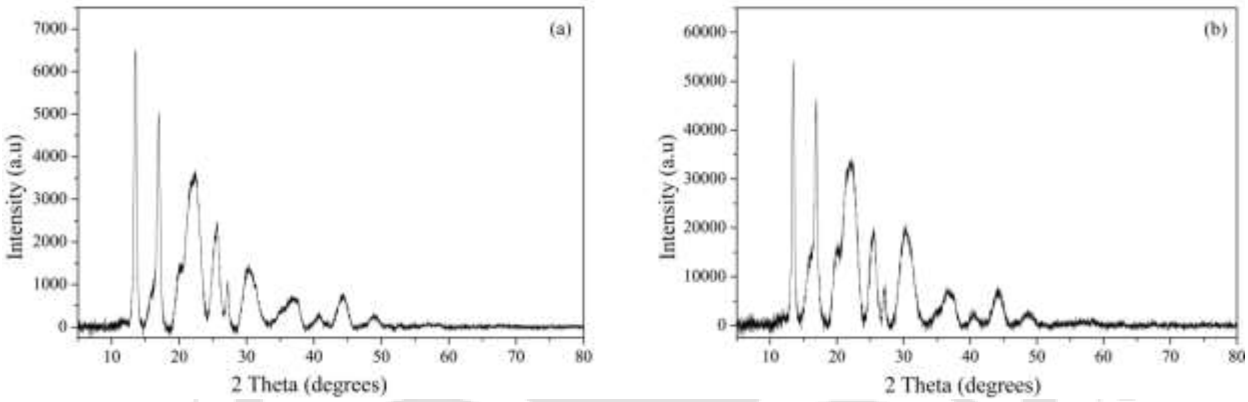


Figure A2.4: Powder X-ray diffraction spectrum of PHB: (a) BR1 and (b) BR2

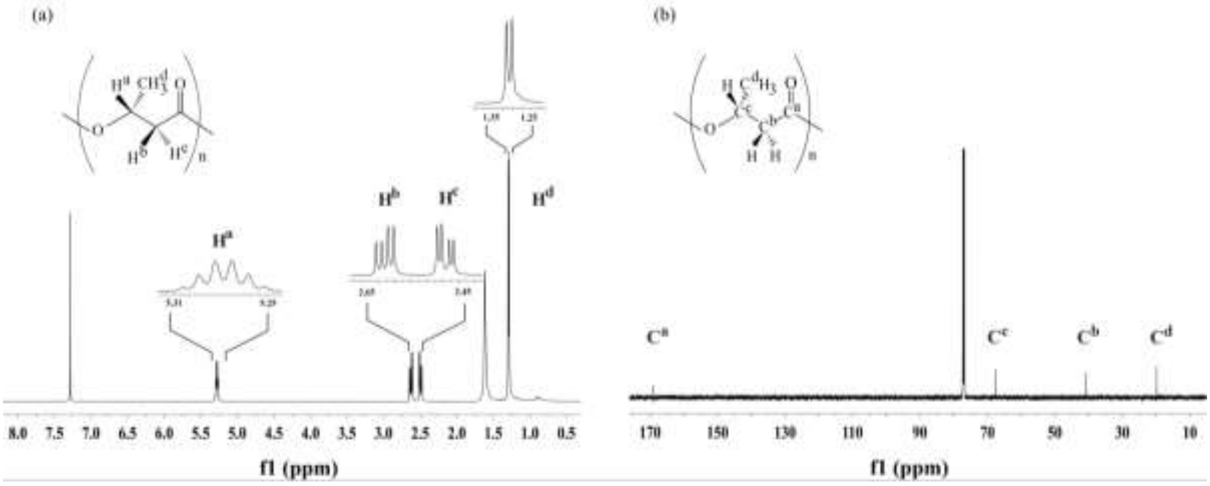


Figure A2.5: Nuclear Magnetic Resonance imaging of PHB extracted from BR1: (a) 1H (b) 13C

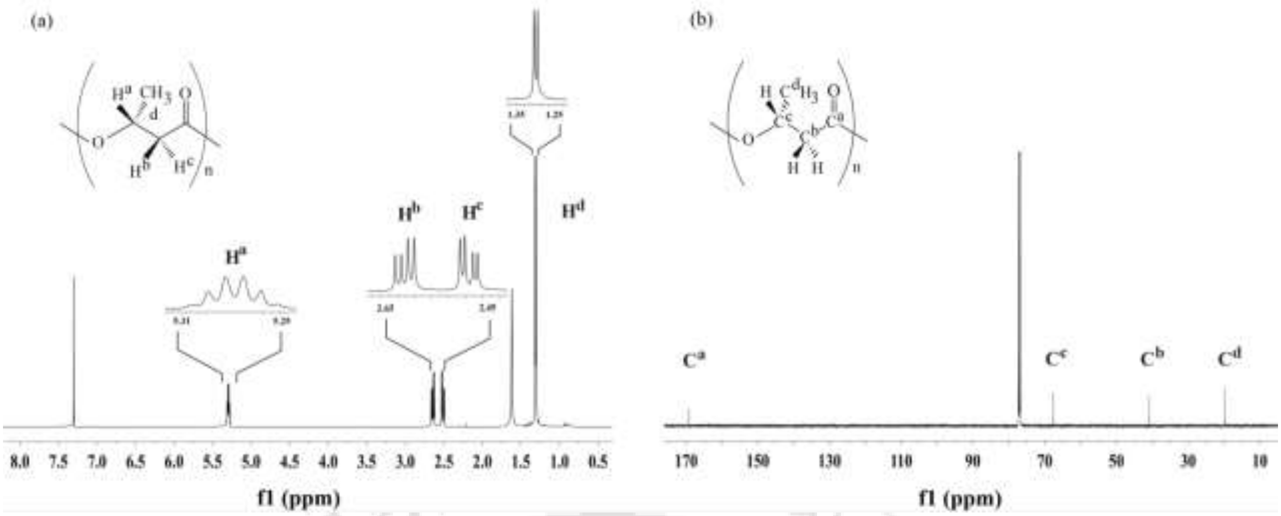


Figure A2.6: Nuclear Magnetic Resonance imaging of PHB extracted from BR2: (a) 1H (b) 13C

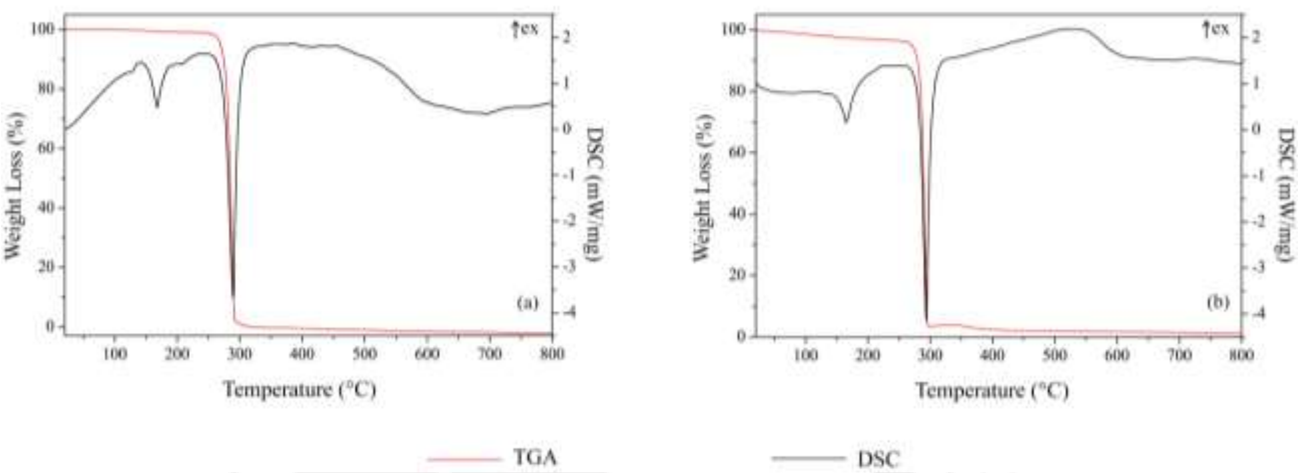
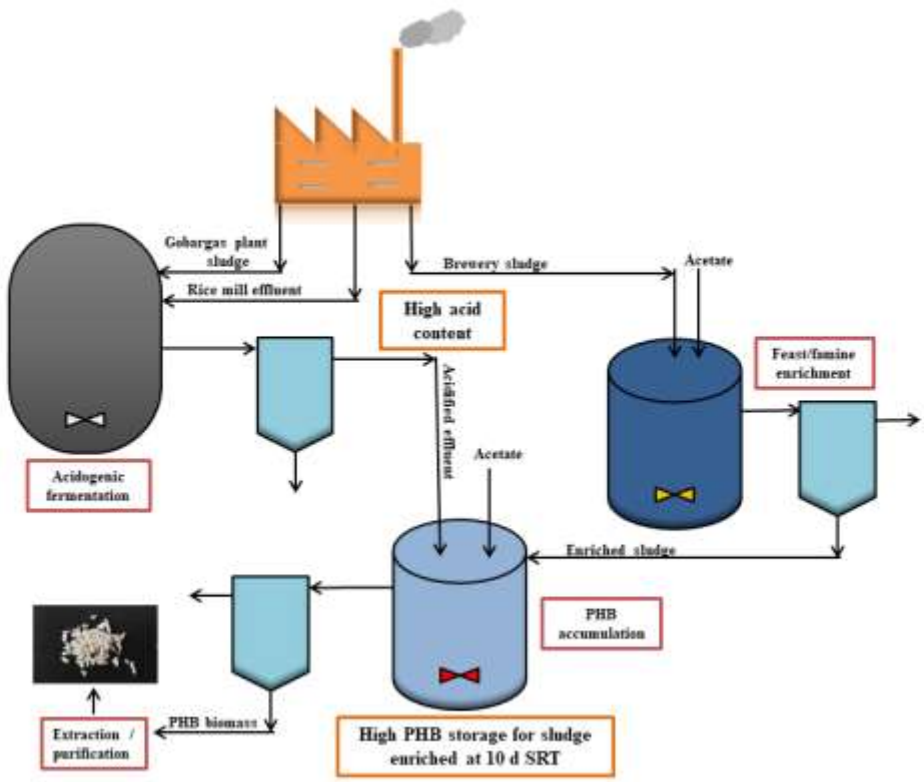


Figure A2.7: Thermogravimetric analysis of PHB: (a) BR1 and (b) BR2

Chapter 6

Polyhydroxybutyrate production from rice mill effluent by influencing sludge retention time during short-term feast/famine enrichment of activated sludge



6.1. Background and research gap

In the present day, plastic pollution is a major concern the waste management initiatives confront due to their unchecked or overuse and improper disposal. Removal of plastic from the market will be disastrous as conventional (fossil fuel based) plastics are being used in a myriad of applications, from highly specific medical use to packaging and automobile parts. Hence, along with reuse and recycle, reduction in its usage is of utmost necessity through minimizing and substitution. Polyhydroxyalkanoate (PHA), touted as the most promising replacements for conventional plastics, are condensed polymer of hydroxylated fatty acids produced as lipophilic inclusion in microorganisms like bacteria, archaea, microalgae, etc. Polyhdyroxybutyrate (PHB) and poly (hydroxybutyrate-co-hydroxyvalerate) (PHBV) are the most industrially valued and investigated PHAs with more emphasis on PHB content. PHB possesses similar property to polypropylene and polyethylene, while the PHBV with higher PHB contains some favorable mechanical properties like higher young's modulus. Even with these exceptional correlations, the higher cost of polymer restricts its widespread applications. Operational costs like culture, bioprocess control, substrate, etc., account for the major share of the cost. Presently, PHA-based materials are mostly used in specific applications like pharmaceutical and therapeutical (Nguyenhuynh et al., 2021).

Activated sludge is one of the cheapest and abundant inoculum source extensively explored for making PHA affordable. A significant portion of the wastewater treatment in the world relies on the activated sludge process. They are exposed to a multitude of variability in the operational conditions like physical, nutrient, substrate, etc., stress factors that develop PHA storing phenotypes. Activated sludge as the inoculum culture enables the use of various waste feedstocks as carbon sources for PHA production like effluents, solid waste (hydrolysates), industrial side streams, landfill leachate, etc. This enables the reduction in PHA production cost through the non-axenic culture, simple bioprocess condition, cheaper carbon source, etc., along with waste treatment. The approach in this direction concentrated on the specificity of waste feedstocks and PHA accumulating potential (PAP) of activated sludge in the form of a three-step method (Nguyenhuynh et al., 2021).

Enrichment of activated sludge is the fundamental step chosen to alter the PAP by coercing different microbial communities in the mix microbial culture (MMC) to divert a larger portion of influent carbon for storage without compromising biomass growth. Aerobic dynamic feeding (ADF) strategy exposes activated sludge to cyclic feast and famine conditions, enhancing the sludge's PHA accumulating potential. Many operating parameters like pH, temperature, dissolved oxygen, organic loading rate, hydraulic retention time, etc., play a vital role in deciding the efficiency of ADF strategy. An earlier study had shown the nutrient excess condition to result in a lower feast to famine ratio eliciting stronger ecological selective pressure that enriched MMC with proper cell machinery to maximize PHA accumulation (Chapter 5). A crucial parameter regulating ADF is sludge retention time (SRT) that dictates the growth rate of MMC that in turn decides stability and storage performance.

Various studies exist that deliberate the effect of SRT on activated sludge in terms of biomass growth, substrate removal, etc. Studies on the ADF process have primarily employed SRT ranging between 1-10 d (Nguyenhuynh et al., 2021). However, extensive investigations into SRTs influence on PHA accumulation are scarce. Johnson et al. (Johnson et al., 2010) had earlier assessed a combinatorial influence of shorter SRTs (0.5, 1, 4 d) and carbon to nitrogen ratio (C/N) (6,11, 13.2, 24) on PHA production. They found shorter SRTs efficient with excess nitrogen condition, especially SRT of 4 d and C/N ratio of 13.2, leading to a dual limitation. Similarly, the enrichment system at SRT of 4 d fed with thermally hydrolyzed sludge fermentate had a storage response of 51 % under phosphorus limitation (Tu et al., 2019). At the same time, an SRT of 1 d with an additional settling phase after feast phase during enrichment with acidified cooked mussels wastewater had a storage of 60 % (Argiz et al., 2020). However, another study found that sludge exposed to 5- 10 d SRT showed no significant variation in PHA storage, and SRT of 15 days had a highly reduced storage response (Mokhtarani et al., 2012). Chen et al. (Chen et al., 2017)analyzed the effect of SRT, 5 d and 10 d, initial biomass, and carbon concentration on the ADF process with fermented molasses. They found SRT of 5 d had better operational stability and PHB storage. Meanwhile, an SRT of 10 d enriched MMC with high PAP such that maximum storage of 60.3 % resulted from sludge acidified at pH 10.0 as substrate (Liu et al., 2020). A pilot-scale study had operated sludge enrichment at different SRT (1 d and 2 d) to cycle length (CL) (0.25 d and 0.5 d) ratios (SRT/CL) of 2, 4, and 8 under high organic loading rate with the organic fraction of municipal sewage waste – waste sludge fermentate (fOFMSW-WS) as substrate. They found high-intermittent ratios of 2 and 4 to have higher storage yield than the ratio 8, indicating a relation between SRT/CL and organic loading (Moretto et al., 2020). These studies depict cross-talk between SRT and other parameters to alter the enrichment process. The impact of SRT without the interference from other factors or other selective pressure hasn't been scrutinized yet, with special attention to short-term enrichment.

The specificity of waste feedstock for bioconversion of organic content to PHA also dictates the efficiency of the process. Non-specific components in the substrate reduce the polymer production for inoculum enriched in sequential feast/famine conditions. The thermal hydrolysis liquor could only raise the accumulation to 24.1 % (Liao et al., 2018), while alkaline fermented sludge liquor produced high storage of 60.3 % (Liu et al., 2020). Hence, acidogenic fermentation (AF) is essential for PHA production by utilizing solid or liquid waste feedstock due to the conversion of non-specific components into polymer precursors, volatile fatty acids (VFAs), i.e., acetate (HAc), propionate (HPr), butyrate (HBu), and valerate (HVa). In the same way, the efficiency of acidogenesis plays a vital role in the overall productivity of the three-step process. Feedstock pretreatment and regulation of operational parameters like pH, temperature, organic loading rate, etc., have been the traditional routes to enhance VFA production from waste(Ramos-Suarez et al., 2021). The anaerobic inoculum pretreatment is an actively investigated area for biohydrogen production (Pachapur et al., 2019). Meanwhile, a perspective in terms of VFA production is yet to be properly constructed, especially for PHA production. Only a few pieces of literature have connected anaerobic inoculum pretreatment to PHA production from feast/famine enriched activated sludge. A heat pretreatment of inoculum at 100 °C for 1 h produced a high organic acid fraction of 203 mmol/L from second cheese whey and 169 mmol/L from concentrated cheese whey resulting in PHA accumulation of 66 % and 55 %, respectively from enriched activated sludge (Colombo et al., 2019). Acidogenic fermentation of high-pressure thermal hydrolyzed waste sludge using anaerobic inoculum pretreated at 110 °C for 1 h produced fermentate with high VFA content of 8.93 gCOD/L. The fermentate under phosphorus limitation led to a maximum PHA accumulation of 51 % from enriched activated sludge (Tu et al., 2019). Other inoculum pretreatment methods with different waste feedstocks also need to be evaluated regarding VFA content/profile and PHA production/composition.

Amongst a wide spectrum of substrates used, medium-strength effluents are least examined for PHA production via the AF-ADF process. Anaerobic inoculum pretreatment opens a route for such substrates by enhancing their specificity like VFA content and composition (Chapter 3). Paper board mill wastewater (Farghaly et al., 2017) and brewery wastewater (Ben et al., 2016) are some of the medium-strength effluent investigated for PHA production. Rice mill effluent (RME) is once such effluent produced in copious amount in Assam, India, which falls under the “orange category” of highly polluting industries(Assam Govt./Environment and Forest, 2019). Hence, the AF-ADF route can be a feasible strategy for an effluent treatment system generating a value-added product, thereby balancing the cost of treatment and reducing PHA cost.

Anaerobic inoculum pretreatment can also refine the VFA profile, which influences the polymer composition. The up-gradation of acetate-butyrate pathway due to pretreatment leads to higher even-numbered fatty acid production as observed for heat shock pretreated sludge fermenting cheese whey (Colombo et al., 2019). Recent research had shown that the correct combination of anaerobic inoculum (heat-acid pretreated) and substrate enhances VFA and even-numbered fatty acid production (Chapter 3). Fermented RME had an even to odd ratio of 20.91 mg/mg after heat-acid shock pretreatment of inoculum (Chapter 4). These outcomes can lead to the production of higher PHB content in PHA that can have favorable physicochemical properties. Therefore, the potential of such anaerobic inoculum pretreatment for enhancing PHB production from waste feedstock is yet to be adequately explored. This study investigates the possibility of efficient and enhanced synthesis of PHB, from RME. Firstly, RME is acidified through acidogenic fermentation for better VFA production. Secondly, activated sludge is enriched through feast/famine enrichment by influencing the SRT. Thirdly, PHB is produced from acidified RME using enriched sludge by different feeding regimes, thereby analyzing the waste biorefinery approach for waste management.

6.2. Results and discussion

6.2.1. Acidogenic bioconversion of rice mill effluent into VFA rich fermentate

Acidogenic fermentation consumes the organic content in RME, resulting in the production of H_2 and VFAs. These VFAs are precursors of PHB that induce production. Hence, it becomes necessary to increase the VFA content to enhance the RME-PHB conversion. For this purpose, an anaerobic biomethanation seed sludge, gobar gas plant sludge, was used that contains a wide spectrum of microbes ranging from hydrolytic, acidogenic, acetogenic, hydrogenoclastic-homoacetogenic, acetoclastic, methanogenic coexisting in the mix culture (Bhushan et al., 2019). Our earlier study showed that pretreated sludge had superior performance compared to untreated sludge in the acidification of RME, generating an HAc-dominated HAc-HBu fermentate. The heat-acid shock pretreatment effectively removes methanogens or VFA consuming microbial community, enriching the sludge with spore-forming/acid-tolerant hydrogen producers. At the same time, the combination of a medium-strength effluent and inoculum pretreatment with a self-buffering capacity restricted the pH in a narrow acidic range (5.0-6.0), indicating the possibility of an uncontrolled pH system (section 3.2.7).

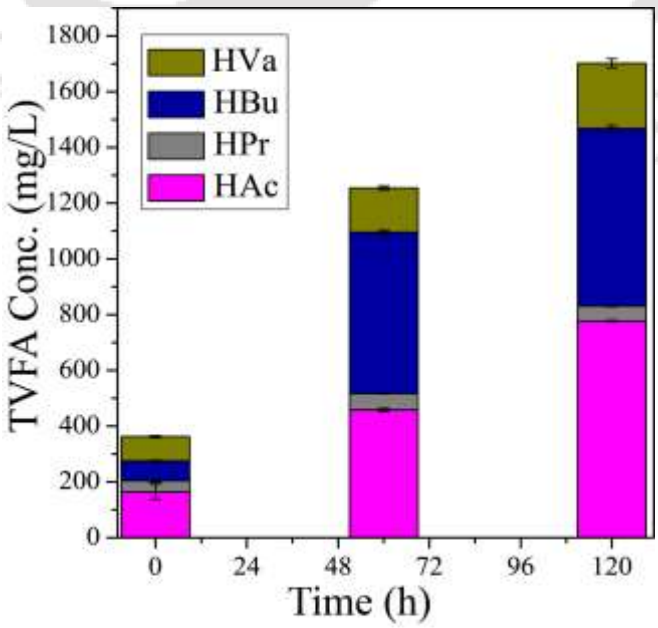


Figure 6.1: Acidogenic fermentation of rice mill effluent

The acidogenic fermentation showed interesting outcomes concerning PHB production. The VFA content in RME (12 %) and that produced during reviving the sludge contributed to the initial point of the process (Fig. 6.1). The bioconversion process picked up swiftly, converting a major portion of the initial COD into VFAs. The acidogenesis process produced fermented RME (fRME) with a total VFA content of 1701.9 mg/L, 79.4 % degree of acidification, and TVFA yield of 0.86 gCOD/gCOD with a decrease in pH from 6.0 to 5.22 (Table 6.1). The VFA profile, HAc/HPr/HBu/HVa::45.6/3.2/37.5/13.7, was similar to raw RME, indicating that substrate plays a vital role in acidogenic output. The pretreatment had directed the metabolism towards the HAc-HBu route indicated by the even to odd ratio of 4.9 mg/mg, which is twice2x that of RME (Table 6.1). Similarly, the acidification of urban biowaste by heat-shock pretreated anaerobic inoculum had HAc-HBu dominated hydrolysate at the peak acidification (Blasco et al., 2020). However, the HAc production was lower than HBu by 60 h, which then reverted by 120 h, increasing 8 % over HBu.

Table 6.1: Performance of the acidogenic fermentation regime for the bioconversion of rice mill effluent

Effluent	RME	fRME
COD (mg/L)	3200±0.0	2880±0.0
TVFA Conc. (mg/L)	263.1±0.0	1701.9±0.003
Degree of acidification (%)	12.2±0.004	79.4±0.005
VFA profile #	41.6/15.3/29.9/13.2	45.6/3.2/37.5/13.7
Even to odd ratio (mg/mg)	2.6±0.03	4.9±0.05
$Y_{TVFA/nonVFA}$ (mgCOD/mgCOD)	-	0.86±0.0
$Y_{HAc/nonVFA}$ (mgCOD/mgCOD)	-	0.28±0.01
$Y_{HBu/nonVFA}$ (mgCOD/mgCOD)	-	0.44±0.01
$N\text{-}NH_4$ (mg/L)	12.0±0.08	6.4±0.1

±%SD, # HAc/HPr/HBu/HVa, RME: RME, fRME: fermented RME

The acidification of RME without pH control can be viewed as inclined towards butyrate production. The higher HBu yield of 0.44 mgCOD/mgCOD compared to the HAc yield of 0.28 mgCOD/mgCOD indicates this. During the acidification of molasses wastewater with heat-shock treated inoculum, the variation of pH within 4.0-5.0 had led to higher HBu production than HAc(Yun and Cho, 2016). At the same time, acidification of food waste-primary sludge mixture using untreated sludge produced caproic acid dominated fermentate with high even-numbered VFA content under pH uncontrolled condition (Perez-Zabaleta et al., 2021). The decrease in pH increases the reducing equivalents in the system, which can lead to chain-elongation. Hence, the acidogenic microbial community self-adjust to changing microenvironment when pH is left uncontrolled. Besides, the buffering capacity of the fermentation system and substrate retains the pH to a narrow range. This promotes a stable performance of the acidification system without controlling the pH. Uncontrolled pH condition could be advantageous on an industrial scale by saving huge capital on chemical consumption (Sarkar et al., 2021). Nonetheless, the acidogenic fermentation successfully enhanced the VFA content in RME to be an ideal substrate for PHB production.

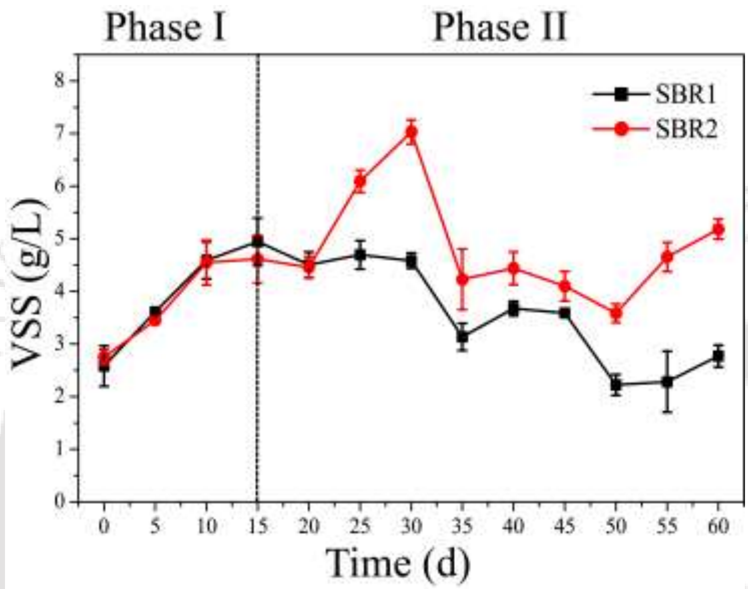


Figure 6.2: The volatile suspended solids fluctuations during the enrichment of brewery sludge

6.2.2. Effect of SRT on reactor operation and performance

Sludge retention time influences microbial growth and thereby the selective pressure in the sequential feast/famine enrichment. An SRT regulation induces a weeding effect into the system. Depending on the SRT, the MMCs with required growth rates are retained, consume the substrate, and generate a short feast phase. These MMCs get exposed to the feast/famine selective pressure, contributing to the PHB storage response. Meanwhile, the microbial community with lower growth rates compared to the set SRT eventually gets washed out. Some of the PHB accumulators present in raw brewery sludge also get removed. The ADF process regulated SRT either by wasting a fixed volume of sludge alone or combined with a settling phase. Wastage of a larger volume of mixed sludge results in lower SRT (5 d), while higher SRT requires wasting of smaller sludge volume (10 d). Some investigations maintained an SRT/HRT of 1 d by wasting 75-83 % of the working volume and adjusting the cycle length without a settling phase (Marang et al., 2016). This additional pressure enriches PHB accumulators with different storage responses. The

HRT (5d) kept higher compared to earlier investigation reduces any additional selective pressure for the investigation of feast/famine enrichment under the sole influence of SRT. Similarly, the settling time and C:N:P ratio kept constant also avoids any other influence. Activated sludge from different origins, petroleum refinery sludge, and brewery sludge had similar PHB accumulation when enriched under ADF. However, the PHB storage of BS was less affected by sludge wasting initiated by the 30^{th} day compared to petroleum refinery sludge. At the same time, long-term ADF enrichment of BS at SRT of 10 d resulted in high PHB accumulation. Hence, BS was preferred in the present study to investigate the effect of different SRT on accumulation response.

In phase I, no sludge wasting and thereby no SRT control was imposed on SBR1/SBR2 for 15 days. Both enrichment systems performed similarly with near biomass growth by consuming the provided substrate (Fig. 6.2). This similarity is reflected even in terms of PHB storage and the f/f ratio (Fig. 6.3). The MMC present naturally in the sludge multiplied and converted the given acetate into PHB with its innate PHB accumulating potential (PAP). An exponential increase in VSS content was observed between 0-15 days (2.6-4.9 g/L). Also, both reactors showed a settleability of more than 50-75 % of working volume in phase I (even though the volume exchange ratio was 25 %). Both systems start to part away in phase II once the SRT control is established after 15 days (day 16), and their growth pattern split. The MMCs in SBR1/SBR2 had lower growth rates in the range of 0.01-0.03 d^{-1} than the growth rate required by the SRTs (0.2d^{-1}/0.1d^{-1} for 5 d/10 d). The larger sludge wastage in SBR1 might have retained only a smaller fraction of the initial MMC that matched the required growth rate. Meanwhile, the smaller sludge wastage in SBR2 retained a significant portion of MMC in the inoculum that aligned with the necessary growth rate that included the microbes with higher and lower growth rates. This resulted in an average VSS content of 2-3g/L in SBR1 and 4-5g/L in SBR2 (Fig. 6.2). A pilot-scale enrichment of activated sludge with fOFMSW-WS also observed a comparable trend under SRTs of 1 d and 2 d (Moretto et al., 2020). However, the different microbial communities in the activated sludge matched the necessary growth rate under SRT of 5 d and 10 d that the enrichment with fermented molasses had similar biomass concentration and yield (Chen et al., 2017). Other than transitional fluctuation, the constant aeration retained a stable and lower f/f ratio throughout the analysis indicating proper substrate removal (Fig. 6.3b).

However, the settleability of sludge SBR1 affected within 2-3 d SRTs of sludge wastage initiation in phase II. Even after the settling time, more of biomass remained suspended than settled. The VSS content never went below 2g/L even though 400 mL mixed sludge was wasted every day and only 100 mL decanted after 40 mins of settling. Probably, the biomass flocs are unable to grow heavier due to the disparity between the actual and the imposed growth rate of the sludge (excessive wastage) that the major portion of biomass remained suspended. This might be the reason that studies with SRT 1 d and 4 d run SBRs without a settling phase (Marang et al., 2018)(Coats et al., 2016). Meanwhile, SBR2 had a higher biomass concentration, leading to larger flocs that settle below 50% of reactor volume with intermittent fluctuation corresponding to the ambient condition (SVI 50-100 mL/g). A sludge enrichment using fermented molasses having SRT of 5 d and 10 d also had good settleability due to the influence of other parameters and similar biomass concentration (Chen et al., 2017). The volume of sludge wasted was also adjusted for biomass content in the decant phase to maintain the SRT in SBR2. Hence it is necessary to standardize the biomass growth rate per day of the activated sludge to fixed the SRT to consider the system's feasibility to be integrated into a conventional effluent treatment plant (sedimentation unit).

6.2.3. Storage performance of the enrichment systems

The initial factor that induces carbon conversion to polymer in SBR1/SBR2 is the substrate used, acetate. VFAs are the precursors for PHB forming the primary backbone of the polymer through the PHB synthesis pathway, especially acetate and butyrate. Also, specific enzymes can divert intermediates during fatty acid synthesis and degradation pathway towards 3-hydroxyacyl-CoA that is polymerized by PHB synthetase to PHB. Moreover, the coenzyme-A (CoASH) content in the microbial cytoplasm decreases due to acetyl CoA formation by acetyl-CoA synthase and β-oxidation pathway, releasing inhibition on polymer synthesis pathway (Vallari et al., 1987)(Tan et al., 2014). Therefore, acetate acts as an ideal carbon source for enrichment that will augment biomass growth through increased functioning of the TCA cycle (shortened pathway without glycolysis) and elevated PHB production.

SBR1 and SBR2 maintained similar PHB storage till day 15 during phase I (Fig. 6.3a). Both systems maintained similar storage similar to raw sludge during the phase (5-7 %) without much effect from feast/famine enrichment. The biomass built up nearly twice from 0 -15 days leading to faster substrate consumption and a lower f/f ratio (Fig. 6.3b). Therefore, phase I can be said to be devoid of proper enrichment with an emphasis on biomass growth. Both reactors started to show a difference in their storage response after the initiation of sludge wastage (day 16) in phase II. The PHB storage increased to 11.7 % for SBR1 and 9.9 % for SBR2 within 10 days of sludge wastage due to feast/famine selective pressure rather than biomass growth (Fig. 6.2,6.3a). This indicates that a washout condition was absent for 25 days even after starting sludge wasting in SBR1. However, a continuous decline in the PHB storage was observed from 30-45 days, with storage reaching 4 %. The washing out of innate PHB accumulators that survived until 25 days can correlate with the decline, as shown in Fig. 6.2. Nonetheless, % PHB stabilized quickly by 60 days with a shorter feast phase than SBR2. Ambient temperature had a negative impact on the settling capacity of the sludge resulting in slower sedimentation and higher suspended biomass in the decant during colder climate. So, the sludge wasted varied between 100-200 mL in order to maintain an SRT of 10 d. This caused a brief increase in biomass and % PHB (17.3 %). However, sludge wastage brought down storage response by 45 days and stabilized above SBR1 in the range of 9-10 %. The f/f ratio also showed inconsistency during the same period stabilizing after 45 days.

In a conventional ADF, the organic load is adjusted such that the biomass can grow faster by consuming the excess nutrient provided, resulting in a higher substrate uptake rate which in turn caters to a shorter feast phase. The cycle length set according to biomass yield and substrate uptake rate enforces a lower f/f ratio to elicit strong ecological selective pressure. The feast phase gets extended, resulting in higher f/f ratio when substrate consumption and organic loading don't align. However, an ADF enrichment had reported an f/f ratio in the range 0.3-0.4 during enrichment using fermented cheese whey under high organic loading using uncoupled carbon and nitrogen feeding strategy (Oliveira et al., 2017). A comparative study also found such a feeding strategy to have better enrichment than conventional ADF in a shorter period(Ahmadi et al., 2020). Aerobic dynamic discharge is a modification of ADF that can establish a longer famine phase manually for higher organic loading (Inoue et al., 2021). In the study, a medium organic content (3 g/L) and cycle length of 24 h aided in maintaining the longer famine phase with a lower f/f ratio (on avg.) of 0.2 during the short enrichment period.

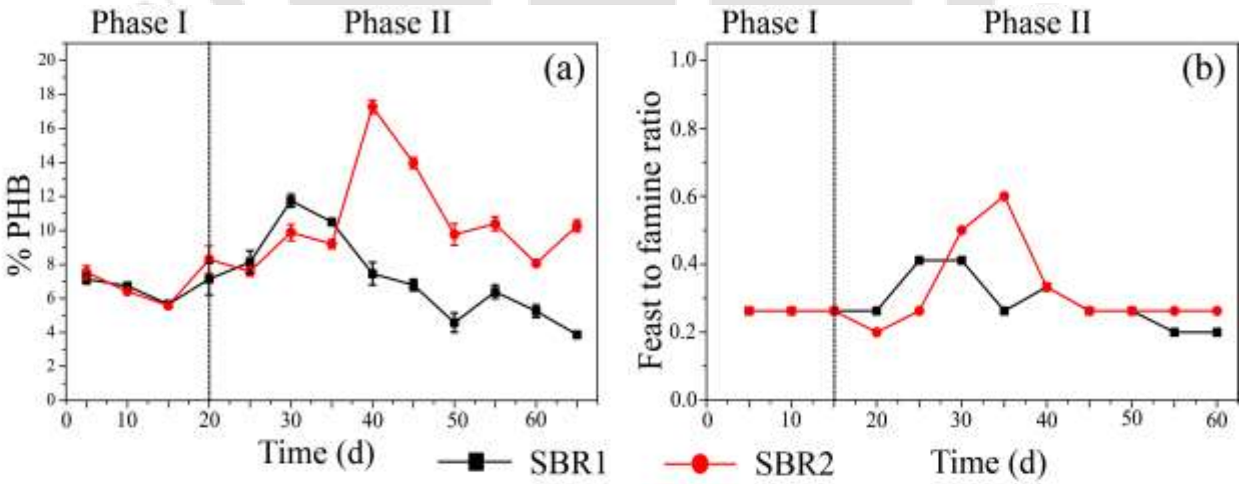


Figure 6.3: The enrichment performance of SBR1 and SBR2 in terms of (a) PHB storage response, and (b) feast to famine ratio

During phase I, the exponential biomass growth resulted in an f/f ratio of 0.2 as higher biomass concentration resulted in faster substrate consumption without much impact on storage response (Fig. 6.3b). SBR1 had a transient increase in the f/f ratio (0.41) during 25-30 days. The considerable sludge wastage caused a decrease in biomass concentration leading to slower substrate removal resulting in a longer feast phase. However, the f/f ratio stabilized quickly as the MMCs in SBR1 started to grow faster under the influence of SRT. A transient built-up of biomass due to lower sludge wastage in SBR2 (to fix SRT at 10 d due to irregularity in sedimentation) causes a rise in f/f ratio to 0.6 without any drastic impact on PHB storage. The set air flow rate (2 L/min) might have been insufficient to meet the oxygen uptake rate during the transient increase in biomass concentration in SBR2. However, lower biomass concentration in SBR1 ensured surplus oxygen available for growth resulting in a stable and low f/f ratio (0.2) from day 40. Similarly, the ratio decreased for SBR2 from 40 days after stabilization of biomass concentration (Fig. 6.2,6.3b)

During the short enrichment of brewery sludge, SRT of 10 d had retained a major portion of the initial accumulators and increased the PAP of BS better than SRT of 5 d. The MMCs in SBR2 have prolonged exposure to enrichment conditions than SBR1 within 60 days. Higher wastage in SRT of 5 d might have washed out initial accumulators, exposing other MMCs with relatively low storage response towards the selective pressure. Besides, MMCs in SBR1 might be less influenced by the selective pressure during such a short enrichment period due to higher wastage. Hence, they would require an extended period to increase the PAP as many studies used shorter SRT for culture enrichment (Nguyenhuynh et al., 2021). Another critical factor for a lower f/f ratio is that the supplied airflow rate needs to sync with biomass concentration (oxygen uptake) to ensure faster growth and substrate uptake.

The analysis of the profile of various parameters in a single (batch) cycle during the enrichment divulges the mechanism of the feast/famine enrichment (Fig. A3.1). Proper nutrient levels ensured faster substrate consumption leading to a shorter feast phase. The low f/f of 0.2 impounded a strong ecological selective pressure and thereby an acceptable conversion of 13-18 mgCOD/mgCOD (at the end of the feast) in a short enrichment period (Table A3.1). Also, maintaining different SRT in the SBRs affected the biomass content and growth. The shorter SRT in SBR1 required mix culture to maintain a faster growth rate (r_X) of 630.5 mgCODx/h that was twice that of SBR2 (352.1 mgCODx/h) with longer SRT. The culture in SBR2 had enough time to replace the amount lost during sludge wastage. Meanwhile, SBR1 had to increase its growth rate to maximally consume the carbon and grow. This nature of biomass is also indicated by higher q_S of 0.21 gCOD$_S$/gCODx for SRT of 5 d compared to 0.1 gCODS/gCODx h for SRT of 10 d. Similarly, quick removal of substrate requires faster uptake of nitrogen, as indicated in Tabel A3.1. A modest difference in $Y_{X/S}$ indicates the capacity of the cultures to divert substrate towards biomass growth.

However, the specific PHB consumption rate ($-q_p$) for SBR2 is 1/10th of the $-q_p$ of SBR1(Table A3.1). Active consumption of the accumulated PHB occurs in SBR1 for microbial growth (stationary phase-reduced microbial growth), cellular maintenance, etc. Meanwhile, a lesser amount of PHB is depolymerized and consumed for cellular metabolism in SBR2. The slow-growing nature of culture in SBR2 might demand slower PHB consumption during the famine phase to prepare for the next cycle. The shorter SRTs render the sludge with biomass having potency towards growth, requiring faster $-q_p$ to speed up the process in the next cycle. This might reason for the difference observed in certain parameters (Table A3.1). Thus, clearer feast/famine phases are generated under 5 d SRT than 10 d SRT.

Therefore, the feast/famine cycle must be manifesting the selective pressure diversely in both enrichment systems. In SBR1, higher sludge wastage coerces different microbial communities to step up their growth to utilize maximum carbon and nitrogen for the primordial objective of survival. This exposes the culture towards a shorter feast phase and longer famine phase that slowly transit the system towards a balanced growth-storage mechanism. In addition, a lower feed to microbe ratio created due to lower biomass content creates a carbon excess microenvironment. The microbes would prefer growth over storage under this environment of high substrate and nutrients. Also, a substantial portion of the initially available accumulators gets washed out with subsequent replacement over the period of enrichment. The present study points out that shorter SRTs can lead to better storage capacity under the specific requirement of an extended enrichment period or other additional selective pressure.

In SBR2, a larger portion of initial accumulators gets retained due to lower sludge wastage sustaining a higher biomass concentration. The higher biomass concentration reflects a higher feed to microbe ratio ($\geq$1.0), creating a micro-carbon limitation that forces the culture to consume substrate quicker. This micro-environment leads to a shorter feast phase even though SBR2 has a comparatively reduced r_x/qs than SBR1. The carbon limited-nutrient excess condition also drives the system towards storage oriented than growth-oriented culture. In addition, a broad spectrum of accumulating/non-accumulating microbial communities gets extended exposure to feast-famine phases under 10 d SRT. These factors, in combination, yield better storage response in a shorter period of the enrichment in SBR2. PHA storage pattern of both SRTs in an extended period of enrichment (years) could fully reveal their characteristics and applicability in the real environment of the wastewater treatment plant. By the time the reactors were stopped, both reactors had similar storage capacity pointing out the requirement of long-term studies. Chen et al. (Chen et al., 2017) also observed no significant difference in PHA storage capacity between both the SRTs during the enrichment period (100 days).

6.2.4. PHB production from acetate and RME using enriched BS

The present study focuses on the bioconversion of RME into PHB, where RME is medium-strength effluent with COD in the range of 3g/L. All the accumulation assays had the same organic load to utilize RME for polymer production. Hence, for the accumulation assays, feed with COD in the similar range was mixed with enriched BS at the end of the famine phase by maintaining an inoculum to feed ratio of 1:2 (w/w), ensuring dilution of 1.5x of the feed. The ratio maintained feed to microbe ratio of 4.2-3.5 in BR1 and 2.1-1.3 in BR2 during the accumulation studies. The reason for this difference is the biomass concentration in SBR1and SBR2 (Fig. 6.2). A lower inoculum volume would have resulted in very low biomass loading in BR1 than BR2 due to the difference in biomass concentration in the SBRs. At the same, higher inoculum volume from SBR1/SBR2 would shift the fixed SRT, thereby affecting the stability of the SBRs.

The previous study had found nitrogen limitation as the ideal condition to elicit a growth imbalance necessary for peak accumulation. The nitrogen excess condition set in the present study had an average residual nitrogen content of 41 % and 35 % (initial nitrogen) at the end of the feast phase in SBR1 and SBR2, respectively. Thus, inoculum to feed ratio in accumulation studies was enough to provide the necessary limiting condition. However, the extent of limitation could differ between the cultures in accumulation assay owing to reduced biomass concentration in BR1 than BR2. Due to the nitrogen limitation, the DO concentration increases within 2 h of operation and stabilizes by 4 h, indicating the saturation phase (no further microbial activity) or the death phase. The reactions are terminated at this point, and the samples taken for analysis. The accumulation process being a stressed condition, maximum conversion can occur during the final phase. The PHB synthesis becomes a secondary metabolism compared to a growth-assisted during enrichment.

6.2.4.1. Accumulation assays using acetate as carbon source

Initially, the batch accumulation assay was conducted to determine the maximum PHB production capacity of the BS enriched under short feast/famine enrichment (Fig. 6.4a,b). The maximum PHB storage for BR1 was 4 % and 28.8 % for BR2 (Table 6.2). Similar results were observed for PHA production (31.3 %) using waste sludge collected from municipal wastewater treatment plant with a medium COD_{AC} (6 g/L) (Zheng et al., 2021). For PHB accumulation, carbon excess condition under nutrient stress caters to higher storage capacity (Marang et al., 2018). This might not have established in the batch accumulation due to lower organic load (2.08 and 2.4 g/L), such that highly nutrient stress conditions could not saturate the cells with PHB. Probably the inoculum to feed ratio was also not able to provide carbon excess as expected. Besides, the shorter enrichment period might have reduced the maximum accumulation that MMC could achieve. A longer enrichment period had produced high storage of more than 70 % with BS during the nitrogen limited accumulation study (section 5.3.6). However, MMC enriched under 10 d SRT had higher storage than 5 d SRT. The MMCs in SBR2 were more exposed to the ecological selective pressure during the shorter enrichment period, and that most of the initial accumulators could be retained in SBR2. MMCs in SBR1 are comparatively exposed less to the selection pressure and, in combination with high washout, might have led to lower storage during batch accumulation. The $Y_{P/S}$ for BR2 (0.22 $mgCOD_{PHB}/mgCOD_S$) was ten-fold higher than BR1 (0.02 $mgCOD_{PHB}/mgCOD_S$). This again indicates the bioconversion efficiency of MMC from SBR2 over SBR1. Similar is the case of q_P. Therefore, batch accumulation is more preferrable in case of substrate concentration high to the non-inhibitory level, whereby a carbon excess/nitrogen deficient condition can bulk the cells with polymer.

Table 6.2: Kinetic and stochiometric parameters of PHB production during different accumulation assays.

Accumulation regime	Feeding strategy	Subs-trate	Inoculum enriched in SBR1						
			PHB storage	$Y_{P/S}$	q_p	q_s	Overall $Y_{P/S}$	$Y_{P/TVFA}$	$Y_{P/HAc+HBu}$
Batch	Batch	Acetate	4.0 ±0.01	0.02 ±0.04	0.008 ±0.05	0.39 ±0.01	0.02 ±0.04	0.02 ±0.04	0.02 ±0.04
Fed-batch	Continuous	Acetate	6.8 ±0.05	0.02 ±0.01	0.009 ±0.02	0.55 ±0.01	0.02 ±0.01	0.02 ±0.01	0.02 ±0.01
	Pulse	Acetate	16.6 ±0.06	0.03 ±0.09	0.01 ±0.01	0.62 ±0.01	0.01 ±0.01	0.01 ±0.01	0.01 ±0.01
		RME	13.6 ±0.07	0.07 ±0.02	0.02 ±0.02	0.25 ±0.02	0.02 ±0.07	0.46 ±0.01	0.66 ±0.01
		fRME	22.1 ±0.01	0.20 ±0.02	0.06 ±0.03	0.34 ±0.01	0.08 ±0.09	0.17 ±0.04	0.22 ±0.03
Accumulation regime	**Feeding strategy**	**Subs-trate**	**Inoculum enriched in SBR2**						
			PHB storage	$Y_{P/S}$	q_p	q_s	Overall $Y_{P/S}$	$Y_{P/TVFA}$	$Y_{P/HAc+HBu}$
Batch	Batch	Acetate	28.8 ±0.03	0.22 ±0.08	0.1 ±0.04	0.25 ±0.01	0.22 ±0.08	0.22 ±0.08	0.22 ±0.08
Fed-batch	Continuous	Acetate	22.4 ±0.07	0.16 ±0.01	0.07 ±0.07	0.38 ±0.01	0.16 ±0.01	0.16 ±0.01	0.16 ±0.01
	Pulse	Acetate	60.4 ±0.0	0.34 ±0.01	0.30 ±0.01	0.48 ±0.01	0.2 ±0.02	0.2 ±0.02	0.2 ±0.02
		RME	12.3 ±0.06	0.05 ±0.2	0.03 ±0.06	0.17 ±0.01	0.02 ±0.1	0.33 ±0.01	0.40 ±0.004
		fRME	29.5 ±0.01	0.32 ±0.02	0.13 ±0.01	0.28 ±0.01	0.11 ±0.04	0.32 ±0.03	0.37 ±0.01

±%SD, %: PHB storage, q_P: $mgCOD_{PHB}$ /mg COD_X h, q_s: mg COD_{Ac} / $mgCOD_X$ h, $Y_{P/S}$, $Y_{P/TVFA}$, $Y_{P/HAc+HBu}$: mg $COD_{PHB}/mgCOD_S$, fRME: Fermented RME

The study also explored fed-batch processes to determine the saturation potential of the enriched MMCs for PHB accumulation. In fact, a fed-batch is an ideal system to generate higher cell density. Fed-batch with continuous feeding of the substrate creates a micro feast-famine condition similar to the feed-on-demand strategy (Fig. 6.4c,d). Also, a dual carbon/nitrogen limitation is formed in the system. The low substrate concentration at an instant with each drop of feed under a particular flow rate (very short time interval) creates a carbon limited condition due to a lower feed to microbe ratio (Valentino et al., 2015). Thereby, substrates are removed much faster for storage than growth. The nitrogen availability swiftly moves from basal level to limitation, along with an increase in volume due to continuous feeding. This sudden shift in nitrogen availability also causes a shift in available carbon to PHB. The flow rate was set such that the entire working volume was filled by 4 h retention time obtained from the previous batch study. However, the PHB storage did not show any improvement over the batch accumulation. Yet, the maximum PHB accumulated in FBR2 (22.4 %) was higher than FBR1 (6.8 %) (Table 6.2). The $Y_{P/S}$ and q_p of FBR2 were eight-fold that of FBR1. The micro-level carbon/nitrogen limitation was unable to increase the production beyond that of the batch, and that storage for FBR2 also got slightly reduced compared to batch-mode. The reason could be the same as batch study, lower organic load, and the shorter enrichment period. Thus, neither a micro-level carbon/nitrogen limitation for fed-batch nor batch condition was unable to maximize PHB production. Yet, fed-batch with continuous feeding can be an ideal regime for high organic loading to circumvent any substrate inhibition and to have higher productivity due to higher cell density.

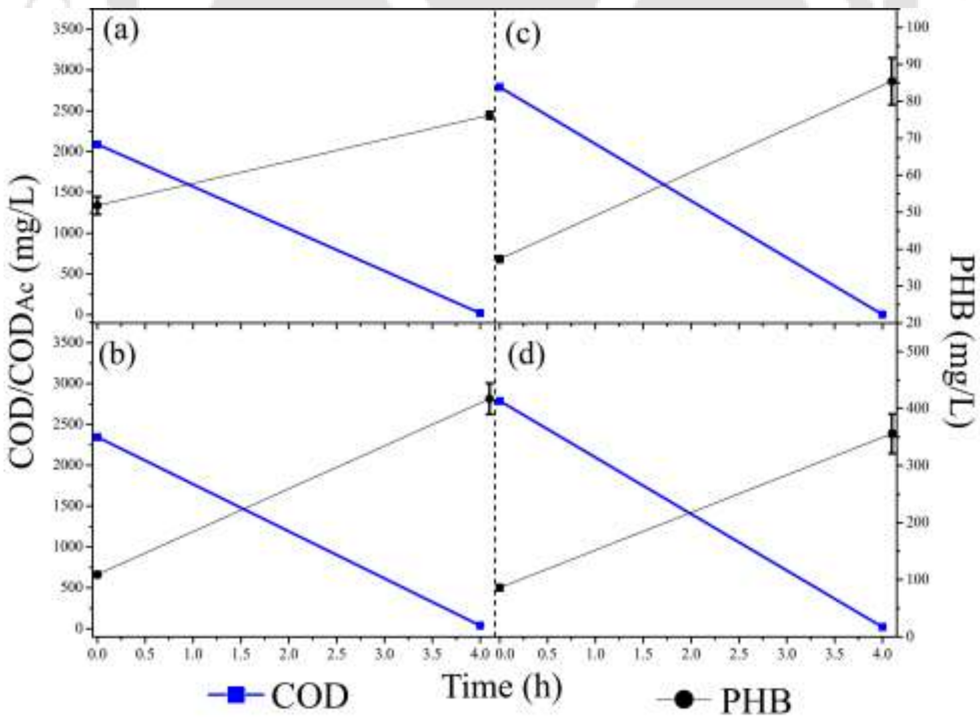


Figure 6.4: PHB accumulation under batch- (a) R1 and (b) R2 and fed-batch with continuous feeding- (c) R1 and (d) R2 with acetate as carbon source.

All these accumulation studies were unable to bring about the maximum storage capacity of the enriched culture. Hence, a fed-batch operation with pulse-wise addition of media exposed the culture to higher organic content directing maximum storage capacity. The fed-batch reactor (FBR2) inoculated with sludge from SBR2 had the highest PHB storage of 60.4 % in the present study. Inoue et al. (Inoue et al., 2021) were also able to obtain storage of 68 % through fed-batch accumulation using sludge enriched under aerobic dynamic discharge and acetate as carbon source. Similarly, the highest accumulation for sludge from SBR1 with acetate as carbon also came with the pulse-wise addition at 16.6 %. The first pulse is terminated after 4 h without any loss in the accumulated PHB that gets added up after the second pulse to give a higher PHB accumulation depending on the MMCs capacity. The slight dips in Fig. 6.5a,b is due to the loss of some biomass during settling, centrifugation, and reinoculation. Similar to other accumulation studies, the yield coefficient is much higher (10x) for FBR2 at 0.34 $mgCOD_{PHB}/mgCOD_S$ compared to FBR1 at 0.03 $mgCOD_{PHB}/mgCOD_S$. Under two pulses of the substrate, culture from SBR2 performed better compared to culture from SBR1. Interestingly, FBR2 had the highest q_P of 0.30 $gCOD_{PHA}/gCOD_Xh$ in the present study indicating a higher production capacity of SBR2 culture. q_P for sludge from SBR1 was also better in pulse-wide fed assay compared to other accumulation regimes. The efficiency of the process is also indicated by the overall yield of fed-batch accumulation of 0.2 $mgCOD_{PHB}/mgCOD_S$ for FBR2. Therefore, accumulation assay with acetate points out the higher PAP of 10 d SRT under short-term enrichment than SRT of 5 d.

6.2.4.2. Accumulation assays using rice mill effluent as substrate

The complexity of the substrate impacts the storage capacity of the enriched MMC. Simple and specific carbon sources like acetate increment PHB production while effluents have comparatively reduced production as observed for RME from Table 6.2. The effluent matrix has a regulatory effect on bioconversion depending on the fermentable contents. Besides, the presence of non-VFA components has a negative impact on PHB production(Tamang et al., 2021).

The pulse-wide fed-batch accumulation has a significant difference in PHB production between raw (Fig. 6.5c,d,) and fermented (Fig. 6.5e,f) RME. Accumulation using fRME had a two-fold increase in the PHB storage for sludge from SBR1 and SBR2 compared to raw effluent. The PHB storage increased from 13.6 % and 12.3 % with RME to 22.1 % and 29.5 % with fRME in FBR1and FBR2, respectively. A fed-batch accumulation study using food waste-primary sludge fermentate (even-numbered VFA rich) acidified without pH control and enriched activated sludge had similar PHB storage (≈30 %) to that of FBR2 (Perez-Zabaleta et al., 2021). Similarly, enriched MMC had PHA storage (23.7 %) similar to FBR1 using fermented food waste(Amulya et al., 2015).The $Y_{P/S}$ showed three-fold increase for FBR1 (0.07 to 0.20 $mgCOD_{PHB}/mgCOD_S$) and six-fold increase for FBR2 (0.05 to 0.32 $mgCOD_{PHB}/mgCOD_S$) while using fRME than RME for PHB accumulation. Similarly, accumulation with fRME had higher q_p of 0.06 and 0.13 $gCOD_{PHA}/gCOD_Xh$ for FBR1 and FBR2, respectively. Thus, the organic content in fRME was converted more into PHB than RME. This huge difference is impelled by the acidogenic fermentation of non-VFAs into VFA, which under nitrogen limited condition get converted to PHB (Fig. 6.5). The raw RME with non-specific components diverts COD into growth and cellular maintenance. The study signifies the transformation brought into the system through acidogenesis, making PHB production from RME a feasible and effective process.

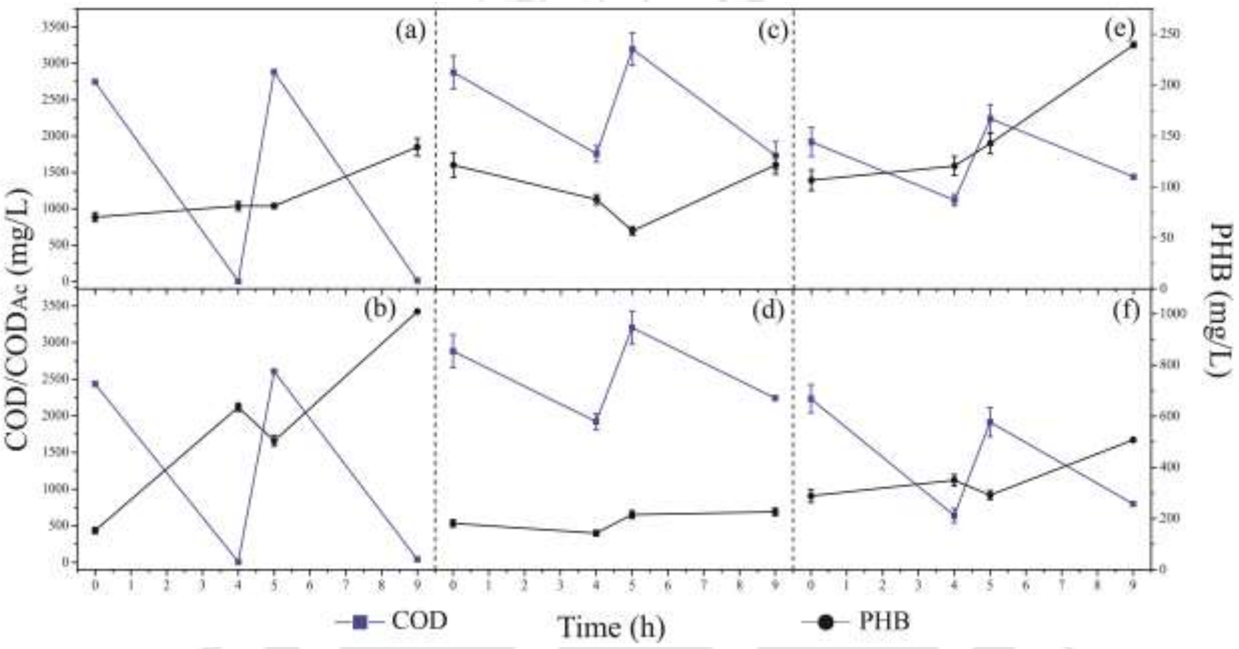


Figure 6.5: PHB accumulation under fed-batch by pulse feeding with substrates: sodium acetate-(a) R1 and (b) R2, RME -(c) R1 and (d) R2, and fRME-(e) R1 and (f) R2

Enrichment of BS under different SRTs also brought about the required improvement in PHB production from RME. Sludge inoculated from SBR2 had storage of 29.5 % compared to 22.1 % for sludge from SBR1 using fRME. The $Y_{P/S}$ and overall yield were also higher for FBR2 than FBR1. The similar values of $Y_{P/TVFA}$ to $Y_{P/S}$ indicates that PHB produced is majorly from VFA in the fRME, which is higher compared to RME. In assays with RME, the non-VFAs contribute (2.8 g/L) more towards PHB production, indicated by lower $Y_{P/TVFA}$ value (Table 6.2). As expected, even-numbered VFA directs PHB production from fRME indicated by $Y_{P/HAc+HBu}$ = $Y_{P/S}$, non-VFAs leads to lower PHB production without acidogenesis ($Y_{P/HAc+HBu}$>$Y_{P/S}$). The similarity between the yield coefficient of the fed-batch (pulse-wise) accumulation in FBR2 with that of *Cupriavidusnecator* using fermented waste paper (0.3 mg/mg) points towards the bioconversion capacity attained by the present system(Al Battashi et al., 2021). There are dips and rises in Fig. 6.5 between pulses due to the difference in biomass concentration. The settling, centrifugation, and re-inoculation unintentionally caused differences in the biomass dilution between pulses. Interestingly, the specific substrate consumption has closer values for FBR1 and FBR2 in all the accumulation assays. A preference of FBR2 towards storage conversion might be the reason for comparatively slower uptake, while carbon consumed for growth and storage results in faster uptake as in for FBR1 (Table 6.2).

The SRT/CL ratio also points out the performance of the present system. Earlier studies had identified a lower SRT/CL ratio to elicit stronger internal growth limitation leading to higher PHA production. Meanwhile, a higher ratio tends to have better enrichment under a higher organic loading rate (Moretto et al., 2020). The present study with fixed HRT and CL has an SRT/CL ratio of 5 for SBR1 and 10 for SBR2. The accumulation assays point out SRT/CL ratio of 10 to have better PHB accumulation potential than SRT/CL of 5 for short-term enrichment. The MMCs that retained longer had better exposure to the selective pressure ensuring the required internal growth limitation. Moretto et al. (Moretto et al., 2020) found higher-intermittent SRT/CL ratios to enrich activated sludge with higher storage response using fOFMSW-WS. The difference between the studies could be due to the higher organic loading rate, the SRT and CL assessed, or the complexity of the substrate. Meanwhile, the VFAs present in RME regulates the bioconversion of the organic content into the polymer. Lower VFAs in RME result in lower production, while higher acid content (acidogenesis) in fRME enhanced PHB production by two-fold. Moreover, the PHB produced is entirely from even-numbered fatty acids in the fRME, similar to acetate in other assays. Besides, the PHB storage value using RME is closer for FBR1 and FBR2 than other accumulation experiments, indicating longer exposure, i.e., an extended enrichment period could bring both systems to a comparable level. The overall yield also projects the PHB accumulation using activated sludge enriched under SRT of 10 d using fRME as a feasible process under the current operational conditions. To our knowledge, the present research would be the first of the kind to report PHA production from rice mill effluent. The efficiency of the PHB production system is depicted in Table 6.3.

6.2.5. Physiochemical characteristics of PHB produced from RME

Functional group identification by FTIR: Interpreting the unique pattern of vibrational bands in the FTIR spectrum and the functional group identified aids in confirming the compound of interest. Simultaneous FTIR analysis of chloroform extract, along with crotonic acid assay, asserted PHB production from RME (Fig.6.6). The FTIR spectrum pattern and the presence of major peaks generated from bending and stretching of groups like ester, methylene, hydroxyl directly indicate the characteristic functionality of PHB. A major absorption peak at 1728 cm^{-1}

resembles the stretching of carbonyl carbon (C=O) in the ester linkage between monomers and the terminal carboxyl group. The peaks ranging from 979-1280 cm^{-1} depict the stretching of the C-O bond present in the ester groups, including the asymmetrical stretching of the C-O-C bond near 1187 cm^{-1}. The alkyl group (C-H) backbone of PHB can be identified from the vibrational bands of the spectrum in the range 2925-2855 cm^{-1} for C-H stretching (alkyl), asymmetric bending at 1455 $cm^{-1,}$ and symmetric bending at 1380 cm^{-1}. The broader peak at 3443 cm^{-1} represents the terminal hydroxyl group (O-H) and traces of moisture (Singh et al., 2021)(Naresh Kumar et al., 2021).

Derving the chemical structure arrangement by NMR: The NMR spectrum represents a unique structural arrangement at the atomic level that can identify a particular molecule. The peak parameters in NMR can also determine the type of polymer, block or random, formed from biological or chemical reactions (Arcos-Hernández et al., 2013). The respective peak areas can identify the number of hydrogen molecules present at each position in the structure constructed. Fig. 6.7 depicts the 1H and 13C NMR spectrum of polymer extracted from FBR1 and FBR2 and the predicted structure. The 1H NMR spectrum of the extract from both reactors had three groups of prominent chemical shifts (δ) (Fig. 6.7 a,b). The chemical shift at δ=5.31-5.25 ppm is associated with the multiplet of -CH group (methine) positioned near the carbonyl end of the monomer (H^a). The multiplet of $-CH_2$ group (methylene) is at δ=2.65-2.45 ppm, diastereotopic protons positioned (H^b and H^c) as shown in the figure. The peak area at δ=1.35-1.25 ppm is thrice to that of peak indicating proton at position H^a, implying the presence of a doublet $-CH_3$ group (methyl) (H^d) that can be a side chain of the polymer.

Similarly, the 13C NMR spectrum of the extract had four separate chemical shifts indicating the position of each carbon in the structure thus constructed, as shown in Fig.6.7c,d. The carbon present in the carbonyl group (-C=O) is placed further in the spectrum at δ=169.2 ppm due to its strong electronegative nature. The peak at δ=67.6 ppm indicates a functional group under the influence of -C=O (carbonyl group of the adjacent unit) at position C^c related to methine carbon. The peak with the lowest chemical shift of δ=19.8 ppm representing carbon moiety having reduced electronegative interaction is the side chain methyl group at C^d position. Similarly, the peak at δ=40.7 ppm sandwiched between the peaks of position C^c and C^d indicates the presence of methylene carbon at C^b. This designation of the alkyl functional groups of carbon foundation is possible by comparing the respective peaks or positions with the 1H spectrum. Finally, the structure constructed from the peak position of NMR confirms the extract from FBR1/FBR2 to be PHB (or PHA). The observation of the present study falls in line with the outcomes of other available literature (Pagliano et al., 2020) (Naresh Kumar et al., 2021).

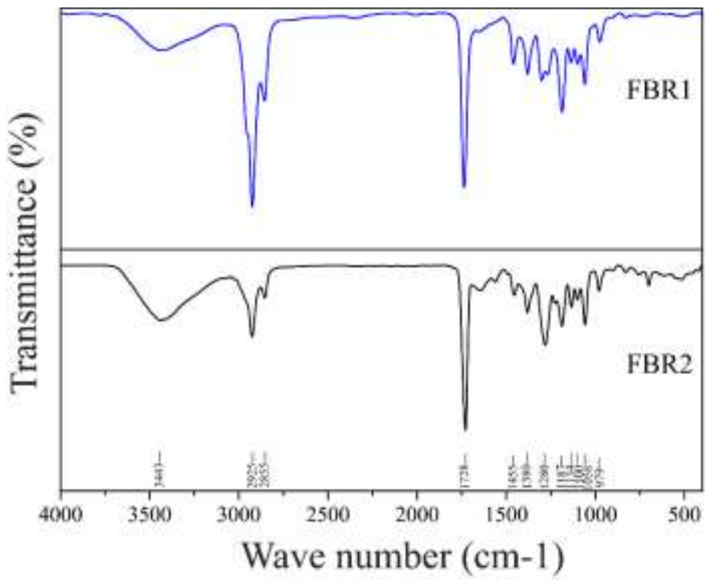


Fig 6.6: FTIR spectrum of the extract from FBR1 and FBR2 after fed-batch accumulation using fRME as substrate.

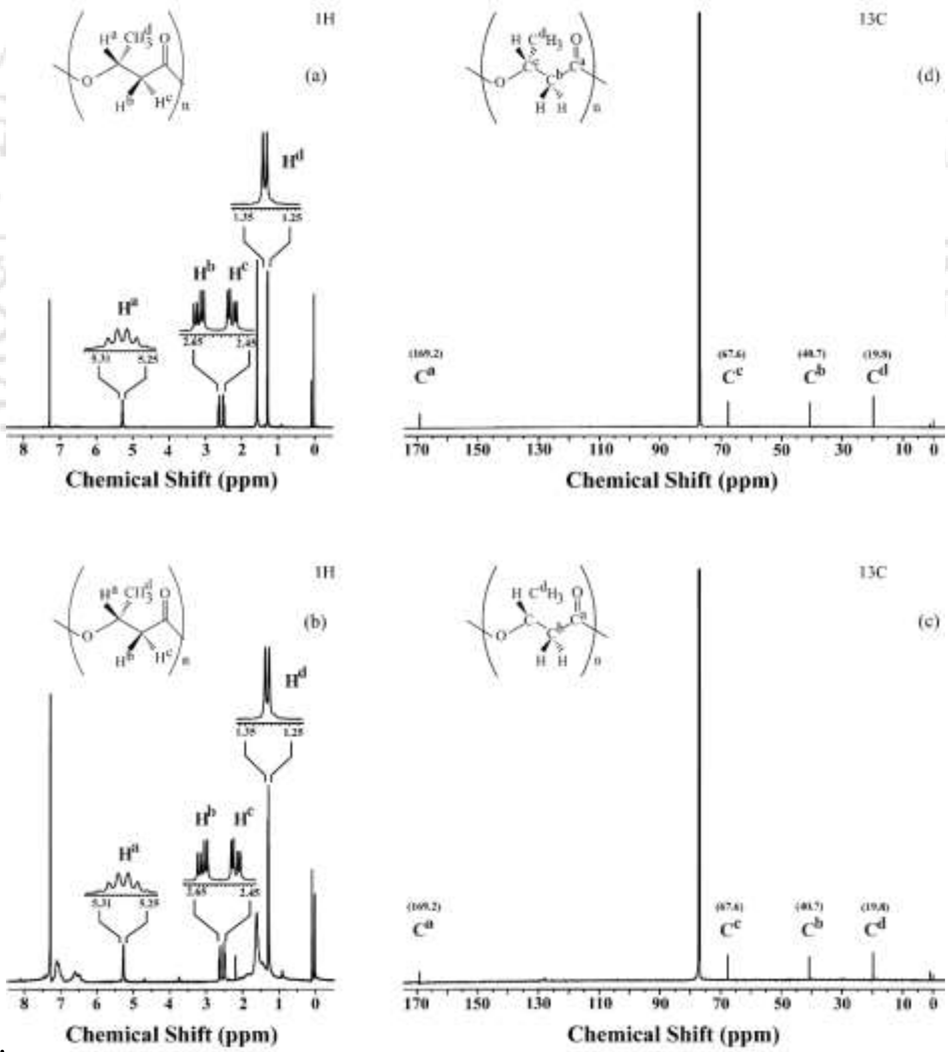


Fig 6.7: 1H and 13C NMR spectrum of the extract from FBR1(a,d) and FBR2 (b,c) after fed-batch accumulation using fRME as substrate

Molecular structure determination by PXRD: The Powder XRD employed assists in analyzing the molecular structure of produced PHB. X-ray diffraction pattern from the atoms arranged in different lattice planes determines the positions or arrangements that identify the material from the signature pattern and its crystalline or amorphous nature. The peaks from the crystallogram at 2Θ values are 13.5°, 17.0°, 22.1°, 25.6°, 27.2°, 30.3° and 44.3° with corresponding orthorhombic crystal planes (020), (110), (021), (111), (121), (040), (200) and (222) (Fig. 6.8a,b). These values are similar to PHB, as evident from the available literature.

The crystallinity indices (CI) determined from the crystallogram are 53.4 % and 44.5 % for FBR1 and FBR2 extracts, respectively. The CI of FBR2 is slightly lower than the FBR1. Some impurities remaining in the extracted PHB from BR2 might have hindered the crystal growth. A similar effect can also be observed from thermal analysis as well (Fig. 6.8c,d). A higher temperature of PHB extraction (100 °C) and a longer period of drying or storage at 50 °C could have favored the development of such a crystalline nature. These exposures might have imparted higher thermal energy that helped in the formation and growth of crystal structure during extraction. Also, complete solvent removal might have prevented the mobility of polymer chains that improved crystal growth. This indicates that more thermal energy leads to ordered structure (Anbukarasu et al., 2015). Similar outcomes were observed for PHB produced from vegetable waste fermentate (Naresh Kumar et al., 2021). Nonetheless, the semi-crystalline nature projects the possible application in areas requiring mechanical strength.

Analysis of thermal properties TGA: Thermal analysis of a polymer indicates its capacity to be cast and employed into the desired application without any deterioration. The PHB extracted from FBR1 and FBR2 had two well-defined stages in its degradation as indicated by the derivative thermogravimetric (DTG) curve (Fig. 6.8c,d). The major phase change for PHB from FBR1 occurs at 274 °C with 65 % (peak point) weight loss, i.e., extensive thermal conversion of polymer into gaseous or other by-products. This phase change terminates at a weight loss of 84 % onset from 231°C to 284 °C (TG curve). The residues of the first stage are then further transformed at 405 °C with a weight loss of 27 % at the peak point. This stage ends with a weight loss of about 8 % by 433 °C (Fig 6.8c).

Similarly, the peak inflection point for the transformation of PHB-FBR2 occurred at 285 °C with a weight loss of 29 % at the peak point (Fig 6.8d). Meanwhile, the degradation of 43 % was attained with a rise in temperature from 240-308 °C. The thermal residue left got transformed at 416 °C with a weight loss of 28 % at peak point. Also, the terminal weight loss attained in the second stage was 46 %, with a temperature rise from 308-440 °C. The absence of a third DTG peak indicates no further change inducing any severe weight loss. At the second stage, the overall weight loss was 92 % and 89 % for PHB extracted from FBR1 and FBR2, respectively. The 6-7 % mass left gets slowly removed to total loss by 800 °C. A similar thermal degradation profile of PHB was observed in other studies using pure or mix culture using VFA as substrate (Pagliano et al., 2020) (Naresh Kumar et al., 2021) (Singh et al., 2021).

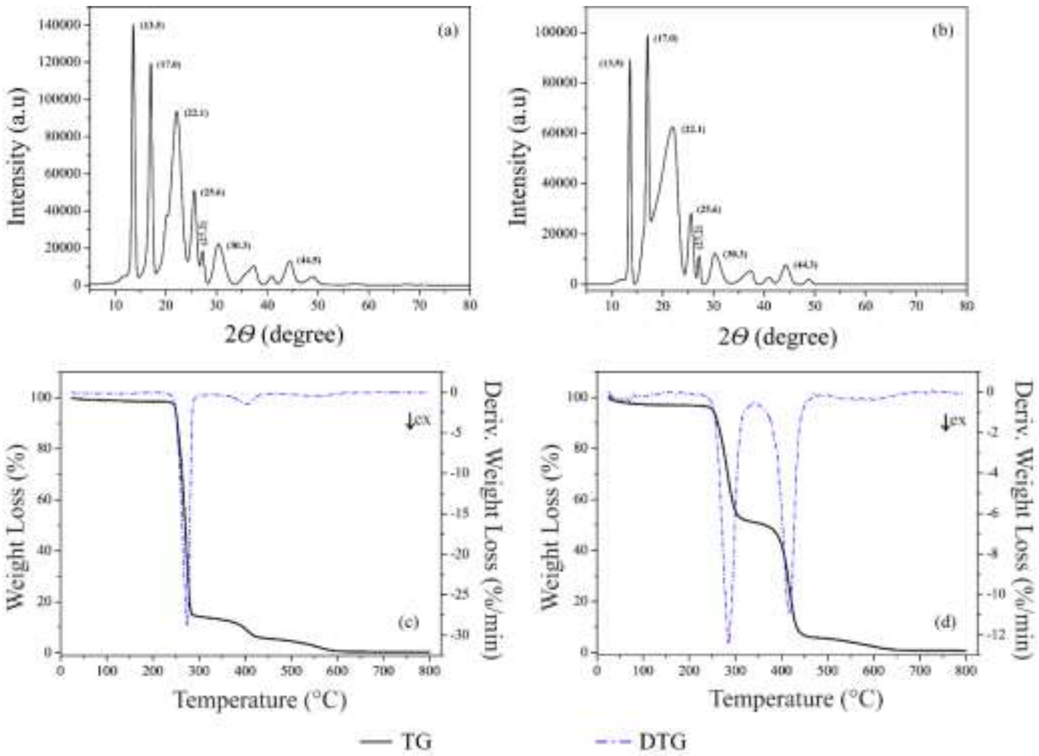


Fig 6.8: PXRD and Thermal analysis of the extract from FBR1(a &c) and FBR2 (b&d) using fRME as substrate.

Many attributes could be pointed out to assess the particular patterns observed in the present thermal analysis. Random chain scission occurs during thermal degradation of PHB. The initial degradation step might involve carboxylic acids and ester moieties with the further deconstruction of by-products into propylene, aldehydes, etc., in the subsequent step (Pagliano et al., 2020). In addition, an earlier study observed that residues left behind after precipitation with ice-cold methanol can influence the thermal degradation pattern, initiate degradation at a lower temperature, lower melt temperature, but improve melt viscosity and shear thinning. Besides, the feeding pattern might also have influenced the degradation pattern as the block copolymer structure of PHA was reported from pulse-wise feeding (Hilliou et al., 2016).

6.3. Conclusion

The present study explores the approaches for enhancing PHB synthesis from rice mill effluent and brewery sludge for a cost-effective production strategy. Acidogenic fermentation converted a significant portion of the influent organic content into VFA, especially even-numbered VFA, through anaerobic inoculum pretreatment compared to raw RME. The pH uncontrolled condition

Table 6.3: A comparative outlook of PHB production performance from acidified waste feedstocks using feast-famine enriched activated sludge in the present study with available literature.

Substrate	Regime	Organic content (g/L,COD or C)	Temperature (°C) /pH	PHB storage# (%)	PHB yield (g/g, COD or C)#	Reference
Molasses +cheese whey	Fed batch	37/43/50 [b]	23-25 /n.c	30.8	0.49	(Duque et al., 2014)
Bio-oil	Fed batch	30 [b]	23-25/n.c	13	0.41[a]	(Fidalgo et al., 2014)
Olive mill effluent	Semi-Continuous	11	27/n.c	24.1	n.a	(Ntaikou et al., 2014)
Food waste	Batch	11	n.a/n.c	14.6	0.1	(Amulya et al., 2015)
Brewery wastewater	Fed batch	2.1 (total)	30/7.0	26.5	0.45[a]	(Tamang et al., 2019)
Primary + Excess sludge (thermal hydrolysed)	Semi-Continuous	5.0	35/n.a	27.1	n.a	(Zhang et al., 2019)
Organic fraction of municipal waste	Fed batch	1.5	n.a	15.2	0.53	(Papa et al., 2020)
Primary sewage+ food waste (70:30)	Fed batch	2 (gVFA/L)	n.c	30.5	n.a	(Perez-Zabaleta et al., 2021)
Mussels cooking wastewater	Fed batch	70[b]	30/n.c	28.6[c]	n.a	(Roibás-rozas et al., 2021)*
Vegetable waste	Sequential batch	13.4	35/n.c	28	n.a	(Naresh Kumar et al., 2021)
Cheese Whey	Fed batch	64[b]	30/n.c	32.3[a]	0.32	(Portela-Grandío et al., 2021)*
Rice mill effluent	**Fed batch**	**3.2**	**n.c**	**29.5**	**0.32**	**This study**

a: C-mmol/C- mmol, b: C-mmol/Lor C-mmol/Ld, *: one among the outcomes of PHA accumulation study, #: mostly approximated from data given in the respective research articles, n.c: not controlled, n.a: not available, nitrogen and phosphorus source are not added creating limited or starved condition, in fed-batch accumulation the organic content given is for each pulse

led to higher butyrate yield. Hence, fRME had higher PHB production than RME. Fed-batch accumulation with pulse-wise feed addition gave superior results than other assays due to effective carbon excess-nitrogen limited condition. Meanwhile, Brewery sludge enriched under SRT of 10 d accumulated a maximum PHB content of sodium acetate and fRME as substrate. The MMCs enriched under 10 d SRT had better exposure to selective pressure, leading to stronger internal growth limitations and better physical property. The smaller sludge wastage retained most of the initial PHB accumulators and improved storage response. Meanwhile, larger sludge wastage for 5 d SRT led to washout condition due to disparity in required and available growth rate that reduced storage response. This led to a weaker internal growth limitation for SRT of 5 d as they have limited exposure to feast/famine selective pressure. An extended enrichment period could increase PHB production of sludge selected at SRT of 5 d. Moreover, the settling capacity of sludge is also affected by the short SRT. Therefore, a combination of fermented RME and sludge enriched at SRT of 10 d can result in higher PHB production under the short-term enrichment. These outcomes point towards the feasibility of the PHB production strategy to be integrated into the existing waste treatment system.

References

Ahmadi, F., Zinatizadeh, A.A., Asadi, A., 2020. The effect of different operational strategies on polyhydroxyalkanoates (PHAs) production at short-term biomass enrichment. J. Environ. Chem. Eng. 8, 103531.

Al Battashi, H., Al-Kindi, S., Gupta, V.K., Sivakumar, N., 2021. Polyhydroxyalkanoate (PHA) Production Using Volatile Fatty Acids Derived from the Anaerobic Digestion of Waste Paper. J. Polym. Environ. 29, 250–259.

Amulya, K., Jukuri, S., Venkata Mohan, S., 2015. Sustainable multistage process for enhanced productivity of bioplastics from waste remediation through aerobic dynamic feeding strategy: Process integration for up-scaling. Bioresour. Technol. 188, 231–239.

Anbukarasu, P., Sauvageau, D., Elias, A., 2015. Tuning the properties of polyhydroxybutyrate films using acetic acid via solvent casting. Nat. Publ. Gr. 5, 17884

Arcos-Hernández, M. V, Laycock, B., Donose, B.C., Pratt, S., Halley, P., Al-luaibi, S., Werker, A., Lant, P.A., 2013. Physicochemical and mechanical properties of mixed culture polyhydroxyalkanoate (PHBV). Eur. Polym. J. 49, 904–913.

Argiz, L., Fra-Vázquez, A., del Río, Á.V., Mosquera-Corral, A., 2020. Optimization of an enriched mixed culture to increase PHA accumulation using industrial saline complex wastewater as a substrate. Chemosphere 247, 125873.

Assam Govt./Environment and Forest, 2019. Category of Industries [WWW Document]. URL https://environmentandforest.assam.gov.in/information-services/category-of-industries-0 (accessed 8.24.20).

Ben, M., Kennes, C., Veiga, M.C., 2016. Optimization of polyhydroxyalkanoate storage using mixed cultures and brewery wastewater. J. Chem. Technol. Biotechnol. 91, 2817–2826.

Bhushan, S., Rana, M.S., Mamta, Nandan, N., Prajapati, S.K., 2019. Energy harnessing from banana plant wastes: A review. Bioresour. Technol. Reports 7, 100212.

Blasco, L., Kahala, M., Tampio, E., Vainio, M., Ervasti, S., Rasi, S., 2020. Effect of inoculum pretreatment on the composition of microbial communities in anaerobic digesters producing volatile fatty acids. Microorganisms 8, 581.

Chen, Z., Huang, L., Wen, Q., Zhang, H., Guo, Z., 2017. Effects of sludge retention time, carbon and initial biomass concentrations on selection process : From activated sludge to polyhydroxyalkanoate accumulating cultures. J. Environ. Sci. 2, 76–84.

Coats, E.R., Watson, B.S., Brinkman, C.K., 2016. Polyhydroxyalkanoate synthesis by mixed microbial consortia cultured on fermented dairy manure : Effect of aeration on process rates / yields and the associated microbial ecology. Water Res. 106, 26–40.

Colombo, B., Calvo, M.V., Sciarria, T.P., Scaglia, B., Kizito, S.S., D'Imporzano, G., Adani, F., 2019. Biohydrogen and polyhydroxyalkanoates (PHA) as products of a two-steps bioprocess from deproteinized dairy wastes. Waste Manag. 95, 22–31.

Duque, A.F., Oliveira, C.S.S., Carmo, I.T.D., Gouveia, A.R., Pardelha, F., Ramos, A.M., Reis, M.A.M., 2014. Response of a three-stage process for PHA production by mixed microbial cultures to feedstock shift : impact on polymer composition. N. Biotechnol. 31, 5–11.

Farghaly, A., Enitan, A.M., Kumari, S., Bux, F., Tawfik, A., 2017. Polyhydroxyalkanoates production from fermented paperboard mill wastewater using acetate-enriched bacteria. Clean Technol. Environ. Policy 19, 935–947.

Fidalgo, R.M., Ortigueira, J., Freches, A., Pelica, J., Gonçalves, M., Mendes, B., Lemos, P.C.,

2014. Bio-oil upgrading strategies to improve PHA production from selected aerobic mixed cultures. N. Biotechnol. 31, 297–307.

Hilliou, L., Machado, D., Oliveira, C.S., Gouveia, A.R., Reis, M.A., Campanari, S., Villano, M., Majone, M., 2016. Impact of fermentation residues on the thermal, structural, and rheological properties of polyhydroxy(butyrate-co-valerate) produced from cheese whey and olive oil mill wastewater. J. Appl. Polym. Sci. 133, 42818.

Inoue, D., Fukuyama, A., Ren, Y., Ike, M., 2021. Optimization of aerobic dynamic discharge process for very rapid enrichment of polyhydroxyalkanoates-accumulating bacteria from activated sludge. Bioresour. Technol. 336, 125314.

Johnson, K., Kleerebezem, R., van Loosdrecht, M.C.M., 2010. Influence of the C/N ratio on the performance of polyhydroxybutyrate (PHB) producing sequencing batch reactors at short SRTs. Water Res. 44, 2141–2152.

Lee, W.S., Chua, A.S.M., Yeoh, H.K., Nittami, T., Ngoh, G.C., 2015. Strategy for the biotransformation of fermented palm oil mill effluent into biodegradable polyhydroxyalkanoates by activated sludge. Chem. Eng. J. 269, 288–297.

Liao, Q., Guo, L., Ran, Y., Gao, M., She, Z., Zhao, Y., Liu, Y., 2018. Optimization of polyhydroxyalkanoates (PHA) synthesis with heat pretreated waste sludge. Waste Manag. 82, 15–25.

Liu, Yuanjun, Guo, L., Liao, Q., Ran, Y., Hu, F., Gao, M., She, Z., Zhao, Y., Jin, C., Liu, Yiping, Wang, G., 2020. Polyhydroxyalkanoate (PHA) production with acid or alkali pretreated sludge acidogenic liquid as carbon source: Substrate metabolism and monomer composition. Process Saf. Environ. Prot. 142, 156–164.

Marang, L., van Loosdrecht, M.C.M., Kleerebezem, R., 2018. Enrichment of PHA-producing bacteria under continuous substrate supply. N. Biotechnol. 41, 55–61.

Marang, L., van Loosdrecht, M.C.M., Kleerebezem, R., 2016. Combining the enrichment and accumulation step in non-axenic PHA production : Cultivation of Plasticicumulans acidivorans at high volume exchange ratios. J. Biotechnol. 231, 260–267.

Mokhtarani, N., Ganjidoust, H., Farahani, E.V., 2012. Effect of process variables on the production of Polyhydroxyalkanoates by activated sludge. J. Environ. Heal. Sci. Eng. 9, 1–7.

Moretto, G., Lorini, L., Pavan, P., Crognale, S., Tonanzi, B., Rossetti, S., Majone, M., Valentino, F., 2020. Biopolymers from urban organic waste: Influence of the solid retention time to cycle length ratio in the enrichment of a Mixed Microbial Culture (MMC). ACS Sustain. Chem. Eng. 8, 14531−14539.

Naresh Kumar, A., Katakojwala, R., Amulya, K., Mohan, S.V., 2021. Polyhydroxybutyrate production from dark-fermentative effluent and composite grafting with bagasse derived α-cellulose in a biorefinery approach. Chemosphere 279, 130563.

Nguyenhuynh, T., Yoon, L.W., Chow, Y.H., Chua, A.S.M., 2021. An insight into enrichment strategies for mixed culture in polyhydroxyalkanoate production: feedstocks, operating conditions and inherent challenges. Chem. Eng. J. 420, 130488.

Ntaikou, I., Peroni, C.V., Kourmentza, C., Ilieva, V.I., Morelli, A., Chiellini, E., Lyberatos, G., 2014. Microbial bio-based plastics from olive-mill wastewater : Generation and properties of polyhydroxyalkanoates from mixed cultures in a two-stage pilot scale system. J. Biotechnol. 188, 138–147.

Oliveira, C.S.S., Silva, C.E., Carvalho, G., Reis, M.A.M., 2017. Strategies for efficiently selecting PHA producing mixed microbial cultures using complex feedstocks : Feast and famine regime and uncoupled carbon and nitrogen availabilities. N. Biotechnol. 37, 69–79.

Pachapur, V.L., Kutty, P., Pachapur, P., Brar, S.K., Le Bihan, Y., Galvez-Cloutier, R., Buelna, G., 2019. Seed pretreatment for increased hydrogen production using mixed-culture systems with advantages over pure-culture systems. Energies 12, 1–26.

Pagliano, G., Gugliucci, W., Torrieri, E., Piccolo, A., Cangemi, S., Angelo, F., Giuseppe, D., Robertiello, A., Faraco, V., Pepe, O., Ventorino, V., 2020. Polyhydroxyalkanoates (PHAs) from dairy wastewater effluent : bacterial accumulation , structural characterization and physical properties. Chem. Biol. Technol. Agric. 7, 29.

Papa, G., Pepe, T.S., Carrara, A., Scaglia, B., Imporzano, G.D., Adani, F., 2020. Implementing polyhydroxyalkanoates production to anaerobic digestion of organic fraction of municipal solid waste to diversify products and increase total energy recovery. Bioresour. Technol. 318, 124270.

Perez-Zabaleta, M., Atasoy, M., Khatami, K., Eriksson, E., Cetecioglu, Z., 2021. Bio-based conversion of volatile fatty acids from waste streams to polyhydroxyalkanoates using mixed microbial cultures. Bioresour. Technol. 323, 124604.

Portela-Grandío, A., Lagoa-Costa, B., Kennes, C., Veiga, M.C., 2021. Polyhydroxyalkanoates production from syngas fermentation effluents: Effect of nitrogen availability. J. Environ. Chem. Eng. 9, 106662.

Ramos-Suarez, M., Zhang, Y., Outram, V., 2021. Current perspectives on acidogenic fermentation to produce volatile fatty acids from waste, Reviews in Environmental Science and Biotechnology. Springer Netherlands.

Roibás-rozas, A., Val, Á., Hospido, A., Mosquera-corral, A., 2021. Strategies for the valorisation of a protein-rich saline waste stream into polyhydroxyalkanoates (PHA). Bioresour. Technol. 124964.

Sarkar, O., Rova, U., Christakopoulos, P., Matsakas, L., 2021. Influence of initial uncontrolled pH on acidogenic fermentation of brewery spent grains to biohydrogen and volatile fatty acids production: Optimization and scale-up. Bioresour. Technol. 319, 124233.

Singh, S., Sithole, B., Lekha, P., Permaul, K., Govinden, R., 2021. Optimization of cultivation medium and cyclic fed - batch fermentation strategy for enhanced polyhydroxyalkanoate production by Bacillus thuringiensis using a glucose - rich hydrolyzate. Bioresour. Bioprocess. 8, 11.

Tamang, P., Banerjee, R., Köster, S., Nogueira, R., 2019. Comparative study of polyhydroxyalkanoates production from acidified and anaerobically treated brewery wastewater using enriched mixed microbial culture. J. Environ. Sci. 78, 137–146.

Tamang, P., Arndt, C., Bruns-Hellberg, J., Nogueira, R., 2021. Polyhydroxyalkanoates production from industrial wastewaters using a mixed culture enriched with Thauera sp.: Inhibitory effect of the wastewater matrix. Environ. Technol. Innov. 21, 101328.

Tan, G.-Y., Chen, C.-L., Li, L., Ge, L., Wang, L., Razaad, I., Li, Y., Zhao, L., Mo, Y., Wang, J.-Y., 2014. Start a Research on Biopolymer Polyhydroxyalkanoate (PHA): A Review. Polymers (Basel). 6, 706–754.

Tu, W., Zhang, D., Wang, H., 2019. Polyhydroxyalkanoates (PHA) production from fermented thermal-hydrolyzed sludge by mixed microbial cultures: The link between phosphorus and PHA yields. Waste Manag. 96, 149–157.

Valentino, F., Karabegovic, L., Majone, M., Morgan-Sagastume, F., Werker, A., 2015. Polyhydroxyalkanoate (PHA) storage within a mixed-culture biomass with simultaneous growth as a function of accumulation substrate nitrogen and phosphorus levels. Water Res. 77, 49–63.

Vallari, D.S., Jackowski, S., Rock, C.O., 1987. Regulation of pantothenate kinase by coenzyme A and its thioesters. J. Biol. Chem. 262, 2468–2471.

Yun, J., Cho, K.S., 2016. Effects of organic loading rate on hydrogen and volatile fatty acid production and microbial community during acidogenic hydrogenesis in a continuous stirred tank reactor using molasses wastewater. J. Appl. Microbiol. 121, 1627–1636.

Zhang, D., Jiang, H., Chang, J., Sun, J., Tu, W., Wang, H., 2019. Effect of thermal hydrolysis pretreatment on volatile fatty acids production in sludge acidification and subsequent polyhydroxyalkanoates production. Bioresour. Technol. 279, 92–100.

Zheng, Y., Guo, L., Liu, Y., She, Z., Gao, M., Jin, C., Zhao, Y., 2021. Effects of chemical oxygen demand concentration, pH and operation cycle on polyhydroxyalkanoates synthesis with waste sludge. Environ. Technol. 42, 1922–1929.

Annexure 3

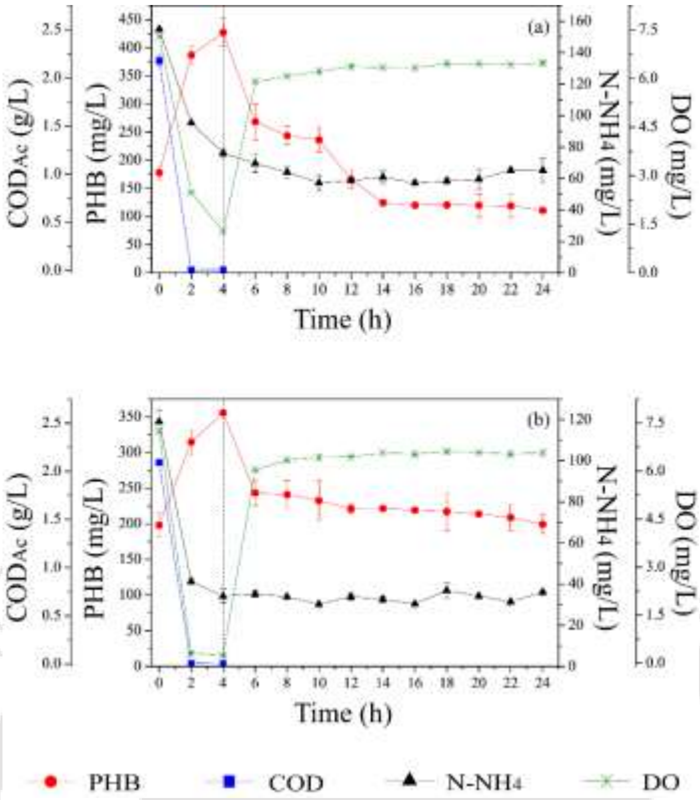


Figure A3.1: The profile of various parameters in a single cycle of (a) SBR1, and (b) SBR2.

Table A3.1: The stoichiometric and kinetic parameters in a single cycle of sequential enrichment of BS.

Parameters	SBR1	SBR2
Feast/Famine ratio (f/f)	0.2	0.2
ΔPHB (%)	2.05±0.01	2.03±0.005
$Y_{P/S}$ (mgCOD/mgCOD)	0.18±0.09	0.13±0.006
q_p ($mgCOD_{PHA}$/mgCODx h)	0.007±0.03	0.006±0.06
$-q_s$ ($mgCOD_S$/mgCODx h)	0.21±0.03	0.1±0.09
$Y_{X/S}$ (mgCOD/mgCOD)	0.78±0.04	0.65±0.02
r_x (mgCODx/h)	630.5±0.02	352.1±0.02
$-q_n$ ($mgCOD_N$/mgCODx h)	0.016±0.006	0.006±0.02
$-q_p$ ($mgCOD_{PHA}$/mgCODx h)	0.004±0.09	0.0004±0.1

ΔPHB= PHB storage (final)-PHB storage (initial), r_x= Rate of biomass production, $-q_n$- Ammoniacal-nitorgen consumption rate

Conclusion and future prospects

Waste biorefinery approaches are widely accepted as a feasible strategy to deal with the ever-increasing waste management issue. Value addition with concomitant waste treatment has the potential to manage the economic aspect of both processes. Microbial production of polyhydroxybutyrate (PHB) production is one such waste biorefinery approach widely explored due to its industrial valuation as a substitute to conventional plastics. Furthermore, activated sludge as a source of inoculum further opened up the possibility of PHB production using waste feedstocks as a renewable carbon source, thereby balancing polymer cost by reducing the operational cost and waste treatment. The present study attempts to extend the opportunity of the process by incorporating novel bioprocess strategies to enhance PHB production through a three step process.

Firstly, acidogenic fermentation of waste feedstocks, rice mill effluent (RME) and brewery effluent (BE) was performed using gobar gas plant sludge as inoculum to improve the specificity by increasing volatile fatty acids (VFAs) in waste feedstocks. The heat-acid shock pretreatment of anaerobic inoculum sludge enhanced the total VFA (TVFA) content and refined the VFA profile of both effluents than without pretreatment. Meanwhile, the fermentate had acetate, and butyrate has the dominant VFA. The pretreated system had minimal consumption and maximum retention of organic content as acids than the untreated system, indicated by stable organic content, pH, and VFA profile. Besides, the acidogenesis of RME with pretreated sludge had higher TVFA content and a better VFA profile compared to BE.

However, brewery anaerobic sludge as inoculum led to a lowered VFA production after pretreatment. The pretreatment regime employed might have attenuated the acidogenic microbial community that could not raise the TVFA content of the pretreated system above the untreated system. Even then, the pretreated system possessed reduced consumption of organic content with stable pH and VFA profile. Interestingly, pretreatment enhanced the even to odd ratio than without pretreatment. The pretreated system with RME had a nearly 20-fold increase, and BE had a two-fold increase in the even to odd ratio compared to the untreated system. These characteristics of the fermentation system cater to higher PHB production. Hence, acidification of RME with pretreated sludge had better acidification performance than BE concerning the potency for inducing PHB production suing both sludges. This is due to simpler and readily biodegradable components available like carbohydrates in RME than BE. Thus, pretreatment effectively removed hydrogenoclastic/acetoclastic methanogens from both the inoculum sludge used that aided in improving the acidogenic process. Also, RME can be considered as a better candidate for improved VFA production and hence an ideal media for PHB production.

The PHB accumulation potential activated sludge from different origins, petroleum refinery sludge (PRS) and brewery sludge (BS) was improved by enriching using aerobic dynamic feeding strategy. The sequential feast/famine enrichment of PRS and BS under nitrogen excess and limited conditions had similar storage response after an extended period. Proper growth ensures a shorter feast phase and stronger ecological selective pressure in nitrogen excess conditions. However, growth imbalance triggered metabolic stress that leads to higher storage conversion during nitrogen limited condition. A long term enrichment under limited condition can cause filamentous growth leading to poor mass transfer in the system that could nullify the storage response. Meanwhile, nitrogen limited condition enhanced PHB storage and yield for PRS and BS during batch accumulation compared to nitrogen excess or starved condition. A growth imbalance rather than starvation diverts carbon better towards storage. In addition, sludge enriched under nitrogen excess conditions had higher PHB storage and yield than sludge enriched under nitrogen limited. The nitrogen available in excess promoted appropriate growth maintaining well-functioning cell machinery that could maximally utilize the limited condition to increase the storage response. On the other hand, severe metabolic stress due to improper growth drained microbial cell essential functioning molecules like proteins, enzymes, etc., that weaken their ability to withstand further stress during batch accumulation. In addition, inoculum origin had no significant influence over PHB production as PRS and BS had similar storage capacity. Prolonged exposure to feast/famine conditions ensures required internal growth limitation that might have converged different microbial communities into similar PHB accumulating phenotypes. Also, the polymer produced was a homopolymer of PHB that had property similar to commercial grade, pointing towards its broad spectrum of applicability. Therefore, feast/famine enrichment ensures a system devoid of influence from inoculum origin under nitrogen excess condition. Brewery sludge had similar PHB accumulation potential with reduced impact from sludge retention time (SRT) control. Besides, the easy availability of the sludge makes it an ideal candidate for further PHB production studies.

Acidogenic fermentation of RME with pretreated gobar gas plant sludge improved TVFA content and degree of acidification of the acetate-butyrate-dominated fermentate. The pH uncontrolled condition led to higher butyrate yield than acetate yield. Meanwhile, an SRT of 10 d improved the accumulation potential of BS compared to 5 d SRT with proper physical properties for short term enrichment. The lower sludge wastage at 10 d SRT provides better exposure to the selective pressure compared to higher sludge wastage at 5 d SRT. PHB accumulation under a fed-batch condition with pulse wise feeding had higher PHB storage than batch and fed-batch with continuous feeding under nitrogen limitation. Similarly, the sludge enriched under SRT of 10 d had the highest PHB storage and yield than sludge enriched under 5 d using sodium acetate and RME as substrate. The PHB storage nearly doubled with a six-fold increase in production yield with fermented RME had substrate than raw RME. Therefore, brewery sludge feast/famine enriched under nitrogen excess condition and an SRT of 10 d can produce high PHB content using fermented RME. The process outcomes show the feasibility of a PHB production system integrated into an existing effluent treatment facility.

Further studies could improve the prospect of the process's applicability. A comprehensive study is necessary with a continuous acidogenic fermentation system producing acidified RME continuously. The system's performance in terms of variation in grains for generating RME, hydraulic and sludge retention time, degree of acidification, VFA profile needs to be analyzed. At the same time, other suitable and untapped industrial effluents or solid waste should also be explored to widen the scope of the process. In aerobic dynamic feeding strategy, the process intensification could be investigated using additional restrictions like uncoupling carbon/nitrogen addition, settling phase after feast phase, higher volume exchange ratio coupled with shorted settling phase at the end of the cycle, etc. Also, sludge from different origins exposed to various conditions like treating specific effluents, environmental fluctuation, the ecological niche could hold unexpected outcomes. Lastly, running the whole process under actual environmental conditions integrated into the effluent treatment facility or with industry along with an techno-economic analysis to understand the process outcomes, capital outlay, and market distribution could make the process acceptable to stakeholders. Thereby, the waste biorefinery approach through PHB production could be finally made a reality.

Curriculum vitae

Name: Jayakrishnan U

Address: Krishna Kripa, Thiruvankulam,

Ernakulam, Kerala-682305

Contact: +91 9007475569/8638521926

Email: jayakrishnanu91@gmail.com

Academic history

- 2015-2022 Doctor of philosophy from Indian Institute of Technology Guwahati (Thesis to be submitted)

  Thesis title-“**Novel bioprocess strategy for poyhydroxybutyrate production from agro-industrial effluents: A waste biorefinery approach**.”

- 2013-2015 Master of Technology in biotechnology from the Maulana Abul Kalam Azad University of Technology, Kolkata, West Bengal. (CGPA-8.45)

- 2009-2013 Bachelor of Technology in biotechnology and biochemical engineering from Mohandas College of Engineering and Technology, under Kerala Technical University (earlier affiliated to Kerala University), Thiruvanthapuram, Kerala. (CGPA-7.2)

- 2006-2009 Higher Secondary (10 +2), Biology-Maths, Kendriya Vidyalaya No. 1 (CBSE), Kochi, Kerala (80 %)

Work Experience

- Handled an anaerobic-acidogenic fermenter to derive volatile fatty acids from wastewater.
- Operated sequencing batch-fed batch reactor combination for the production of polyhydroxybutyrate from volatile fatty acid.
- Worked with different bacterial strains for removing nitrate from different waste streams (biological nutrient removal).

Skill Summary

- Design and fabrication of appropriate reactor systems for treatment and valorization.

- Operating and troubleshooting for high-end instruments high-pressure liquid chromatography, Gas chromatography, flame photometer, Kjeldahl nitrogen.
- Conceptualization, investigation, validation, and data curation of potential environmental issues, the research gap, formal analysis, and writing scientific articles.
- Coordination and active participation in the team for effective achievement of professional or research objectives involved.

Research Interest

- An earnest desire to work towards preventing and mitigating environmental pollution and issues.
- Exploring new opportunities to derive value-added products from broad-spectrum of waste and aim for market induction.
- Delve into the biological and physiochemical process for waste treatment and valorization.
- Work with a pilot-scale operation for treatment and valorization of waste.
- Improving the availability and implementation of existing as well as newer technologies for dealing with the present waste management problems.

Publications

- U. **Jayakrishnan**, D. Deka, G. Das, 2019. Enhancing the volatile fatty acid production from agro-industrial waste streams through sludge pretreatment, Environ. Sci. Water Res. Technol. 5, 334–345.
- U. **Jayakrishnan**, D. Deka, G. Das, 2020. Influence of inoculum variation and nutrient availability on polyhydroxybutyrate production from activated sludge, Int. J. Biol. Macromol. 163, 2032–2047.
- U. **Jayakrishnan**, D. Deka, G. Das, 2020. Regulation of volatile fatty acid accumulation from waste: Effect of inoculum pretreatment, Water Environ. Res. 93, 1019–1031.
- U. **Jayakrishnan,** D. Deka, G. Das, 2021. Waste as feedstock for polyhydroxyalkanoate production from activated sludge: Implication of aerobic dynamic feeding strategy and acidogenic fermentation, J. Environ. Chem. Engg. 5, 105550.
- Polyhydroxybutyrate production from rice mill effluent: Effect of sludge retention time on feast/famine enrichment. (Manuscript under preparation).

Conferences

- Presented poster at International Conference on Waste Management (**Recycle 2016**), Indian Institute of Technology Guwahati, April 1-2, 2016
- Presented poster at **Research Conclave**, Indian Institute of Technology Guwahati, March 2017.
- Presented poster at Recent Advancements in Environmental Research (**RAER 2017**), Indian Institute of Technology Guwahati, June 5, 2017
- Poster presented at Bioprocessing India **(BPI 2017)**, Indian Institute of Technology, Guwahati, December 2017
- Presented poster at **Recycle**, Indian Institute of Technology Guwahati, February 2018.
- Presented Poser at **BioSd**, International conference on biotechnological research and innovation for sustainable development, CSIR-Indian Institute of Chemical Technology, Hyderabad, November 22-25, 2018.
- National Conference on Issues and Challenges in Water Treatment and Allied research for Sustainable Environment (**WATER2020**), Indian Institute of Technology Guwahati, 23-25 January 2020
- Flash talk and poster presentation at International Conference on Biotechnology for Resource Efficiency, Energy, Environment, Chemicals and Health (Hybrid) (**BRE3CH 2021**), Indian Institute of Petroleum, Dehradun & Biotech research society of India. December 1-4.