UNIVERSITY OF BOLOGNA

BIOMATERIALS FROM RENEWABLE SOURCES: BIOSURFACTANTS AND BIOPOLYMERS

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Declaration of Authorship and copyright

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ABSTRACT

Surfactants are a wide class of compounds used in a broad spectrum of household and industrial applications. However, two of their main concerns lie in their non-biodegradability and the fact that they are low-dose permanent toxicants in the water ecosystem, arising from the widespread usage. An aspect of this thesis involves the synthesis and biodegradability assessment of a new family of biobased surfactants that could be potential green alternatives to traditional anionic and non-ionic surfactants (e.g. alkylphenol ethoxylate, APE). They were prepared from itaconic acid and fatty amines (molecules industrially obtained from renewable resources) under mild conditions (solventless) and in excellent yields. Biodegradability tests confirmed that many of them are biodegradable.

The second part of this thesis is focused on the production of polyhydroxyalkanoates (PHAs) from organic renewable resources: paper wastes and microalgae. PHAs are biodegradable, immunological inert and very versatile bioplastics with properties similar to worldwide used petro-based plastics. They have broad applications in many industrial fields: pharma and biomedical, food and beverage packaging, fibres, electronics and automobiles. Recent research on PHAs has focused on developing cost-effective production using low-value or industrial waste as carbon source for PHA-producing bacteria. In this study, pyrolysis oil from paper waste (bio-oil) and aqueous phase obtained from hydrothermal liquefaction of two microalgae species (*Scenedesmus almeriensis* and *Nannochloropsis gaditana*) were investigated as substrates for microbial conversions with the aim of PHA production. Both substrates were first upgraded via acidogenic fermentation to obtain liquid streams enriched with volatile fatty acids (VFAs, which are considered excellent platforms for PHA production) and di/triethylcitrates (DEC and TEC, which are value-added chemicals with wide range of applications, e.g. plasticizers). After this step, acidogenic products were extracted and used for PHA production in a subsequent aerobic fermentation.

KEY WORDS: r*enewable, surfactant, itaconic acid, bio-oil, polyhydroxyalkanoates, volatile fatty acids.*

ABSTRACT

I tensioattivi sono una vasta classe di composti utilizzati in molteplici applicazioni domestiche e industriali. Tuttavia, sono spesso sostanze non biodegradabili e tossiche a bassi dosaggi, causando danni permanenti negli ecosistemi acquatici. La tesi si articola in due parti: una riguarda la sintesi e la valutazione della biodegradabilità di una nuova famiglia di tensioattivi a partire da fonti rinnovabili, potenziali sostituti ecocompatibili dei tensioattivi anionici e non ionici tradizionali (es etossilato alchilfenolo, APE). Questi tensioattivi sono sintetizzati a partire dall'acido itaconico e da ammine a lunga catena (industrialmente ottenute da risorse rinnovabili), usando blande condizioni di reazione (es assenza di solvente) e in ottime rese. Inoltre, le prove di biodegradabilità confermano la biodegradabilità della maggior parte dei composti sintetizzati.

La seconda parte di questa tesi è incentrata sulla produzione di poliidrossialcanoati (PHA) da fonti rinnovabili: scarti di cartiera e microalghe. I PHA sono bioplastiche biodegradabili molto versatili, con proprietà simili a plastiche di amplissimo uso ottenute da fonti petrolifere. Hanno un vasto potenziale di applicazioni in campo farmaceutico, biomedicale e alimentare. I più recenti studi sul PHA sono stati incentrati sull'utilizzo di fonti di carbonio a basso costo come substrato per la crescita di batteri PHA-produttori, nell'ottica di abbassarne i costi di produzione. In questo studio, l'olio di pirolisi di rifiuti cartacei (bio-olio) e la fase acquosa ottenuta mediante liquefazione idrotermale di due specie di microalghe (*Scenedesmus almeriensis* e *Nannochloropsis Gaditana*) sono stati studiati come substrato di conversioni microbiche anaerobiche e aerobiche volte alla produzione di PHA. Entrambi i substrati sono stati sottoposti a fermentazione acidogenica per ottenere fasi arricchite di acidi grassi volatili (VFA), eccellenti substrati per la produzione di PHA; e di- e trietil citrati (DEC e TEC) sostanze chimiche ad alto valore aggiunto, con un'ampia gamma di applicazioni come plastificanti. I prodotti acidogenici stati poi estratti ed utilizzati per la produzione di PHA mediante una successiva fermentazione aerobica.

PAROLE CHIAVE: risorse rinnovabili, tensioattivi, acido itaconico, bio-olio, poliidrossialcanoati, acidi grassi volatili.

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ADSS	-	Aerobic digestion spent solution,
AF	-	Acidogenic fermentation
AP	-	Aqueous phase
BOD	-	Biochemical oxygen demand
BSTFA	-	Bis-trimethylsilyltrifluoroacetamide
COD	-	Chemical oxygen demand
ChIR	-	Erasmus Mundus Master Course in Chemical Innovation and Regulation
DEC	-	Diethylcitrate
DGME	-	Diethylene glycol mono methyl ether
DMC	-	Dimethylcarbonate
FAs	-	Fatty acids
FASs	-	Fatty alcohol sulfates
F & F	-	Feast and famine
FOHs	-	Fatty alcohols
GC-MS	-	Gas Chromatography – Mass Spectrometer
HB	-	Hydroxybutyrate
HRT	-	Hydraulic retention/residence time
HTL	-	Hydrothermal liquefaction
HTL-AP	-	Aqueous phase obtained from hydrothermal liquefaction
IA	-	Itaconic acid
Mcl-PHAs	-	Medium chain length PHAs
MMC	-	Mixed microbial cultures
PCR-DGGE	-	Polymerase chain reaction denaturing gradient gel electrophoresis
PHAs	-	Polyhydroxyalkanoates
PHB	-	Polyhydroxybutanoate

P(3HB)	-	Poly(3-hydroxybutanoate)
P(3HHx)	-	Poly(3-hydroxyhexanoate)
P(3HO)	-	Poly(3-hydroxyoctanoate)
P(3HV)	-	Poly(3-hydroxyvalerate)
P(3HB-co-3HX)	-	Poly (3-hydroxybutyrate-co-3-hydroxyhexanoate)
P(3HB-co-3HV)	-	Poly (3-hydroxybutyrate-co-3-hydroxyvalerate)
P(3HHx-co-3HO)	-	Poly(3-hydroxyhexanoate-co-3-hydroxyoctanoate)
pTsOH	-	p-Toluenesulfonic acid
R1	-	Sequencing batch reactor fed with triethylcitrate substrate
R2	-	Sequencing batch reactor fed with synthetic VFA
R3	-	Sequencing batch reactor fed with ethanol substrate
SBR	-	Sequencing Batch Reactor
TEC	-	Triethylcitrate
ThOD	-	Theoretical oxygen demand
TLC	-	Thin layer chromatography
TMCS	-	Trimethylchlorosilane
VFAs	-	Volatile fatty acids (VFAs)

1. INTRODUCTION

1.1 GREEN CHEMISTRY AND RENEWABLE RESOURCES

Directly or indirectly, many chemicals such as agrochemicals, biocides, petrochemicals, paints and coatings, polymers, surfactants, cleaning and personal care products, to mention a few, are released in the environment. Some are non-(bio)degradable while some are toxic to either/both humans or/and the environment. Moreover, this has resulted in a negative perception of chemistry and chemical activities to a lay person and the mass media. Over the years until the last decade, the images associated to the social perception of chemistry were those of polluted rivers, the smog in the city, the black smoke of a chimney, acid rain, chemical explosion, to mention a few; the word "chemicals" is used everyday as short-hand to refer to harmful or potentially dangerous substances [1]. Beyond doubt, this is one of the problems still faced by Chemists – the social perception and acceptance of Chemistry. However, it is also true that there is a social perception of the need for Chemists to evaluate the health and environmental side effects of chemical activities.

With the dire need to reduce pollution and its effects on both human and the environment, a new and safer concept to obtain chemical products and processes known as "Green" or "Sustainable" Chemistry has been developed. The United States Pollution Prevention Act of 1990 set the platform for Green Chemistry: its focus is the prevention of pollution at the source rather than the treatment of pollutants after they are formed. This goal became one of the objectives of the EPA in 1991. Paul Anastas coined the term "green chemistry" the same year, and with John Warner in 1993 developed the twelve principles of Green Chemistry [2].

The field of Green/Sustainable Chemistry is a fast emerging one that strives to achieve sustainability at the molecular level. It is defined as "the design of chemical products and processes to reduce or eliminate the use and generation of hazardous substances [2,3]. A critical and imperative aspect of Green Chemistry is the concept of 'design' – a purposeful human effort and not something by accident. This concept includes innovation, novelty, planning and systematic conception [3].

The principles of Green Chemistry are based on the framework summarized below [3]:

1. Green chemistry designs across all stages of the chemical life-cycle.
2. Green chemistry seeks to design the structure of the chemical products and processes to reduce their intrinsic hazard.

3. Green chemistry works as a coherent system of principles or design criteria.

The "twelve principles of Green Chemistry" [2] which act as guidelines for the above framework are:

1. **Prevention**: It is better to prevent waste than to treat or clean up waste after it is formed.
2. **Atom Economy**: Synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product.
3. **Less hazardous chemical synthesis**: Whenever practicable, synthetic methodologies should be designed to use and generate substances that pose little or no toxicity to human health and the environment.
4. **Designing safer chemicals**: Chemical products should be designed to preserve efficacy of the function while reducing toxicity.
5. **Safer solvents and auxiliaries**: The use of auxiliary substances (e.g. solvents, separation agents, etc.) should be made unnecessary whenever possible and when used, innocuous.
6. **Design for energy efficiency**: Energy requirements of chemical processes should be recognized for their environmental and economic impacts and should be minimized. If possible, synthetic methods should be conducted at ambient temperature and pressure.
7. **Use of renewable feedstocks**: A raw material or feedstock should be renewable rather than depleting whenever technically and economically practicable.
8. **Reduce Derivatives**: Unnecessary derivatization (use of blocking groups, protection/deprotection, temporary modification of physical/chemical processes) should be minimized or avoided if possible, because such steps require additional reagents and can generate waste.
9. **Catalysis**: Catalytic reagents (as selective as possible) are superior to stoichiometric reagents. Catalysts are effective in small amounts and can carry out a single reaction many times, unlike the use of stoichiometric reagents that are required in excess amount and carry out a reaction once.
10. **Design for degradation**: Chemical products should be designed so that at the end of their function they break down into innocuous degradation products and do not persist in the environment.
11. **Real-time analysis for pollution prevention**: Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances.

12. **Inherently safer chemistry for accident prevention**: Substances and the form of a substance used in a chemical process should be chosen to minimize the potential for chemical accidents, including releases, explosions, and fires.

Poliakoff et al. further came up with a simpler way to commit these principles to memory and a pleasant way to communicate them. The acronym "PRODUCTIVELY' was devised to capture the twelve principles of Green Chemistry [4] in just two to five words, though not in the order above.

P – Prevent wastes
R – Renewable materials
O – Omit derivatization steps
D – Degradable chemical products
U – Use of safe synthetic methods
C – Catalytic reagents
T – Temperature, pressure ambient
I – In-Process monitoring
V – Very few auxiliary substances
E – E-factor, minimize feed in product
L – Low toxicity of chemical products
Y – Yes, it is safe

Consequently, it is interesting how the concept of Green Chemistry is gradually increasing the social acceptance of chemistry, especially in developed countries. In a recent survey by the Royal Society of Chemistry (RSC) on the public attitudes to chemistry in the UK [1], the public perception of chemistry, Chemists and chemicals is actually more positive than what was expected. For instance, 75% disagreed that all chemicals are dangerous and harmful and about 70% agreed that everything including water and oxygen can be toxic at certain dose. In general, the respondents showed their definition of chemicals were multiple and context-dependent, hence the negative stereotyped view of chemicals and chemistry is gradually becoming nuanced and multifaceted to the lay man; thanks to the concept of Green Chemistry which has been an excellent way to communicate chemistry to the public, as a tool for sustainable development.

With respect to Green or Sustainable chemistry, this thesis focuses on principle 7, the use of renewable feedstocks, with good consideration of principles 1, 2, 3, 5, 8, 9, 10 and 12.

From the beginning of modern chemical industry in the last three decades of the XIX century, till date, the bulk of manufacturing products are derived from natural gas and petroleum feedstock which are non-renewable and not equally distributed [2,5]. Moreover, despite the increasing exploitation of alternative fossil sources (e.g. shale gas, deep reservoirs), the availability of fossil feedstocks in the next future will necessarily decrease and their cost will rise [6]. These factors, coupled with their environmental concerns, are key drivers for more sustainable alternatives: the biobased renewable resources.

However, the challenge for a sustainable and biobased economy lies in the production of materials and products based on green and sustainable supply chains [7]. Resources will no longer be sustainable when they are consumed more quickly than produced and waste will lack sustainability when it is produced more quickly than the planet can process it back into useful resources. Hence, the start of any sustainable supply chain requires renewable resources with shortcycle growth/production [7]. In this regard, the most promising source of viable and sustainable alternative to fossil fuel as a source of carbon for both chemical and product needs is biomass. Renewable raw materials are biomass sources from agricultural, forest or marine production and have a relatively short cycle measured in years compared to fossil resources that takes millions of years. ***Fig. 1.1*** connotes how Green Chemistry can be used to close the loop for a biobased economy, using renewable resources as feedstock.

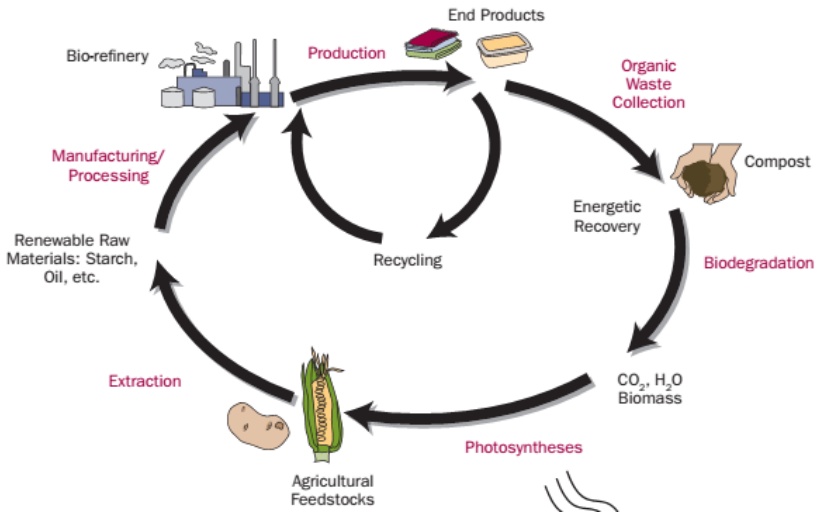


Fig. 1.1: Closing the loop with Green Chemistry [5]
Green Chemistry Principle 7: Use of renewable feedstocks

1.2 THE BIOREFINERY CONCEPT

With the vast majority of worldwide energy carriers and products from fossil fuel refinery, alternative solutions able to mitigate climate change and reduce fossil fuel consumption are being promoted. The replacement of oil with biomass as a raw material for fuel and chemical production is an attractive option and is the driving force for the development of the biorefinery concept [8].

A biorefinery is a facility (or network of facilities) that covers an extensive range of combined technologies aiming to full sustainable transformation of biomass into their building blocks with the concomitant production of fuels, energy and value-added chemicals and materials [8,9]. A biorefinery concept embraces a whole crop approach of biomass conversion pathways leading to a whole portfolio of valuable products, drawing direct similarities to today's petroleum refineries, in which multiple fuels, basic chemicals, intermediate products and sophisticated products are produced from petroleum [8,10].

The long term perspective is the stepwise conversion of large parts of the global economy/industry into a sustainable biobased society (a bioeconomy) having bioenergy, biofuels and biobased products as main pillars and biorefineries as the basis. Of course, this is an enormous challenge as it requires some breakthrough changes in today's production of goods and services: a synergetic effect of biological, chemical, physical and technical sciences.

Biorefinery industries are expected to develop as dispersed industrial complexes able to revitalise rural areas. Unlike petroleum refineries, which almost invariably means very large plants, biorefineries are likely to encompass a whole range of different-sized installations. Consequently, several bio-industries can combine their materials material flows in order to reach a complete utilisation of all biomass components. For instance, the residue from a bio-industry (e.g. lignin form lignocellulosic ethanol production plant) can be used as feedstock for other industries, giving rise to an integrated bio-industrial systems. Furthermore, biomass resources are locally available in many countries and their use may contribute to reduce national dependence on imported fossil fuels [8].

1.2.1 FEEDSTOCKS

Feedstocks refer to raw materials used in biorefinery. Biomass is synthesized via the photosynthetic process that converts atmospheric CO_2 and water into sugars. Plants in turn use the sugar to synthesize the complex materials that are generically called biomass. A vital

concept in a biorefinery system is the provision of a renewable, consistent and regular supply of feedstock. These feedstocks are provided from five different sectors:

- agriculture (dedicated crops and residues)
- forestry
- industries (process residues and leftovers)
- households (municipal solid wastes and wastewaters)
- aquaculture (algae and seaweeds)

The main biomass feedstocks can be further grouped in three wide categories: carbohydrates (from starch, cellulose and hemicellulose) and lignin, triglycerides (from vegetable and animal raw materials), and mixed organic residues.

1.2.2. TECHNOLOGICAL PROCESSES IN BIOREFINERY

The aim of technological process in biorefinery is to depolymerise and sometimes to deoxygenate the biomass components. In order to convert biomass feedstock into valuable products within a biorefining approach, it is essential to integrate several technological processes. They can be divided in four main groups: thermochemical, biochemical, mechanical/physical and chemical processes

Thermochemical processes

Gasification and pyrolysis are the two main thermochemical processes for biomass volarisation. Gasification involves heating biomass at high temperature (> 700^{o}C) with low oxygen levels to produce syngas, a mixture of H_2, CO, CO_2 and CH_4. Syngas can either be used directly as a stationary biofuel or as a chemical intermediate (platform) for the production of fuels (FT-fuels, dimethyl ether, ethanol, isobutene, and so on) or chemicals (alcohols, organic acids, ammonia, methanol, and so on). Pyrolysis on the other hand uses intermediate temperatures (300 – 600 ^{o}C) in the absence of oxygen to convert feedstock into liquid pyrolysis oil (or bio-oil), solid char and light gases similar to syngas [8]. Their yields vary with process conditions and for biorefinery purposes, the treatment which maximises the production of bio-oil is the most desirable (flash pyrolysis). Bio-oil, together with charcoal is generally best suited as fuel for stationary electric power or thermal plants, as its use as transportation fuel is quite problematic. In addition to gasification and pyrolysis, another thermochemical pathway is direct combustion. This is the oldest form of biomass conversion that involves burning biomass in an oxygen-rich environment mainly for the production of heat.

Biochemical processes

Unlike thermochemical processes, biochemical processes occur are lower temperatures and have lower reaction rates. Fermentation and anaerobic digestion are the most common types. Fermentation involves the use of microorganisms and/or enzymes to convert a fermentable substrate into recoverable products (usually organic acids and alcohols). Currently, ethanol is the most required fermentation product, but the production of many other chemical compounds (such as itaconic acid, succinic acid, methanol, among others) is nowadays a subject of many research and development activities. The most frequent fermentation substrates are the hexoses, mainly glucose, while pentoses (sugars form hemicellulose), glycerol and other hydrocarbons require the development of customised fermentation organisms to enable their conversion to ethanol [11]. On the other hand, anaerobic digestion involves the bacterial breakdown of biodegradable organic material in the absence of oxygen over a temperature range from about 30 – 65 ^{o}C. It consist of four main stages (see **section 3.2.3**) with the main end product of these processes as biogas (gas mixture made of CH_4, CO_2 and other impurities), which of course can be upgraded up to >97% methane content and used as a surrogate of natural gas [8].

Mechanical processes

These processes do not change the state or composition of biomass, but only perform a size reduction or a separation of feedstock components. In a biorefinery system, they are usually applied first, because the following biomass utilisation requires a reduction of the material size within specific ranges depending on feedstock specie, handling and further conversion processes. Biomass size reduction is a mechanical treatment that involves cutting or commuting processes that significantly change the particle size, shape and bulk density of biomass [8]. Separation processes entails separating the substrate into its components, while with extraction methods valuable compounds are extracted and concentrated from a bulk and heterogeneous substrate. Mechanical comminution and lignocellulosic pretreatment methods (e.g. the split of lignocellulosic biomass into cellulose, hemicellulose and lignin) fall within this category, even though for the latter, some of the hemicellulose is hydrolysed to single sugars [12].

Chemical processes

These are processes that lead to changes in the chemical structure of the molecule by reacting with other substances. Hydrolysis, transesterification, Fisher-Tropsch synthesis, steam reforming, methanisation, ozonolysis, among others are chemical reactions in biorefining. However, the most common in biomass conversion are hydrolysis and transesterification. Hydrolytic pathway may either involve the use of acids, alkalis or enzymes to depolymerise

polysaccharides and proteins into their component sugars (e.g. glucose from cellulose) or derivate chemicals (e.g. levulinic acid from glucose) [12]. Transesterification is the most common method for biodiesel production today. It is a chemical process by which vegetable oils can be converted to methyl or ethyl esters of fatty acids, also called biodiesel. A by-product of this process is glycerol, a chemical compound with diverse commercial uses in pharmaceuticals, food, personal care, antifreeze, among other applications.

1.2.3. BIOREFINERY PRODUCTS

Biorefinery products can be grouped into two broad categories: energy products and material products. Energy products are used because of their energy content to provide electricity, heat or transportation service. Material products on the other hand are not used for energy generation purposes but for their chemical or physical properties. Meanwhile, products like biohydrogen and bioethanol might be used as either fuels or as chemical compound in chemical synthesis. In this case, it is necessary to identify the addressed market(s).

The products of biorefinery must be able to substitute fossil fuel based products coming from oil refinery, both energy and chemicals. For chemicals, the objective can be met by producing the same chemical species from biomass instead of from fossil (e.g. phenols, ethanol), or producing a molecule having a different structure but a similar function. Concerning fuels, a biorefinery should replace conventional fossil fuels (majorly natural gas, gasoline, diesel and heavy oil) with biofuels coming from biomass upgrading [8]. The most important energy products that can be produced in biorefineries are:

- gaseous biofuels (biogas, syngas hydrogen, biomethane)
- solid biofuels (biochar, lignin, pellets)
- liquid biofuels (bioethanol, biodiesel, FT-fuels, bio-oil)

The most important chemical and material products are:

- biochemicals (fine chemicals, building blocks, bulk chemicals)
- organic acids [succinic, lactic, itaconic, fumaric and other sugar derivatives, majority of which are biomass platform molecules, (bio-PM) – ***Fig. 1.2***]
- polymers and resins (starch-based plastics, polyhydroxyalkanoates, phenol resins, furan resins)
- biomaterials (wood panels, pulp, paper, cellulose)
- food and animal feed
- fertilizers

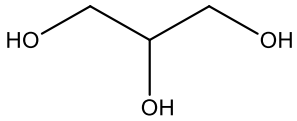
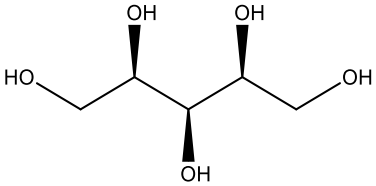
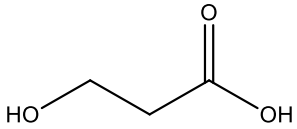
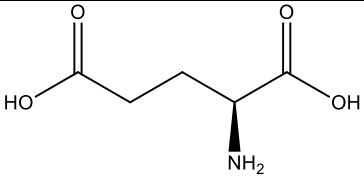
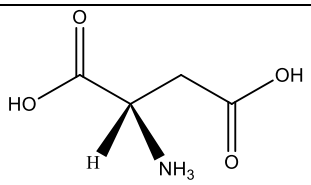
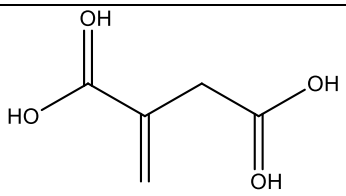
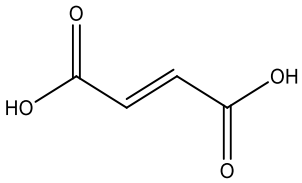
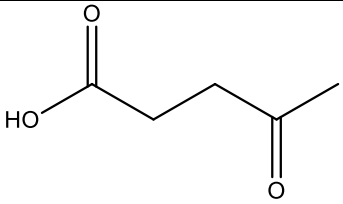
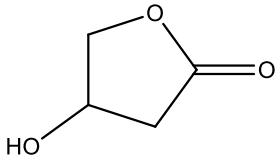
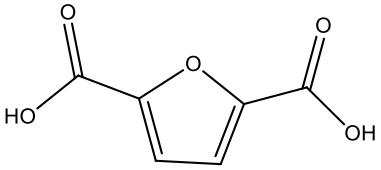
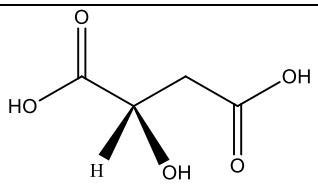
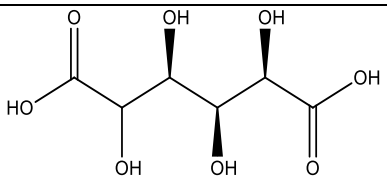
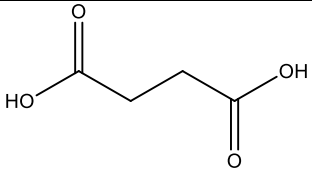
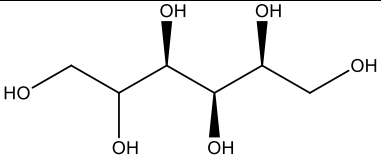
Bio-PM	Structure	Bio-PM	Structure
Glycerol		(S,R,R)-xylitol	
3-hydroxy propionic acid		L-glutamic acid	
L-aspartic acid		Itaconic acid	
Fumaric acid		Levullinic acid	
3-hydroxy butyrolactone		2,5-furan-di-carboxylic acid	
L-malic acid		Glucaric acid	
Succinic acid		Sorbitol	

Fig. 1.2: Biomass platform molecules [13]

1.2.4. THE ROLE OF GREEN CHEMISTRY IN BIOREFINERY

In the quest for a sustainable future production of these biofuels and biochemicals, the integration of green chemistry into biorefineries, along with the use of low environmental impact technologies, is necessary and mandatory. Green chemistry offers a tool kit of techniques and underlying principles that should be applied when developing the next generation of biorefineries. The overall goal of green chemistry with a biorefinery is the production of genuinely green and sustainable chemical processes and products [7].

Green chemistry offers a protocol when developing biorefinery processes and plays a vital role in facilitating sustainable production of commodity from biomass. Chemical processes and product life cycle should incorporate minimisation of energy demands, safe processes, waste prevention or minimisation, avoidance of hazardous chemical use and production. Final product should be non-toxic and degradable into innocuous compounds. Green methodologies and techniques can be used to reach these goals. For instance, supercritical carbon dioxide is recognised as a green solvent, since it is non-flammable, non-toxic, available as by-product of many conversion technologies (e.g. biomass fermentation) and gives no solvent residues [8]. Other technologies with great potential as energy efficient extraction methods are microwaves and ultrasounds [14]. As a matter of fact, the range of chemicals and materials that future biorefineries could produce is extensive, and with further research the selection will become larger.

1.3 OBJECTIVES OF THE THESIS

There are two objectives of this thesis:

SURFACTANTS FROM ITACONIC ACID

The first part of this thesis is to:

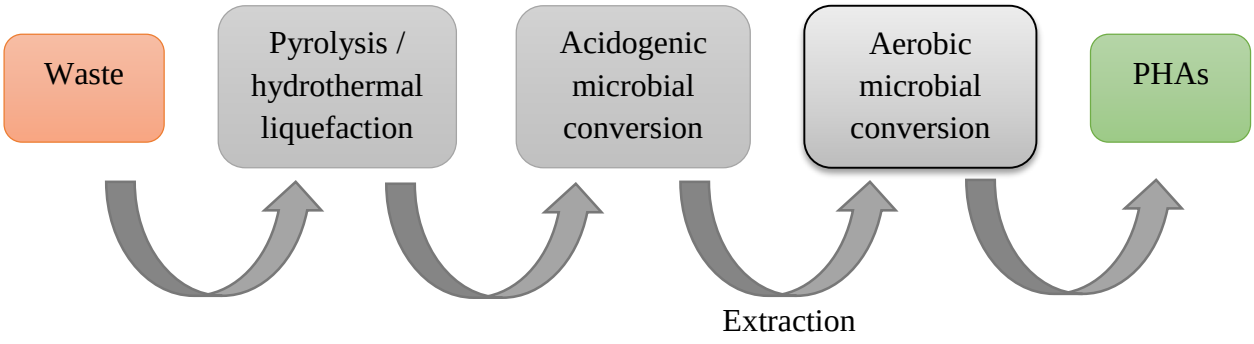
- present the synthesis of a new family of surfactants having C12 alkyl chains obtained from itaconic acid and fatty amine, molecules industrially obtained from renewable resources; and to compare their biodegradabilities with some conventional surfactants.

TRANSFORMATION OF WASTE BIOMASS TO PHA

The second part of thesis is targeted on the study of:

- three biorefinery technological processes in the novel transformation of paper waste and microalgae to PHA: Pyrolysis/hydrothermal liquefaction, acidogenic microbial conversion and aerobic microbial conversion (***Scheme 1.1***) – the integration of thermochemical and microbiological processes in order to harness the advantages of each. These wastes are obtained from renewable resources (7th principle of Green Chemistry).
- Extraction and recovery of fermentation products from the acidogenic broth to the aerobic culture medium.

Scheme 1.1: Biorefinery concept involving the hybrid thermochemical-microbial conversion of waste to PHAs



With respect to the ChIR learning objectives, the first aim of this project is related to ‘design’ and ‘assessment’ while the second is related to ‘design’ and ‘industry’.

1.4 SURFACTANTS FROM RENEWABLE RESOURCES

There has been an unceasing increase in the use of synthetic surfactants since the early 20th century when they were introduced, and a significant development in the last century to produce molecules that have higher efficiency than the fatty acid soaps that have been for over 2000 years [15,16]. Consequently, surfactants are presently among the highest volume synthetic chemicals produced and used globally, with the current estimated production volume over 12 million metric tonnes per annum, and an expected annual growth of 3-4% [16,17]. Surfactants which in this context exclude soaps, have from time pasts been produced from either petrochemical or oleochemical feedstocks, favouring petrochemicals which account for about two thirds of the organic carbon embedded in the final product(s) [18]. More importantly, the main problem of most surfactants lies in their non-biodegradability and the fact that they act as low-dose permanent toxicants in the water ecosystem (e.g. in pharmaceuticals and personal care products).

Surfactants (short form of "surface active agents") are broadly defined as compounds that can change the surface properties of water at an interface, typically the air-water or oil-water interface. Specifically, they can enhance dispersing, emulsifying, cleaning efficiency, wetting, forming/deforming, solvency or/and lubricity of water-based compositions. They lower the surface or interfacial tension between two liquids or between a liquid and a solid. Structurally, surfactants are often composed of a hydrophilic polar head and a hydrophobic non-polar tail, which are mainly sourced from oleochemical (natural) or/and petrochemical (synthetic) raw materials.

Oleochemical/natural surfactants are more often derived from plant oils such as palm oil and coconut oils [17]; from carbohydrates such as sucrose, glucose and sorbitol; or from animal fats such as tallow. In recent years, oleochemical feedstock sources of surfactants have been changing: animal fat is been replaced with vegetable oils and soybean oils - beef tallow has been impacted by mad cow disease concerns while fish oil use has dropped due to high oil prices caused by growing antioxidant demand [19].

Petrochemical/synthetic surfactants are derived from fossil fuel, mainly crude oil. The primary synthetic feedstocks are ethylene, benzene, kerosene and n-paraffins.

The largest end use market for surfactants is as household cleaning detergents - typically formulated based on linear alkylbenzene sulfonate made form petroleum feedstocks – benzene, kerosene and *n*-paraffins. Although they find predominant application as detergents and cleaners, they also find a variety of industrial, agricultural and specialty uses as well (***Fig 1.3***); each application requires unique performance characteristic [16].

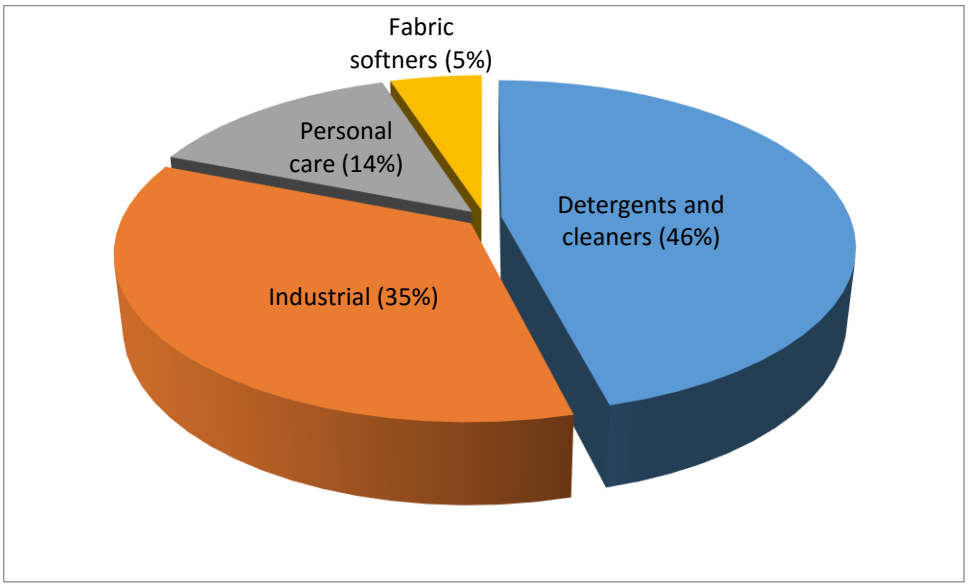


Fig. 1.3: Surfactant applications and their estimated relative market share [20]

The quest for renewable feedstocks for surfactant production is partly driven by the realisation that fossil fuel reserves are finite and oil price fluctuates (***Fig. 1.4***). More so, studies have shown that the utilisation of renewable feedstocks has a significant CO_2 emission reduction associated with the production and use of surfactants. Patel et al. estimated that oleochemicals may lead to greater CO_2 savings when utilised to produce surfactants instead of biodiesel production [21]. There was a further estimate by Patel *et al.* that the complete replacement of petrochemical-based surfactants with surfactants from renewable feedstocks in EU could lead to a total CO_2 emission reduction associated with surfactant production – a reduction as much as 37% [22]. Hence, the full replacement of petrochemical surfactants is desirable from the viewpoint of Greenhouse Gases reduction. Nonetheless, key factors such as cost and performance need to be well considered in determining the success of the biobased surfactant market. As a result, technologies for surfactants from renewable resources should tend toward production from robust, viable and sustainable feedstocks, efficient production process, must have similar or

superior physicochemical properties in comparison with petrochemical surfactants, and low production cost [16].

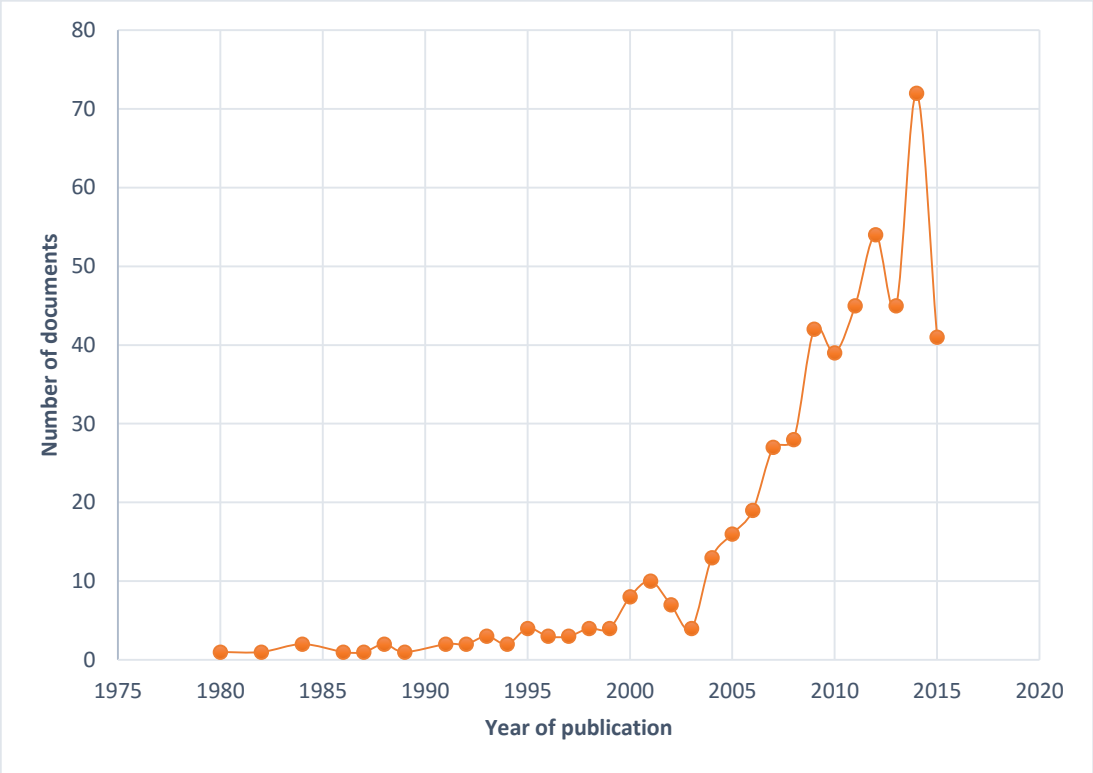


Fig. 1.4: Yearly trend in the research for surfactants from renewable resources [23]

1.4.1 HYDROPHOBES FROM RENEWABLE RESOURCES

These are compounds, typically characterised with long carbon chains and are the oil-loving part of surfactants.

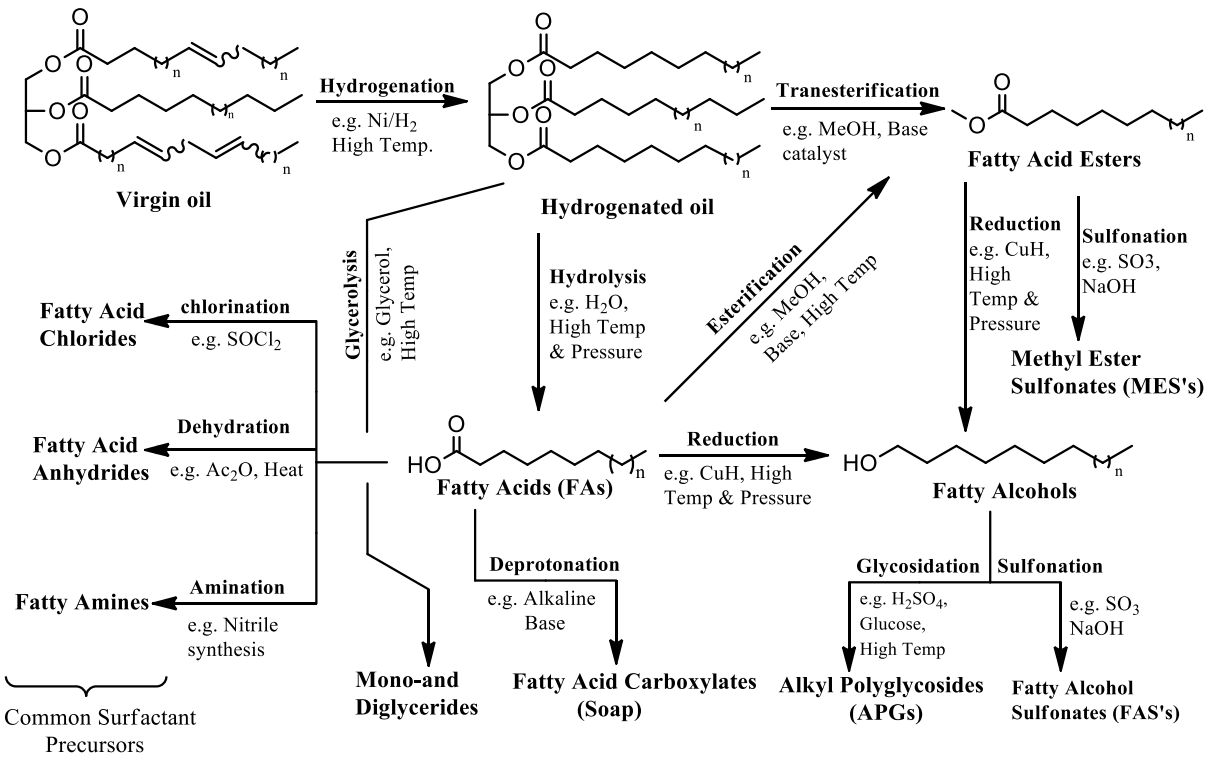
Triglycerides (TGs): Renewable sources of surfactants are derived from fatty acids (FAs) of either plant or animal tallow TGs, with a few exceptions. However, trends are shifting from the use of tallow which has been a historically vital feedstock to the use of plant-based oils as primary feedstock. This shift is partly due to the concern of beef tallow's potential role as a transmission vector for bovine spongiform encephalopathy [16].

The saturated palmtic (C16) and stearic (C18) acids, the unsaturated oleic (C18) and palmitoleic (C16) acids, and the polyunsaturated linoleic and linolenic acids are the most common FAs derived from plant oils; however shorter-chain FAs are required in many surfactant technologies for optimal performance properties. Palm kernel oil (PKO) and coconut oil (CO)

with high percentages (>70%) of FAs and tails 14 carbon or less in length, are among the most important FAs for surfactants production [24,25].

Oleochemical transformations such as hydrolysis, hydrogenation, transesterification and some other transformations specific to surfactant production like glycosidation, reduction, sulfonation, chlorination, etc are various ways TGs from raw oil can be processed to the desired surfactant and surfactant precursors (***Scheme 1.2***) [16].

Scheme 1.2: Surfactant production through common transformations of vegetable oils [16]



Fatty acids methyl ester (FAME): There have been extensive studies on the trans-esterification of TG to produce FAME owing to its role in the manufacture of biodiesel and this is commonly achieved through the use of a strongly basic catalyst such as $NaOCH_3$ in the presence of excess methanol (MeOH) at ambient or slightly elevated temperatures (***Scheme 1.2***) [16]. However, FAME can also be generated from FA at high temperatures (200 – 250 ^oC) in the presence of MeOH vapour and an alkaline catalyst in a counter-current reaction column where water is removed continuously [16,26].

Fatty alcohols (FOHs): One of the most vital intermediates in the production of surfactants is FOHs. The report by World Surfactant Congress in 2004 reports that about 2.5 million tonnes of surfactants from FOH were produced in 2003, with about half of the FOHs got from renewable resources [27]. Alkyl polyglucosides (APGs) and fatty alcohol sulfates (FASs) are two crucial classes of surfactants that are derived directly from FOHs trough acid catalysed glycosylation and sulfonation respectively. FASs find application in a variety of personal care and detergent applications.

FOHs are preferably produced by reduction of FAMEs with H_2 using a copper catalyst (e.g. copper chromite, >2%) at high temperature and pressure (e.g. 250 - 300 bars, 250 - 300 ^{o}C) [16,28] (***Scheme 1.2***).

Fatty acids (FAs): Acid catalysis has been used in the conversion of TGs to FAs, with a preference in the use of steam at varying degrees of high temperature and pressure (***Scheme 1.2***) [16]. FAs can be made to undergo alkaline hydrolysis to directly obtain carboxylates (i.e. soap), condensed with a nucleophile (e.g an amine), or further modified to enhance their reactivity for derivatization with a hydrophile [16].

Fatty amines: The most common route to the production of primary and secondary amines is the three-step Nitrile® process (***Scheme 1.2***) which involves in the reaction of FA with ammonia at elevated temperature (> 250 ^{o}C) in the presence of a metal oxide catalyst (e.g. alumina or zinc oxide). This results in a fatty nitrile which, can be hydrogenated in the presence of a nickel catalyst and excess ammonia to generate primary amines in good yield. Besides, secondary and tertiary fatty amines can be generated directly from the reaction of FOHs with alkylamines [15,16]. However, though fatty amines represent a vital class of renewable hydrophobes, the drawback is that is their downstream derivatization (e.g. to quaternary ammonium salts) often relies on traditionally non-renewable chemicals such as ethylene oxide, dimethyl sulphate, benzyl chloride, and the like [16].

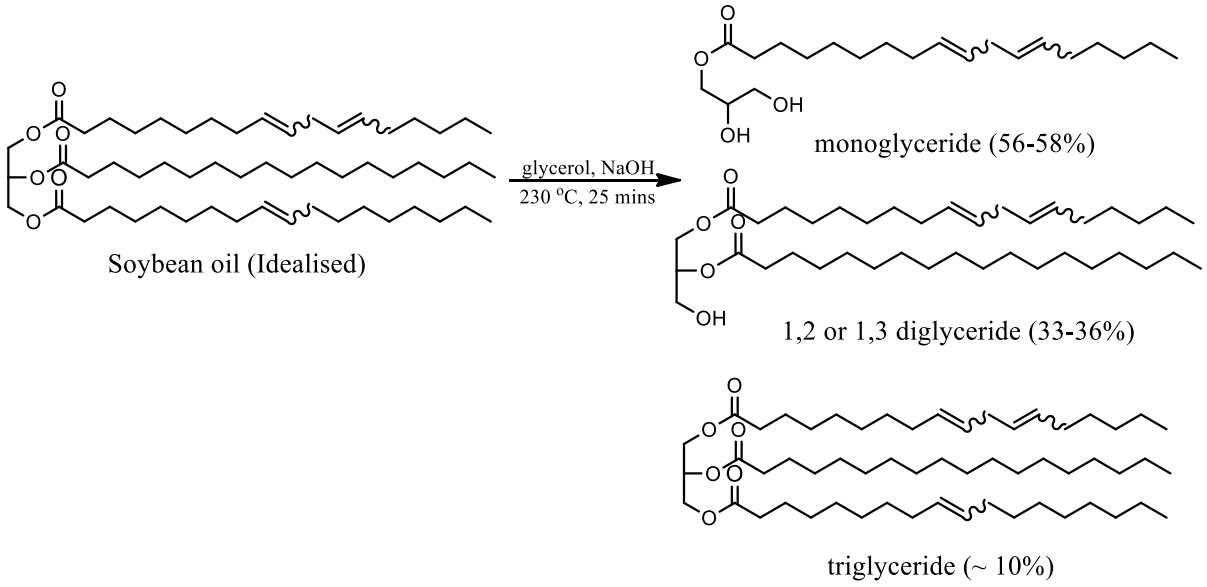
Other hydrophobes: Cost, chemical composition and availability are some factors that can restrict the implementation of a TG feedstock, hence alternative sources have been of interest [16]. Alternative plant crops, engineered plant crops, biomass waste and various biomass byproducts such as tall oil, sterols and sterol derivatives are been explored and harnessed as potential feedstocks for surfactant production [6,16].

1.4.2 RENEWABLE HYDROPHILES FROM RENEWABLE RESOURCES

Glycerol from vegetable oils feedstock, sucrose from sugar cane and sugar beets, and glucose from starch-producing crops such as potato, maize, wheat and tapioca are used directly in surfactant production. Glycerol is primarily produced as a by-product in the refining of glycerides, though can also be derived industrially from glucose. Furthermore, glucose serves as a common precursor to a variety of downstream chemicals (organic acids) that can also be used as hydrophiles [16].

Glycerol: Mono and diglycerides (MGs and DGs) are the common surfactants for food emulsification in the food industry, and accounts for ~75% of the total emulsifier use. MGs and DGs also find application in a variety of other industries such as cosmetics, textiles, pharmaceuticals, plastics and machine oil formulation [16]. A general commercial synthesis for MGs and DGs is the transesterification of TGs in the presence of glycerol and base catalyst such as NaOH or KOH at high temperature (200 – 255 ^{o}C) for about 4 hours [29]. However, this depends on the type of oil and the exact conditions being used, MG, DG and TG are found distributed in the resulting product, with ranges of MG from ~30 – 90% (***Scheme 1.3***) [16]. Meanwhile, a milder alternative is the glycerolysis of FAME which involves a lower temperature (120 – 230 ^{o}C).

Scheme 1.3: Glycerolysis of soybean oil with excess glycerol as described by Noureddini et al. [16,30]



Glucose: The use of glucose as a hydrophile in surfactant synthesis can either be directly or indirectly. Directly, it can undergo glycosidation reaction with a nucleophile such as an alcohol (for instance, FOH), a carbohydrate or a protein to generate APGs [31], an increasing popular class of non-ionic surfactants due to their desirable detergency properties and low toxicity [32]. Hence they are used in personal care products, in pharmaceutical formulations, as self-emulsifiers and as agrochemicals [6]. Despite the popularity of these APGs as surfactants, there have been a variety of synthetic challenges [31] that results in the products being isolated as relatively expensive technical mixtures [32].

Furfural, **5-hydroxymethylfurfural**, **sorbitol** and **cellulose** are carbohydrates that can be derived from waste biomass and have been proposed as starting materials for surfactants [33,34,35,36].

Citric acid: Citric acid is a natural organic acid produced on a large scale mainly through biotechnological processes, that involves the natural action of *Aspergillus niger* (a fungus) on feedstocks such as sucrose, sucrose molasses, glucose syrups, and wood pulping waste. The yeast *Yarrowia lipolytica* also converts carbohydrates and lipid raw materials into citric acid. Though citric acid is one of the most ubiquitous chemicals in commerce, its application for surfactant technologies only account for a small fraction. Other hydrophilic groups such as glycosides, ethoxylates and glycerides have been coupled with citric acid to impart anionic character to ordinarily non-ionic surfactants. For instance, a citrate ester of fatty alcohol glucoside (***Fig 1.5***) having a surface activity superior to that derived from petrochemicals is marketed as a mild, foaming detergent for formulation of household products [16].

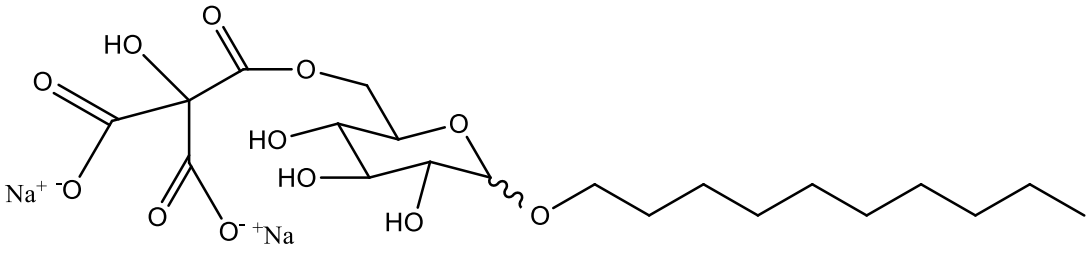


Fig. 1.5: A disodium citrate ester of fatty alcohol glucoside

An interesting group of surfactants reported in recent years contains some examples derived from polycarboxylic acids like maleic acid, itaconic acid, succinic acid, fumaric acid and aconitic acid [37]:

Itaconic acid (IA): IA is a naturally occurring compound, nontoxic and readily biodegradable [6]. It is a thermal decomposition product of citric acid in acidic conditions, but for industrial purposes, it is economically convenient to produce it biotechnologically using various fungi strains (primarily mutants of *Aspergillus terreus* and *Aspergillus itaconicus* [38]). The optimal feedstocks are glucose and sucrose with yield higher than 80g/L [16,39]; nevertheless, materials such as starch, corn syrup, molasses, glycerol, lignocellulose, and citric acid may also be used by fungi [16]. In a typical process, the fermentation broth formed is filtered, concentrated, recrystallized (once or severally) and decolorized.

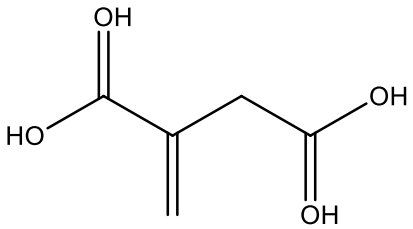


Fig. 1.6: Schematic depiction of itaconic acid

Nowadays, IA is majorly used by the polymer industry where it is employed as a co-monomer for the synthesis of polyesters. It can undergo addition polymerisation, giving polymers with many free carboxyl groups that confer advantageous properties on the polymer [40]. It is a very interesting 'green' starting material for the production of polymers, since it is a potential renewable substitute of fossil-derived acrylic and methacrylic acids. Additionally, its two carboxyl groups allow its use as AA-type monomer for polycondensation reactions [38]. Common end products of IA polymerisation reactions include polyitaconic acid and styrene-butadiene latex made from the polymerisation of styrene, butadiene and IA [40].

IA also finds applications in other industrial products such as detergents, adhesives, synthetic fibres and resins, agriculture, plastics and coatings, paints, to mention a few (***Fig. 1.7***). There was an estimation that over 41000 tonnes of IA were produced in 2011 and a projection to surpass 50000 tonnes by 2020. China, USA, France and Japan were the major IA producing countries in the world. When Cargill and Pfizer in the USA, and the French company Rhodia

stopped its production, China became the largest producer with companies like Qingdao Kehai Biochemistry having an annual output capacity of 20000 tonnes per annum [40]. With a current price of $1800-2000 per tonne, it is not economically favourable to use IA in many applications, however, if the price can fall below $1500 per tonne, a complete replacement of petroleum-based polyacrylic acid with IA will be feasible [41].

Fig. 1.7: Some industrial applications of itaconic acid [40]

Succinic acid (SA): SA or butanedioic acid is also a naturally occurring and nontoxic organic acid like IA. On an industrial scale, it is being produced by catalytic hydrogenation of petrochemically derived maleic acid or maleic anhydride. Moreover, SA produced from carbohydrates by bacterial fermentation is mostly used in the food industry [42]. Combined, 30 000 to 50 000 tonnes of SA are produced annually, and expected to grow at a compound annual growth rate of 18.7% from 2011 to 2016 [43], as a result of the four major existing markets: first and largest is as a surfactant/detergent extender/foaming agent. The second market is as an ion chelator, where it is used in electroplating to prevent corrosion and pitting of metals. Third, in the food and beverage market as an acidulant/pH modifier, a flavouring agent and as an anti-microbial agent. The fourth market is as a precursor to active pharmaceutical ingredients or pharmaceutical additives [43,44].

Succinate-based surfactants such as sulfosuccinates and sulfosuccinamates are valuable anionic surfactants. Alkenyl or alkyl and epoxy derivatives of SA are used in detergent formulations [42]. Furthermore, some novel surfactants with favourable properties have been prepared from succinic anhydride [45].

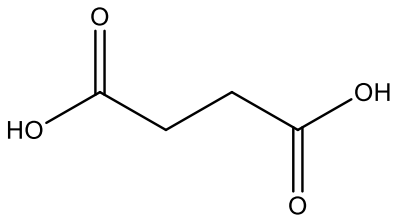


Fig. 1.8: Schematic depiction of succinic acid

Furmaric acid (FAc): FAc or trans-butenedioic acid is also a naturally occurring and nontoxic organic acid like IA. It is an intermediate of the tricarboxylic acid (TCA) cycle, hence can be produced by microbial fermentation [46]. FAc is an industrially important four-carbon chemical due to its structure (a carbon–carbon double bond and two carboxylic acid groups, ***Fig. 1.9***) and this has made it got application as a starting material for polymerisation and esterification reactions to make plasticizers, paper resin, and additives for food and beverage [46,47].

Currently, the major production of FAc is obtained by the oxidation of benzene to maleic anhydride at elevated temperature in the presence of V_2O_5 and MoO_3 as catalysts. This affords only 73% yield and over 20% of the reagents are destroyed by waste stream incineration [48]. More so, benzene is a hazardous chemical, with very low emission standards and a worldwide consensus that its use should be abandoned as much as possible in the future [47]. As a result, the production of FAc by a "green" biobased process has been of increasing interest, with a wide range of study on microbial fermentation processes, using *Rhizopus* species [49] and metabolic engineered *Escherichia coli* [46] on feedstocks such as glucose, sucrose, xylose, molasses, cassava bagasse and other starch containing materials. Submerged fermentation systems coupled with product recovery techniques have achieved economically attractive yields and productivities [49].

FAc finds application in the polymer industry especially in the production of unsaturated polyester resins due to its special properties like greater hardness in the polymer structure [49], and also in the pharmaceuticals (to treat psoriasis – a skin condition in which FAc cannot be produced in the body due to certain biochemical defects that interferes with adequate FAc production on the skin) and agriculture (as a supplement in cattle feed to reduce the emission of methane from cattle, up to 70% reduction) [49].

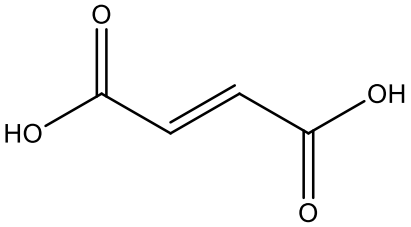


Fig. 1.9: Schematic depiction of fumaric acid

1.5 BIOMASS CONVERSION TO BIOPLASTICS

Bioplastics are plastics derived from renewable biomass sources. The increasing application of bioplastics is vital in the quest for a biobased economy for the nearest future. With the increasing scarcity of fossil raw materials and global warming, the use of renewable raw materials is an obvious solution and promises huge potential. One of the decisive advantages in the replacement of conventional plastics with bioplastics is the contribution to the reduction of atmospheric greenhouse gases, thus plays a major role in in the mitigation of global warming. Another advantage is that most biopolymers are also biodegradable and compostable, thus reducing a major source of pollution, especially for water bodies.

1.5.1 POLYHYDROXYALKANOATES (PHAs)

PHAs are aliphatic polyesters. They represent one of the most versatile fully biodegradable polymers and have received increasing attention in the last decade (***Fig. 1.10***).

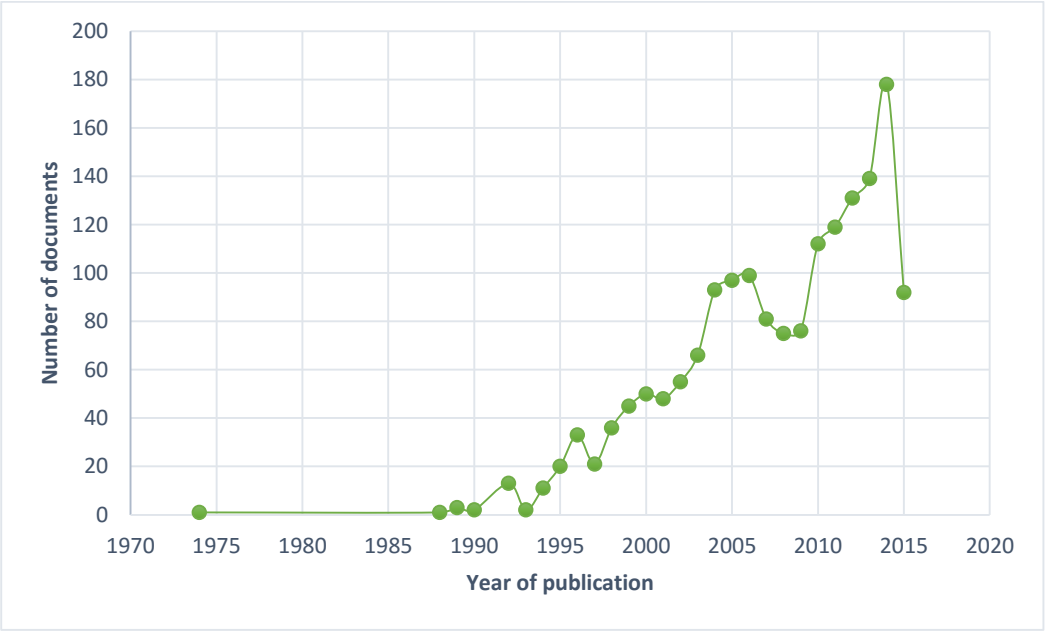


Fig. 1.10: Yearly trend in the research for polyhydroxyalkanoates [23]

PHAs possess highly favourable technological properties similar to conventional petro-based plastics, sometimes superior to other biodegradable polymers such as chemically synthesized plastics (like polylactic acid and polyglycolic acid) and starch-based plastics (such as starch-polyethylene) [50]. Their versatility, biodegradability and immunological inertness have made them potential candidates in several applications, from biomedical/medical fields to food, packaging, textiles and household materials (***Table 1.1***).

Table 1.1: Worldwide PHA producing and research companies, and the commonly used bacterial strains [51]

Company	Strain	Types of PHA	Production scale (t/a)	Applications
ADM, USA (with Metabolix)	*Ralstonia eutropha*	Several PHA	50000	Raw materials
Meredian, USA	Unknown	Several PHA	10000	Raw materials
Tianjin Green Bio-Science (+DSM)	*Escherichia coli*	P(3HB-4HB)	10000	Raw materials & Packaging
Yikeman, Shandong, China	Unknown	PHA (unclear)	3000	Raw materials
Zhejiang Tian An, China	*Ralstonia eutropha*	P(3HB-co-3HV)	2000	Raw materials
ICI, UK	*Ralstonia eutropha*	P(3HB-co-3HV)	300	Packaging
Chemie Linz, Austria	*Alcaligenes latus*	P(3HB)	20 – 100	Packaging & drug delivery
btF, Austria	*Alcaligenes latus*	P(3HB)	20 – 100	Packaging & drug delivery
BASF, Germany	Unknown	P(3HB), P(3HB-co-3HV)	Pilot	Blending with Ecoflex
Tepha, USA	Unknown	Several PHA	PHA medical implants	Medical bio-implants

High molecular mass (HMM) PHAs have been of great interest for industries, thanks to their elastomeric/thermoplastic properties, which are tunable according to actual co-monomer composition [52,53]. Production of PHA is based on microbial fermentation technologies using

a two-stage production process. First, the microorganisms which accumulate PHA intracellularly are selected and grown using a renewable feedstock. Second is the harvesting of the bacterial biomass once the fermentation process is complete. The PHA polymer is extracted from the cells using either a solvent extraction process or an aqueous process in which the non-PHA component of the microbial cell is disrupted either chemically or enzymatically and then removed from the PHA polymer [52]. This approach incurs major cost factors such as the large capital investment required for aerobic fermentation facilities, polymer recovery systems and the cost of the renewable feedstocks. All these make the production cost of PHA relatively high (~ 5 – 6 $/kg) compared to other biobased or petro-based polymers, hence hitherto gained most applications in low volume high cost items and hampering its exploitation as a commodity material [50,53].

STRUCTURE AND PROPERTIES OF PHAs

PHAs are elastomeric or thermoplastic polyesters of hydroxyalkanoic acid monomers (***Fig. 1.11***) biosynthesized by a wide range of Gram-positive and Gram-negative bacteria as intracellular carbon and energy storage compounds [50,54].

In homopolymeric PHAs, only one monomeric unit is present, while more different units are found in heteropolymers.

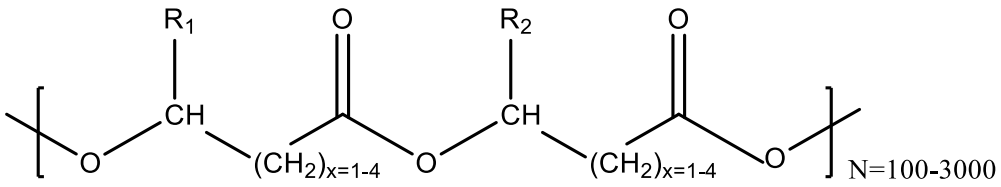


Fig. 1.11: General structure of PHA. R_1 and R_2 are alkyl groups (CH_3 – C_3H_7)

PHAs are classified on the basis of the number of carbon atoms ranging from 4 to 14 and the structure of monomeric units. PHAs with 3–5 carbon atoms are classified as short chain length PHAs (scl-PHAs). Examples include the pioneer P(3HB) and P(3HV). Medium chain length PHAs (mcl-PHAs) contain 6–14 carbon atoms. Homopolymers P(3HHx), P(3HO) and heteropolymers such as P(3HHx-co-3HO) are examples of mcl-PHAs.

The 4-carbon atom homopolymers were the pioneering PHAs with much relevance industrially and are relatively better characterised compared to other PHAs. However, recent reports reveal the more versatility of other types. P(3HB) has high molecular weight and crystallinity with melting point of 180 ^{o}C and an elongation break of 5%, thus very hard and brittle. This is quite similar to one of the forms of the current leading compostable plastics, polylactic acid (PLA), made through the chemical polymerization of enantiopure lactic acid produced from fermentation process. The cost of PLA is lower than PHAs and its uses in the plastic market have increased based on the development of additives to improve its properties [55] (***Table 1.2***). On the other hand, specifically designed copolymers have been used to reduce or eliminate the brittleness and thermal stability of PHB. In fact, mcl-PHAs and their copolymers have low crystallinity (20 – 40%) and do not break easily (extension to break of 300 – 450%) [50], hence have been harnessed for a wide range of applications.

Table 1.2: Comparison between PLA and PHA [51]

	PLA	PHA
Monomer structures	D- L- and *rac*-lactic acids (LA)	At least 150 monomers
Production methods	Bio-production of LA and chemical synthesis of PLA	Totally biosynthesized as intracellular polyesters
Production cost	Comparable with conventional plastics like PET	At least twice that of PLA
Material properties	Can be adjusted by regulating D- and L-LA ratios according to monomeric composition	From brittle, flexible to elastic, fully controllable
Technology maturity	LA production well established, yet LA polymerization to PLA is complicated.	At least 10 companies worldwide produced or are producing PHA up to 2000t/a scale via microbial fermentation

PRODUCTION OF PHA

Factors such as type of microorganisms (e.g. Gram-negative or Gram-positive), media ingredients, fermentation conditions, mode of fermentation (batch, fed-batch, continuous) and recovery, affect the monomer content of PHAs.

Microorganisms

Several varieties of Gram-positive and Gram-negative bacteria such as *Pseudomonas* sp., *Bacillus* sp. and *Methylobacterium* sp. possess the enzymes for the biosynthesis of PHAs and accumulate them in their cytoplasm as both carbon and energy sources, usually in a granular shape. Biosynthesis of PHAs by bacteria usually occurs in response to stress conditions such as nutrient (e.g. phosphate or/and nitrogen) limitation, in the presence of excess carbon source [50,56], although some bacteria such as *Azotobacter vinelandii* UWD and *Azotobacter eutrophus* have the ability to accumulate PHA under non-limiting conditions. Basically, the vital point to put into consideration is the suitability of the bacteria for efficient production of PHAs from low-cost resources. For a viable large-scale production of PHA, higher growth of the PHA producing bacteria is necessary to increase the production rate [50].

Depending on the substrate or desired PHA yield, single or mixed microbial cultures (MMC) may be employed for PHA production. While the former is a pure culture that contains only one species of bacteria, often a clone, the latter consists of two or more species. Mixed cultures can consist of unknown species to the exclusion of all others, or they may be composed of mixtures of unknown species. Moreover, the mixed cultures may be of all of one microbial group – all bacteria or they may consist of a mixture of organisms of bacteria, fungi or/and yeast.

Advantages of mixed-culture fermentation over single-culture fermentation

Mature MMC can be seen as a stable ecosystem obtained through an artificial selection that operates according to Darwin's principles, acting on the microbial community as a whole. Below are some advantages of MMC over single strain culture (SSC):

- MMC enables the utilisation of cheap and impure substrates. They possess a wider range of enzymes and have a wider metabolic potential than single strains such as *Cupriavidus necator* or genetically modified *Escherichia coli*, hence, are able to attack a greater variety of compounds. Likewise, with proper strain selection, they are able to change or destroy toxic or noxious compounds that may be present in the fermentation substrate [53,57].
- MMC require no sterile conditions unlike single-cultures. It offers more protection against contamination and phage infections. In pure-culture commercial fermentations involving bacteria and actinomycetes, an epidemic of phage infections occur which can shut down production. Since MMC have a wider genetic base of resistance to phage,

failures hardly occur, often because if a strain is wiped out, a second or third phage-resistant strain in the inoculum will take over and continue the fermentation. In such processes, especially with a heavy inoculum of selected strains, contamination usually does not occur even when the fermentations are carried out in open containers [57].

If the environmental conditions can be controlled (i.e., temperature, length of fermentation, mass of fermenting substrate and kind of substrate), it is easier to maintain a mixed-culture inoculum indefinitely and to carry out repeated successful fermentations [57].

- MMC are able to bring about multi-step transformations that would be impossible for a single microorganism.
- The growth rate may be higher: In MMC, one microorganism may produce needed growth factors or essential growth compounds such as carbon or nitrogen sources beneficial to a second microorganism. It may alter the pH of the medium, thereby improving the activity of one or more enzymes.
- In some MMC, a remarkably stable association of microorganism may occur.

Limitations of mixed-culture fermentation over single-culture fermentation

- MMC is usually less efficient than SSC in the production of PHA. Indeed, since only part of the microbial community is productive, part of the supply is consumed by non-productive strains for their own survival.
- It is more difficult either to genetically manipulate the organisms, or to apply specific stress aimed to enhance the desired performance, since the community responds globally to the stress.
- The scientific study of MMC is difficult. Obviously, it is more difficult to study the fermentation if more than one microorganism is involved [57].
- Contamination of the fermentation is more difficult to detect and control.

Culture media

The culture medium (which comprises the carbon source and nutrients) is essential to supply optimal conditions in different bacteria for the production of high amount of PHA varieties that will be economically competitive with traditional plastics. The carbon source specifically poses a cost challenge for the production of commodity materials. This is because it is the major cost incurred in the PHA production stage [58]. Thus, significant cost reduction can be achieved if

cheap carbon source with the necessary requirements for high production of PHAs can be used. Cheap sources for fermentation include media containing molasses, starch and starchy waste water, poultry litters, swine waste, effluents from olive mill and palm oil mill, glycerol, grass, to mention a few [50,59]. The choice of carbon source partly depends on if the microorganism is wild or recombinant and whether it needs nutrient limiting conditions or not. In addition, the production of either homopolymers or copolymers is another factor in the choice of media ingredients and composition [50].

Fermentation

Many PHA fermentations are carried out in two stages: the first aimed at the production of a high cell density culture, and the second, which is usually a nutrient limited fermentation process, is aimed at increasing the concentration of PHAs. Conditions for fermentation depends on the demands of the microorganism (usually bacteria) and often at a temperature range of 30 – 37 ^{o}C, together with low stirrer speeds, which results in low dissolved oxygen tension [50]. pH is either regulated linking to the substrate addition or left uncontrolled. Depending on the desired PHA kind and yield, batch (particularly fed-batch) or continuous fermentation can be applied.

Recovery

Recovery of PHA is another stage that adds significantly to the production cost and remains a non-negligible challenge [50].

Following the fermentation, cells containing PHAs are separated by conventional procedures such as centrifugation, filtration or a hybrid flocculation-centrifugation method. After the biomass harvesting, microbial cells are disrupted to recover polymers. The first method developed for PHA recovery involves the use of organic solvents, mostly chlorinated, such as chloroform, methylene chloride, dichoroethane; and propylene carbonate. Due to the high viscosity of even dilute PHA solutions, the ratio of solvent needed to the extracted polymer is 20:1. The large amount of solvent required, besides strongly contrasting with the green chemistry principles, makes this method economically unattractive, even after the recycle of the solvent [54].

Several other protocols have been developed for PHA recovery. They can be grouped into two main strategies: an optional cell pre-treatment under acidic conditions followed by solvent extraction (e.g with acetone or chlorinated compounds), or a treatment with strong oxidant (eg.

NaClO) or bases (NaOH) to disrupt the non-PHA cellular materials (NPCM) and release the polymers stored inside the cells [53]. The use of organic solvents generally provides poor recovery (18 – 30%) but high molecular weight polymers (2.2 MDa) [60]. On the contrary, the use of strong oxidants such as NaClO provides an excellent recovery (80 – 100%) and purity (~ 95%) of short-chain polymer due to cleavage of the polymer bonds [53,54,59]. Samori et al. recently developed a green protocol for PHA recovery from MMC by means of dimethylcarbonate (DMC) or combination of DMC and NaClO. The former affords an overall polymer recovery of 62%, purity of 98% and molecular weight of 1.3 MDa, while the use of a NaClO pre-treatment before DMC extraction increases the overall PHA recovery (82%) but diminishes the molecular weight from 0.6 to 0.2 MDa [53].

1.6 STATE OF THE ART

With respect to other bioplastics that are commercially available and currently produced on an industrial scale (e.g. PLA or starch-based polymers [Mater-Bi®]) as commodity materials, PHA encounters a major challenge which hampers its use as a widespread commodity material: the relatively high cost, in comparison to some other bioplastics and petro-based plastics. This high cost is mainly due to a combination of three factors:

- Use of expensive substrates as carbon source which accounts for approximately 40% of the total production cost.
- Absolute sterile operation conditions necessary for pure culture fermentation.
- PHA extraction and purification drawbacks

Thus, the commercialisation of PHAs is at the moment quite limited to high added value applications. With the dire need to reduce costs, the development of a more cost effective PHA production process is essential, and a multilevel approach is mandatory. In principal, the utilisation of low value substrates such as agro-industrial waste and by-products combined with the use of MMC, and with green and cheap extraction and purification methodologies would lower investments and operating costs for the entire process.

Considering the abundance, diversity and lack of proper treatment approach, use of waste/wastewater for PHA production is a beneficial approach with dual benefits of waste remediation and concurrent value addition. Different types of waste/wastewater such as agro-industrial wastes [61], food waste [62], sugar industry waste [63], tomato cannery wastewater [64], paper mill waste [65,66], spent wash effluents [67] have been used for PHA production.

In order to explore the potential of waste/wastewater for PHA production, many strategies have been proposed and one of them is the integration of anaerobic acidogenic fermentation (or acidogenesis) with aerobic PHA synthetic process. This has emerged a viable strategy of waste remediation with simultaneous value addition [68]. Acidogenic fermentation (AF) of wastes generates effluents rich in volatile fatty acids (VFAs) such as acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid or/and isovaleric acid. These VFAs can be effectively used as source of carbon by aerobic bacteria for their growth and survival during carbon depletion conditions by converting these VFAs into PHAs reservoir.

Meanwhile, a challenging aspect in the use of MMC for PHA production is the ability to select a culture with high storage capacity. The most well-known approach for MMC selection with PHA accumulation capacity is the sequential operation of the system under a short carbon excess phase followed by a long substrate depleted phase, known as "feast and famine" (F&F) or "aerobic dynamic feeding" (ADF). This procedure selects bacterial communities that have the capacity to convert available carbon into storage polymers during the short feast phase and use them during the starvation period as an energy and carbon source for cell growth and maintenance. A two-step process is almost always the case in the use of MMC to produce PHA. First is the selection step where experimental conditions imposed in the reactor allow the selection of a culture with a good and stable PHA storage capacity. Second is the production step where the maximization of PHA storage efficiency is attempted, usually through nutrient or electron donor restriction so that the selected cultures are able to direct most carbon resources for PHA storage [69]. However, MMC store little or no PHA from sugar-based compounds when submitted to F&F conditions [70,69]. Hence, the importance of the additional step of AF to allow the production of VFAs (for PHA production) from sugar fraction present in the substrate [66]. Therefore, a viable option in order to exploit the full potential of waste/wastewater using MMC is the development of an integrated strategy where the effluent obtained from AF can be used for PHA production by aerobic bacteria [71].

1.7 PYROLYSIS: TO OBTAIN CARBON SOURCE FOR PHA PRODUCTION

Lignocellulosic biomass is considered the most abundant and inexpensive sustainable carbon source [72,73] but its utilization in a biorefinery is somewhat challenging. One of the fastest ways to depolymerise these biomass macromolecules to smaller fragments that can be anaerobically fermented by bacteria is pyrolysis. Intermediate pyrolysis is the thermochemical decomposition of organic material at not very elevated temperatures (400 – 500 ^{o}C) and reaction

time of about 10 – 30 seconds, in the absence of oxygen. Due to its low heat transfer and possibility to be readily performed with a simple reactor, it is considered economical also at small scale [74]. It is able to yield solid char (20 – 30%), a non-condensable gas (10 – 20%) and 50 – 60% wt/wt of liquid (called pyrolysis oil or pyrolysis liquid; bio-oil when produced from biomass, which can be upgraded to biofuel) [74]. Several chemicals used in food flavourings, fertilizers and resins production can be derived from bio-oil. Also, the direct use of bio-oil to substitute fuel oils in many static applications such as boilers, engines, furnaces and turbines for electricity generation has been accomplished [72]. However, bio-oils due to their high oxygen content (up to 60% wt/wt), high water content (25 – 50% wt/wt), acidity and the occurrence of phase-separation upon storage, are considered low-quality fuels that cannot be used in conventional gasoline and diesel fuel engines since that are immiscible with petroleum-derived fuels. Bio-oils can with high polarity could be deoxygenated to yield a mixture of organic molecules similar to petroleum-based fuels but the high cost associated with this process hampers its application in this regard [73].

Meanwhile, polar bio-oils usually have high concentrations of aldehydes, ketones, alcohols, carboxylic acids and other polar components, and due to their good water solubility, they are interesting substrates for microbial fermentations. Thus, fermentation of bio-oil as a post-processing biological strategy has been of interest [73], with few papers in the literature about it. Several studies are focused on the use of sugars present in the bio-oil, especially levoglucoscan to accumulate PHA-producing bacteria. Lemos and Moita used bio-oil from chicken beds (fast pyrolysed at 550 ^{o}C, 2 kPa pressure and short residence time) without further pre-treatment as carbon source for selection of scl-PHA-accumulating cultures in an aerobic F&F system, using MMC in a sequencing batch reactor (SBR) [69]. They later upgraded the chicken beds bio-oil by two strategies to maximise carbon utilisation toward higher PHA production: first, AF to produce VFAs for scl-PHA accumulation by MMC. Second, vacuum distillation which produced phenolics and long chain fatty acids (C20 – C23) able to be used by the MMC to produce PHA [73]. Serafim et al. used hardwood spent sulphite liquor (HSSL), a by-product of the paper industry, rich in acetic acid for PHA production by using MMC selected in a SBR under F&F conditions [65].

1.8 HYDROTHERMAL LIQUEFACTION (HTL) OF ALGAE

Among the available biomass feedstocks, microalgae, which is the feedstock for third generation biofuels (first generation biofuels refer to biofuel derived from food feedstocks,

while second generation biofuels are derived from non-edible lignocellulosic biomass) have a good potential for liquid fuel production for the following reasons:

- higher growth rates than terrestrial biomass sources;
- ability to fix CO_2 to organic substances using solar energy while growing in a wide variety of climates and lands;
- capable of storing solar energy into energy-rich compounds such as lipids;
- no direct competition for land agriculture and ability to utilise a wide variety of water sources [75].

Thermochemical processes such as pyrolysis, combustion and dry conventional gasification are utilised for dry biomass valorisation. Hence, any attempt to use these methods for wet biomass such as microalgae will require drying the biomass before the conversion step, which leads to a significant increase in energy costs [75]. Therefore, a wet biomass-handling process, such as HTL is attractive for the production of liquid fuels from wet microalgae, as it avoids the need for complete water removal and associated high energy cost for thermal drying. It is noteworthy that algae concentrations of about 5-20% are suitable for HTL, and this can be achieved with less than 5% the energy cost required for complete drying (by means of thermal drying) [76].

HTL is a process involving the reaction of biomass in water at subcritical temperatures (below 374 ^{o}C) and high pressure (above water vapour pressure) for a certain reaction time with or without the use of a catalyst [77]. Hence, it merely involves the modification of water properties at high temperature and pressure. As water approaches its critical point, the ionic product (K_w) increases, enabling it to act as an acid or base catalyst during reactions. Moreover, the increase in dielectric constant and decrease in Hildebrand solubility parameter indicate that water can increasingly act as an organic solvent, enhancing solubility of organic molecules [78]. The lipid, protein and carbohydrate macromolecules present in algae are susceptible to degradation/cracking and rapid hydrolysis under hydrothermal conditions. Hence, hydrothermal water is well suited for application as a biomass liquefaction medium [79].

Dichloromethane (DCM) is used to extract the maximum amount of product, which is often stuck to the walls of the reactor. This is then vacuum-filtered to obtain filter cake (solid residue) and heterogeneous filtrate (liquid product). To optimise the separation between the DCM bottom phase (defined as the biocrude phase) and the aqueous top phase, the filtrate is centrifuged and both phases can be recovered separately. Subsequently, DCM is evaporated

from the biocrude phase to obtain the oil. In total, the four products obtained are: a biocrude fraction, an aqueous fraction, a gaseous fraction and solid residue [75].

Dote et al. pioneered the work on the HTL of microalgae for high lipid forming *Botryococcus braunii* [80]. High oil yields of 57-65% were reported (at 300 ^{o}C and 60 mins retention time) due to the lipid content of *Botryococcus braunii* cells, already as high as 50%. However, beyond *Botryococcus braunii* (which is known as "slow growing organism" with relatively low resistance to biological contaminants, requiring highly controlled culture conditions, large efforts have [81]), large efforts have been focused on the hydrothermal conversion of other types of microalgae biomass, with fast growth or/and advantageous biological features [59]. Further developments by Minowa et al. reported oil yields of 37% for the lower lipid-containing but more rugged *Dunaliella tertrolecta* algae [82]. *Dunaliella tertrolecta* can utilise inorganic nutrients present in wastewater, saltwater or brackish water along with sunlight to produce biomass using CO_2 as carbon source. Furthermore, it is a motile specie with high tolerance to salt, temperature and light; relatively easy to cultivate and has a relatively high growth rate [83]. The catalyst used for both aforementioned HTL studies was the alkali Na_2CO_3.

Some of the most productive microalgae in terms of biomass production are lower in lipids but contain larger amounts of proteins and carbohydrates. These low lipid content algae are not economical for biodiesel production, hence their alternative processing route via HTL to produce biofuels and chemicals; involving the production and subsequent upgrading of the biocrude. This biocrude may be processed by a conventional refinery and potentially augmented with a petroleum crude. On the other hand, chemicals such as nitrogen heterocycles and n-alkanes can be separated from the biocrude [77].

Meanwhile, the aqueous phase (AP) contains low molecular mass (LMM) organic compounds, nitrogen-containing compounds and nitrogen, phosphorus, potassium (NPK) nutrients [77]. Zhou et al. found acetic acid as the most abundant compound in the AP of HTL of *Enteromorpha prolifera* macroalgae; followed by glycerol, suggesting its formation from the hydrolysis of lipids (triglycerides) contained in the algal cells [84]. They also detected several organic nitrogen-containing compounds. Brilman et al. observed the formation of NH_4^+ in the AP of a hydrothermally treated microalgae, which was not present as nitrogen nutrient in the initial growth medium (containing mainly NO_3^- and PO_4^{3-}) prior to hydrothermal treatment, HTT (called HTT, rather than HTL because experiments were carried out above 374 ^{o}C) [75]. They explained that ammonium was most likely formed by the thermal decomposition of

proteins to give initial yields of amino acids, which were further hydrolysed to produce ammonia (deamination) and after accepting a proton from water, it produced NH_4^+ and OH^-. Furthermore, the presence and formation of nitrogen-containing compounds was observed, and all the phosphorus in the AP was present in the form of PO_4^{3-}, as evidenced by the total phosphorus and PO_4^{3-} determination being nearly equal.

2. MATERIALS AND METHODS

2.1 MATERIALS AND METHODS FOR SURFACTANTS' SYNTHESIS

2.1.1 CHEMICALS

All reagents and solvents used in this study were obtained from Sigma–Aldrich® (purities ≥ 99%) and used without further purification.

2.1.2 INSTRUMENTS AND METHODS

GC-MS ANALYSIS: GC-MS analyses were performed using a 6850 Agilent HP gas chromatograph connected to a 5975 Agilent HP quadrupole mass spectrometer. The GC-MS analysis of compounds **1** and **3** was done by means of silylation: 2 drops of pyridine using a glass pasteur and ~0.1mL BSTFA containing 1% of TMCS were added to the sample to be analysed, heated at 60 ^{o}C for 15 mins, then injected to the GC-MS.

BIODEGRADABILITY TEST: Biodegradation was determined by a ready biodegradability test in an aerobic aqueous medium according to the OECD guideline 301 F, "Manometric respirometry" [85]. The test medium was prepared by adding to distilled water, potassium and sodium phosphates plus ammonium chloride, calcium chloride, magnesium sulphate and iron (III) chloride. The bacterial inoculum (derived from an activated sludge taken from a treatment plant receiving domestic sewage located in Ravenna, Italy) was aerated in mineral medium for 5 days at the test temperature. The biodegradability tests were carried out in sealed bottles for 28 days at 20±2°C. Compounds **1-4** were tested in duplicate, run in parallel with a blank (containing only inoculum). ThOD was calculated under the assumption that nitrogen was eliminated as ammonia. The consumption of oxygen (BOD) was determined by measuring the change in pressure inside the apparatus. Evolved carbon dioxide was absorbed in KOH. The amount of oxygen taken up by the microbial population during the biodegradation of the test substance (corrected for uptake by blank inoculum) was expressed as a percentage of ThOD. BOD-biodegradation is given as (BOD/ThOD) * 100%; where the BOD was measured from the apparatus and the ThOD calculated.

2.1.3 SYNTHETIC PROCEDURES

*Synthesis of Compound **1*** (1-dodecyl-5-oxopyrrolidine-3-carboxylic acid) – ***Scheme 2.1,*** **route A**: IA (2-methylidenebutanedioic acid, 7.69 mmol, 1 eq) was added to a stirring solution of dodecylamine (1.1 eq) in solventless condition and reaction heated at 120 °C for 3 h. The course

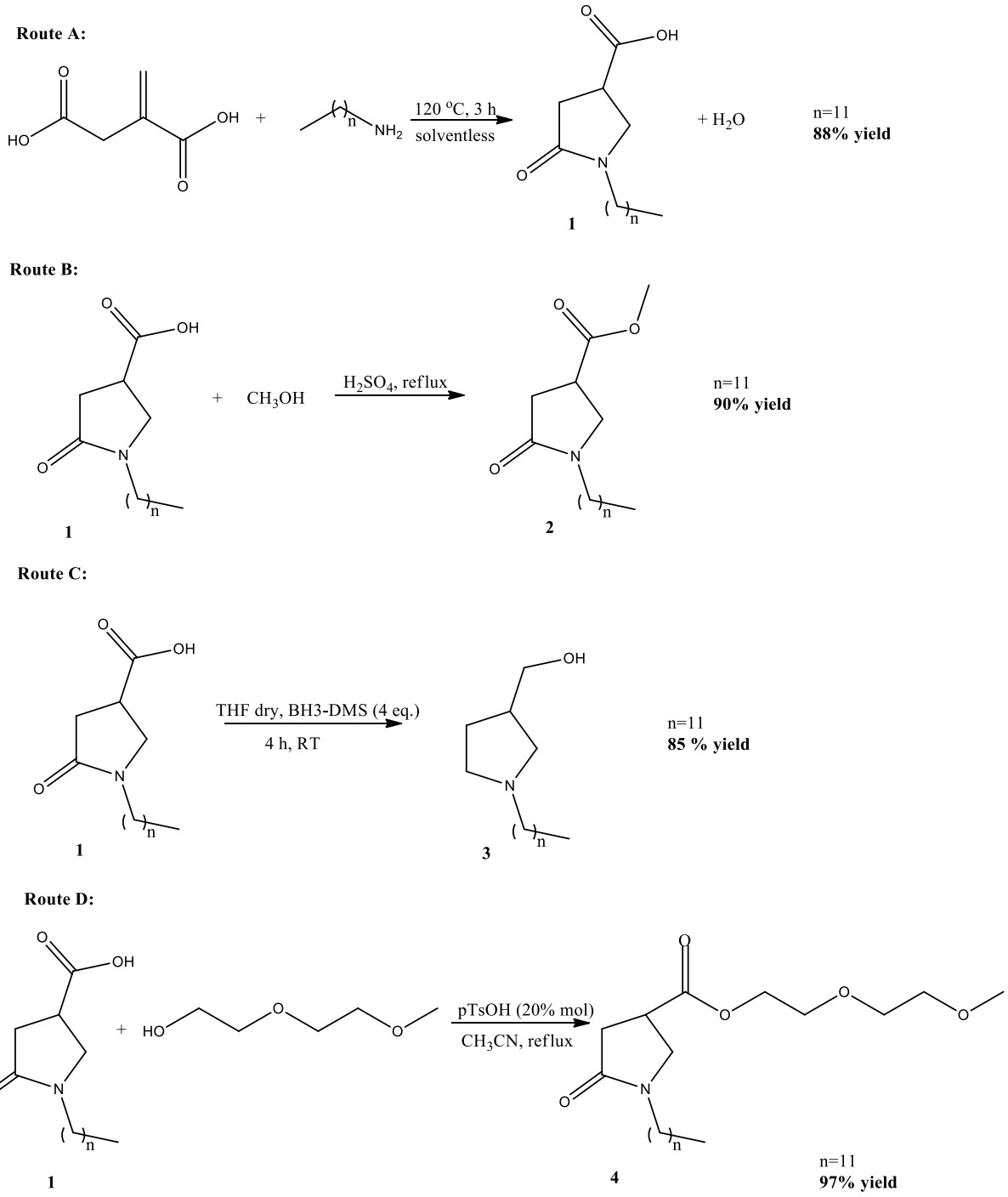
of the reaction was monitored by means of TLC (cyclohexane/ethylacetate 3:7) and GC–MS. Purification was done on a silica gel column chromatography to give a white solid (yield 88%).

*Synthesis of Compound **2*** (1-dodecyl-5-oxopyrrolidine-3-carboxylic acid methyl ester) – ***Scheme 2.1, route B***: Compound **1** (7.69mmol, 1 eq) was added to a stirring solution of CH_3OH (0.1M) and H_2SO_4 (30 % moles) and the solution stirred for 12 h under reflux. TLC (cyclohexane/ethylacetate 3:7) and GC–MS were used to monitor the course of the reaction. CH_3OH was evaporated under reduced pressure and the crude product (**2**) extracted in ethyl acetate (x3) from water. The organic layers were combined, dehydrated over sodium sulphate, filtered and the filtrate was concentrated under reduced pressure to give a solid. Purification was done on a silica gel column chromatography (yield 90%).

*Synthesis of Compound **3*** (1-dodecyl-3-hydroxymethyl pyrrolidine) – ***Scheme 2.1, route C***: Compound **3**, the reduced form of compound **1** was synthesized by the addition of borane dimethylsulfide (BH_3-DMS, 4 eq) to a stirring solution of **1** (7.83 mmol, 1 eq) dissolved in dried THF (0.43M) at O ^oC (ice bath). The ice was removed after addition of remaining borane and reaction left stirred for 4 h at RT. TLC (ethylacetate/methanol 9:1) and GC–MS were used to monitor the course of the reaction. At the end of the reaction, the borane was quenched with the slow addition of HCl/CH_3OH solution (1:3 ratio) for 1 h under reflux. Basification to pH 9 was done with NaOH (1M) after solvent (THF) was evaporated under reduced pressure. The crude product (**3**) was extracted in ethyl acetate (x3) from water. Organic layers were combined, dehydrated over sodium sulphate, filtered and the filtrate was concentrated under reduced pressure Purification was done on a silica gel column chromatography (yield 85%).

*Synthesis of Compound **4*** (1-dodecyl-5-oxopyrrolidine-3-carboxylic acid diethylene glycol mono methyl ether) – ***Scheme 2.1, route C***: Diethylene glycol monomethyl ether (DGME, 1.2 eq) was added to a stirred solution of compound **1** (3.46 mmol, 1 eq) dissolved in acetonitrile (0.1M). p-Toluenesulfonic acid (20% mol) was added, and the reaction was left stirred for 12 h under reflux. TLC (ethylacetate/methanol 9:1) and GC–MS were used to monitor the course of the reaction. Crude product (**4**) was extracted in CH_3CN (x3) from water. The organic layers were combined, dehydrated over sodium sulphate, filtered and the filtrate was concentrated under reduced pressure to give a solid. Purification was done on a silica gel column chromatography (yield 97%).

Scheme 2.1: Reaction schemes for the synthesis of compounds **1** – **4**



2.2 MATERIALS AND METHODS FOR PHA PRODUCTION

2.2.1 MATERIALS

Aside D-sorbitol (with purity 99.5%) which was purchased form Fluka®, purolite® resins purchased from Purolite S.r.l. and biodiesel provided by Novaol S.r.l. (Ravenna, Italy), all other reagents and solvents used in this study were obtained from Sigma–Aldrich® (purities ≥ 99%) and used without further purification..

The aqueous phases (obtained from HTL) of *Scenedesmus almeriensis* and *Nannochloropsis gaditana* microalgae species were provided by Department of Biosystems Engineering, University of Ghent, Netherlands (characterisation provided, see **section 3.2.2**).

2.2.2 PYROLYSIS OF PAPER BIOMASS

Pyrolysis apparatus and conditions

Paper wastes (provided by a local printing company in Modena, Italy) were pyrolysed at 500 ^{o}C for 15 mins on a lab scale pyrolyser. It consists of a tubular quartz reactor (length 650mm, internal diameter: 37 mm) placed cannulaly within a furnace refractory (Carbolite, Italy) equipped with a thermocouple, connected to a nitrogen inlet by means of pressure valve and a flow meter, and connected downstream to an ice trap and a round bottom flask for trapping condensable compounds (***Fig. 2.1***).

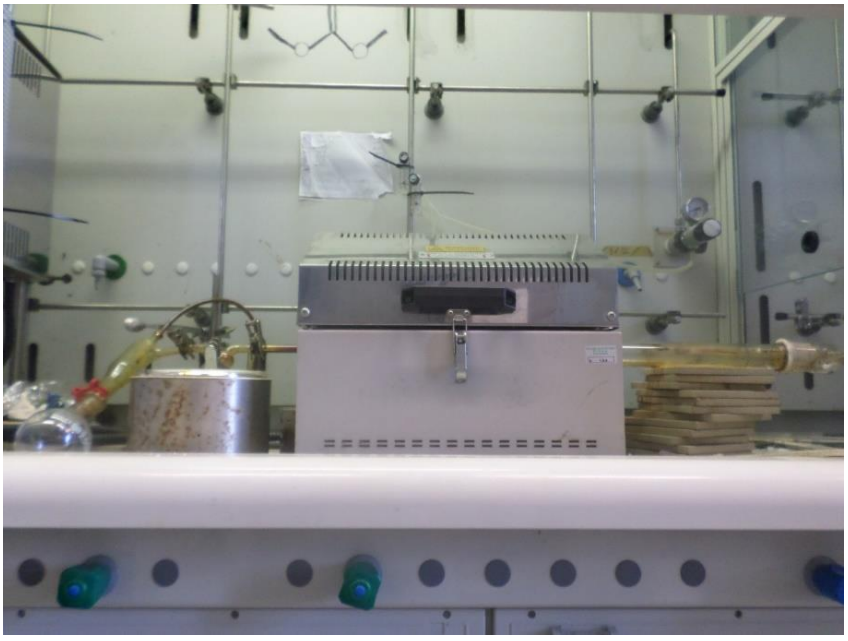


Fig. 2.1: Pyrolyser set-up

40 - 45g paper waste was rolled around the hook of a sliding quartz boat, the nitrogen flow was set at 1500cm^3 min^{-1} and the pyrolyser was turned on. As soon as the temperature inside reached 500 ^oC, the sample was inserted into the central part of the oven for 15 minutes, then retrieved downstream in the colder part of the reactor with the aid of the carrier gas, nitrogen. The bio-oil recovered in the cold trap and flask was weighed and analysed.

Bio-oil analysis and characterisation - solvent fractionation method

The elemental composition of the bio-oil was determined by using an elemental analyzer (Thermo Scientific, Flash 2000, Organic Elemental Analyzer) by means of the flash combustion technique. The water content was determined by Karl Fischer equipment. The analytical characterization was based on the solvent fractionation procedure developed by Oasmaa et al. [86,87], slightly modified. Bio-oil (1 mL) was added drop wise to a 10-fold excess of water (10 mL). The heterogeneous mixture resulting from the addition of water to bio-oil was sonicated for 10 min. The water insoluble (WI) fraction (mainly composed of degraded lignin [LMM and HMM lignin materials], extractives and char) [87] was recovered by centrifugation at 3000rpm for 10 min, filtered and weighed after drying at 80 ^oC.

The aqueous supernatant derived from the procedure described above was washed with 10mL ethyl acetate to remove solvent soluble compounds, mainly aldehydes, ketones, phenols, furans and lignin monomers (known as the water soluble /ethyl actetate soluble fraction, WS-ES); and then with 10mL cyclohexane to wash out residual ethyl acetate in the water phase. The obtained water solution was purged with nitrogen for 30 mins, in order to eliminate the residual organic solvent and analyzed by means of a Brix Refractometer, obtaining the water soluble/ethyl acetate insoluble (WS-EI) fraction [86]. It was assumed that this fraction is mainly composed of sugars: anhydrosugars and anhydrooligomers [86] (***Fig. 2.2***). The amount of high molecular weight sugar derivatives (anhydro/oligosaccharides) not detectable by using GC-MS, was calculated by subtracting the GC-MS detectable silylated derivatives of polar compounds (e.g. levoglucosan) from the total WS-EI [86,88]. This solvent fractionation experiment was carried out in triplicate.

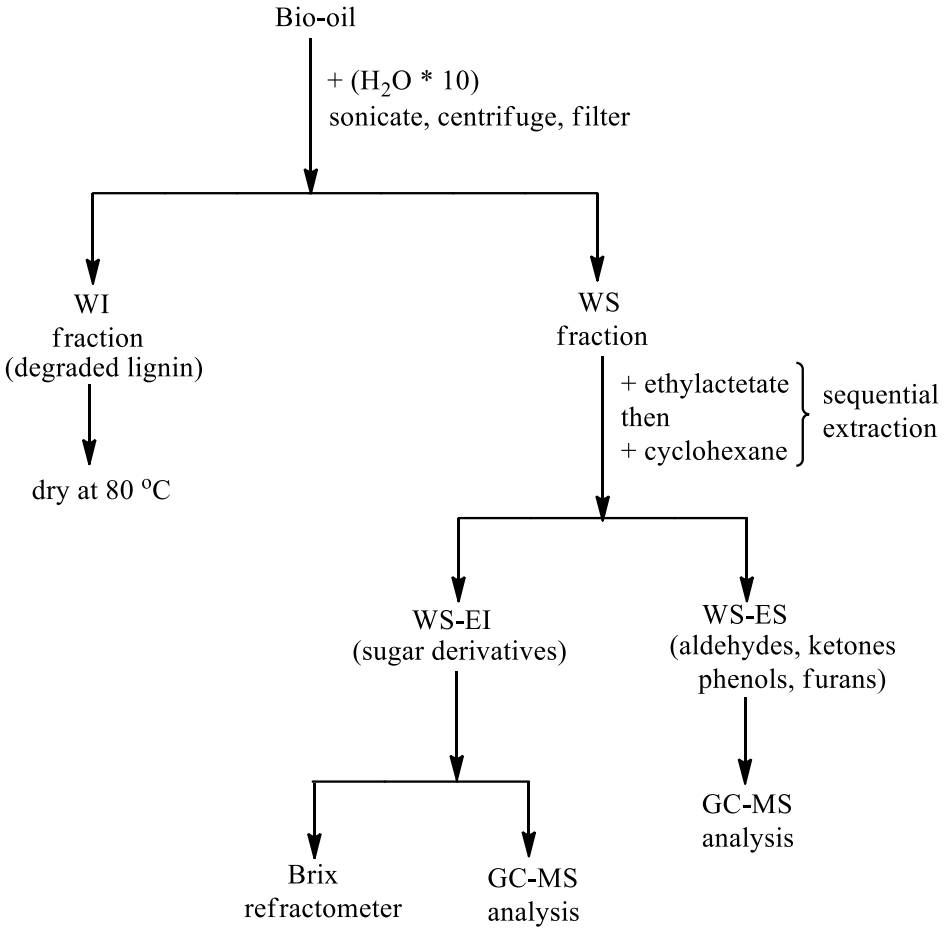


Fig. 2.2: Solvent fractionation scheme and product recovery

GC-MS analysis of the semi-volatile and heavy compounds were performed by using a 6850 Agilent HP gas chromatograph connected to a 5975 Agilent HP quadrupole mass spectrometer, while analysis of volatile compounds (such as VFAs) were performed by means of a 7820A Agilent HP gas chromatograph connected to a 5977E Agilent HP quadrupole mass spectrometer detector. In order to achieve the highest mass balance closure, different GC columns and temperature programs were used for the various semi-volatile compound classes.

Polar volatile substances (e.g. VFAs): The injection port temperature was set at 250°C. Analytes were separated by a DB-FFAP capillary column (nitroterephthalic-acid-modified polyethylene glycol, 30 m, 0.25 mm i.d., 0.25 µm film thickness), with helium as carrier gas (at constant pressure, 33 cm s^{-1} linear velocity at 200°C). Mass spectra were recorded under electron ionization (70 eV) at a frequency of 1 scan s^{-1} within the 33-600 m/z range. The

temperature of the column started from 50°C held for 5 min, then increased up to 250°C at 10 °C min^{-1}, held for 12 mins. Molecule recognition was done by use of VFA standards.

Mildly apolar semi-volatile substances (phenols and hydrocarbons): The injection port temperature was set at 280°C. Analytes were separated by means of a 5HP-MS (Agilent) fused-silica capillary column (stationary phase: poly[5% diphenyl/95% dimethyl]siloxane, 30 m, 0.25 mm i.d., 0.25 µm film thickness) using helium as carrier gas (constant pressure, linear velocity of 33 cm s^{-1} at 200°C). The temperature program consisted in a range from 50°C (5 min) to 325°C (10 min) at 10 °C min^{-1}, and 325°C post-run for 3 min.

Heavy polar GC-MS detectable substances (e.g. levoglucosan and other anhydrosugars) were determined by GC-MS analysis after trimethylsilylation as described by Torri et al. [89]: An aliquot (0.1 mL) of the bio-oil solution (1% by weight in acetonitrile) was added with internal standard (1-methyl-α-arabinopyranoside), bis-trimethylsilyltrifluoroacetamide (0.1 mL) containing 1% of trimethylchlorosilane and pyridine (0.02 mL). The solutions were placed in an incubator at 80°C for 30 min and then analyzed by GC-MS. Thereafter, the same thermal program used for 'mildly apolar semi-volatile substances' was used.

Reactive aldehydes, including acetaldehyde and hydroxyacetaldehyde, were obtained through a dimethyl acetalization with methanol [89]. The bio-oil sample (40 mg) was sonicated for 30 mins with excess methanol (4 mL) in the presence of Amberlyst 15-H (200 mg), then injected under the same GC column and conditions used for polar volatile substances. External calibration was performed applying the same procedure on standard solutions of hydroxyacetaldehyde dimer.

2.2.3 HYDROTHERMAL LIQUEFACTION OF MICROALGAE

The algal biomass species were subjected to hydrothermal liquefaction (HTL) at 350 ^{o}C for 15 min, by a procedure reported by Barreiro et al. [90]. The HTL was carried out in microautaclaves made of stainless steel 1.4571 with a volume of 10mL. 70% of their volume was filled with water containing 10 wt% of algal dry matter. The headspace was flushed with nitrogen, and subsequently pressurized up to 2 MPa, before tightly closing the microautaclaves. They were then placed in a GC-oven and the temperature adjusted to 350 ^{o}C. It took about 18 min to reach the desired temperature inside the reactor, which was then maintained for 15 min. Once the HTL was completed, the autoclaves were submerged in an ice bath for fast quenching. All the experiments were repeated until enough HTL-AP was obtained for fermentation.

2.2.4 ACIDOGENIC FERMENTATION/ACIDOGENESIS

Two up-flow anaerobic sludge blankets (UASB: working volume of 33mL each, ***Fig. 2.3***) were inoculated with sludge obtained from an anaerobic digester of a municipal wastewater treatment plant (located in Ravenna, Italy) and acclimatised to the two carbon source feeds. The systems were fed by a calibrated peristaltic pump, with 1% (10g L^{-1}) paper bio-oil in one reactor and 20% (200g L^{-1}) aqueous phase obtained from hydrothermal liquefaction (HTL-AP) of *Scenedesmus almeriensis* and *Nannochloropsis gaditana*. The paper bio-oil was diluted with tap water supplemented with ammonia and phosphate in order to keep the COD/N/P ratio of 100:5:1 (on a molar basis). The overall substrate concentration was 6.0g COD L^{-1} (60 % of 10 g L^{-1} bio-oil). The supplemented nutrient composition was prepared per litre of tap water:

- 1.34g NH_4Cl
- 0.88g K_2HPO_4
- 2mL trace element solution (composition reported in **section 2.2.6**)

Meanwhile, HTL-AP was made up with only tap water, since it already contains nitrogen and phosphates nutrients [77,90].

Fig. 2.3: Up-flow anaerobic sludge blanket (UASB) reactor used for experiment

The reactor sludge retention time was kept constant while the diluted feedstocks' (paper bio-oil and HTL-AP) flow rate was adjusted to keep a hydraulic retention time (HRT) of 2 days. The reactor was maintained at a temperature of 30 ^{o}C, with no mixing. Effluent was collected into 1L sample air-bags which is capable of measuring both the liquid and gas evolved. Effluent was analysed for VFAs and GC-MS detectable substances, and an aliquot stored at -25 ^{o}C. The remaining effluent was stored at 4 ^{o}C before the extraction of fermentation products from it.

2.2.5 CARBOXYLIC ACIDS EXTRACTION

In order to selectively remove fermentation products (di and tricarboxylic acids. E.g. VFAs) from the acidic broth medium produced during the acidogenic fermentation, and use as substrates for PHA-producing MMC (alkaline medium), it is necessary to "capture" the analytes from the acidogenic broth and "release" in the alkaline aerobic medium, with the aim of possible recycling. Resins, pure amines and amine-biodiesel mixtures [biodiesel (palm oil methyl ester, POME) as diluent], were tried as possible extractants to achieve this aim (***Table 2.1***).

Table 2.1: SPE resins used for extraction experiment

Resin	Description
Purolite® A103 Plus	Macroporous polystyrene crosslinked with divinylbenzene (DVB), having tertiary amine functionality.
Purolite® A847	Gel polyacrylic crosslinked with DVB, having tertiary amine functionality.
Lewatit® VP OC 1065	Macroporous, DVB-crosslinked polymer in spherical bead form, with benzylamine groups.
C18 SPE cartridge	Strongly hydrophobic silica-based bonded phase [surface functionality – $Si(CH_3)_2C_{18}H_{37}$].
Oasis® HLB cartridge	Strongly hydrophilic modified styrene polymer with a unique hydrophilic-lipophilic balance.
Amberlite® XAD-2 cartridge	Hydrophobic cross-linked polystyrene co-polymer resin

Solid phase extraction (SPE) experiment was carried out using the above resins in a cartridge mounted on a SPE manifold. Carboxylic acid extraction on the resin involved passing 3mL broth sample [pH 4, comprising mainly di and triethylcitrates – 1.60g L^{-1} and VFA (acetic and propionoic acids) – 1.56g L^{-1}] through a cartridge containing 1g resin, for *purolite* and *lewatit* resins (standard available commercial cartridges were used for *C18*, *HLB* and *XAD-2*) and extracted under vacuum. The extract was passed through the resin a second time. The final extract was analysed on GC-MS for VFA and ethylcitrate absence level (see **section 2.2.8**). The recovery experiment was carried out by passing 3mL aerobic digestion spent solution, (ADSS, pH 9, composition same as in **section 2.2.6** and in addition, 5.3g L^{-1} $NaHCO_3$) through the above resin-containing cartridge (having captured the fermentation products) under vacuum. This was done thrice sequentially. Citrate and VFA analyses were carried out on GC-MS (see **section 2.2.8**).

Furthermore, pure amines and amine-biodiesel mixtures were used:

- Trioctylamine (TOA)
- Perfluorotributylamine (PTFBA)
- Biodiesel + TOA
- Biodiesel + PTFBA
- Biodiesel + oleylamine (OA)

First VFA extraction experiments using pure amines (TOA and PTFBA) with(out) diluent (biodiesel) were conducted as reported by Yang et al. [87]. It was conducted with 10mL centrifuge tubes at RT. 2mL broth sample (pH 4, containing with 1.6g/L citrates and 1.44g/L VFA) and 2mL amine (2mL TOA, 2mL PTFBA, 1mL TOA + 1mL biodiesel) were added to each centrifuge tube.

The tube containing the mixture was shook for about 1 min and then left to equilibrate for 24h, followed by centrifuging at 1000rpm for 3 min to separate the two phases. The upper organic phase layer was removed and an aqueous-phase sample was then taken from the bottom layer for VFA analysis on GC-MS.

Second extraction experiments were carried out using a separating funnel at RT. Unless otherwise stated, 3mL broth sample and 2mL biodiesel containing 1% amine additive (20mg amine in 2mL biodiesel) were added to each separating funnel. The funnel was shook for 2 min

and then left for 24 h to separate into two phases. The aqueous phase at the bottom was selectively collected and analysed for VFA and citrates on GC-MS while the upper layer (containing biodiesel and 'captured' citrates/VFA on amine) was further used for the recovery experiment. The recovery was carried out twice and sequentially, using the ADSS (12mL, pH 9). In the same vein, the aqueous phase at the bottom was selectively collected from the funnel and analysed for VFA and citrate derivatives.

2.2.6 MICROBIAL BIOMASS CULTIVATION

In order to know if the acidogenic fermentation products are capable of growing and adapting our PHA-accumulating microbial culture, the experimental set-up consisted three single bench-scale reactors under specific conditions.

Lab-scale SBRs (2.5L working volume each) inoculated with an activated sludge obtained from a municipal wastewater treatment plant (located in Ravenna, Italy) were set up for the selection and PHA-enrichment of MMC. The following mineral medium composition was prepared per litre of tap water for each SBR:

- 940mg $MgSO_4.7H_2O$
- 190mg EDTA
- 210mg NH_4Cl
- 170mg $CaCl_2.2H_2O$
- 80mg KH_2PO_4
- 40mg K_2HPO_4
- 7.72mL trace element solution, which comprised (per litre of distilled water):
    - 1500mg $FeCl_3.6.H_2O$
    - 150mg H_3BO_3
    - 150mg $CoCl_2.6H_2O$
    - 120mg $MnCl_2.4H_2O$
    - 120mg $ZnSO_4.7H_2O$
    - 60mg $Na_2.MoO_4.2.H_2O$
    - 30mg $CuSO_4.5H_2O$
    - 30mg KI

First reactor was fed with triethylcitrate (**R1**) to obtain an overall concentration of 6.6 g COD L^{-1} per day, second reactor, which was a form of control, was fed with VFA (Na acetate and propionoic acid) – **R2**, to obtain an overall concentration of 6.8 g COD L^{-1} per day and the third reactor was fed with ethanol (**R3**) to obtain an overall concentration of 6.6 g COD L^{-1} per day; so each gave a C/N/P ratio 100:5:1 as potential chemical energy for the microbial community. Each cycle consisted of an initial feeding phase, an aerobic reaction phase, a sedimentation phase and a withdrawal of depleted water phase. During the overall cycle, the SBR was stirred and aerated by means of an air stone. For bacteria selection, the reactor was operated at a temperature range of 20 – 23 ^{o}C for about 26 days. Biomass slurry was sampled every 7 days for measuring the biomass concentration and freeze-dried at -25 ^{o}C for PHA analysis. This sample was also analysed for the microbial community qualitative characterisation.

2.2.7 QUALITATIVE CHARACTERISATION OF MICROBIAL COMMUNITY

Microbial community DNA extraction from freeze-dried biomass was carried out using a ZR Soil Microbe DNA MiniPrep^{TM}, Zymo Research, according to the manufacturer's instructions. In order to assess indicators of biodiversity, a PCR-DGGE analysis with further gene sequencing was carried out in outsourcing (Micro4yoUsrl, Milan, Italy), according to established protocols [91-93].

2.2.8 ANALYSES

Analysis of VFA

Analysis of VFAs was performed using a polar column 7820A Agilent HP gas chromatograph connected to a 5977E Agilent HP quadrupole mass spectrometer detector (details aforementionedin **section 2.2.2**), optimized for analysis of VFAs. An analysis aliquot of the acidogenic fermentation broth output (0.1mL) was added to 0.1mL of 1ppm internal standard solution (2-ethylbutyric acid in distilled water) and 0.1mL of saturated $KHSO_4$ solution, with 1mL DMC as solvent. It was centrifuged for a min at 3500 rpm to have a conspicuous biphasic system. The upper phase which contains the DMC and VFAs was selectively injected into the GC-MS by means of an auto-sampler. Calibration was performed by applying the same procedure to standard solutions containing known amount of the five VFAs analysed (acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid). The VFA concentration was expressed as COD of VFA (g COD L^{-1}) which was calculated from the ThOD of each VFA using the following formula:

$$COD_{VFA} = \sum_i ThOD_i \cdot VFA_i$$

where $ThOD_i$ (g ThOD g^{-1}) is 1.07, 1.51, 1.82, 2.04 for acetic acid, propionic acid, butyric/isobutyric acid, valeric/isovaleric acid respectively and VFA_i is the concentration of different VFA in g L^{-1}.

Analysis of GC-MS detectable compounds

Heavy polar GC-MS detectable compounds were analysed using a 6850 Agilent HP gas chromatograph connected to a 5975 Agilent HP quadrupole mass spectrometer, after trimethylsilylation: An aliquot (0.2mL) of the acidogenic fermentation broth output was dried at 80 ºC under air, 0.1mL of 100ppm internal standard solution (D-sorbitol dissolved in acetonitile) was added, 0.1mL BSTFA containing 1% of TMCS and 2 drops of pyridine using a glass pasteur. The solution was heated at 60 ºC for 30 mins, then analysed by GC-MS.

Analysis of PHA

This was done under thermal treatment (pyrolysis) of the freeze-dried biomass, on the basis that PHAs are degraded in high yield (>40%, w/w PHA) to the corresponding 2-alkenoic acid (e.g. crotonic acid from PHB). Freeze-dried microbial biomass (4 – 6 mg) was pyrolysed at 350 ºC for 20 mins. The pyrolysate was spiked with 0.05mL internal standard solution (5 µL mL^{-1} 2-ethylbutyric acid in acetonitrile) and acetonitrile (4 mL) was added. It was shook for 1 min and the acetonitrile solution (1 mL) was injected into the polar GC column for 2-alkenoic acids [(*E*)-2-butenoic acid/crotonic acid, (*E*)-2-pentenoic acid, (*E*)-2-hexenoic acid] analysis (see **section 2.2.2**).

Calibration for PHA determination

The amount of PHAs in the biomass sample was determined from the quantity of the most abundant 2-alkenoic acid (Q_{AC}) evolved from the pyrolysis of the corresponding monomer in the polymer chain. The three aforementioned 2-alkenoic acids (ACs) were utilised for quantitation, and Q_{AC} in the pyrolysate was calculated from the following formula:

$$Q_{AC} = Q_{IS} \cdot A_{AC}/A_{IS}$$

Where Q_{IS} is the mass of the internal standard (2-ethylbutyric acid) expressed in mg, while and A_{AC} and A_{IS} are the GC-MS peak areas on the AC and internal standard respectively. The relationship between the quantity of the selected AC (Q_{AC}) and the corresponding quantity of the monomer of the pyrolysed sample ($X_{HA} \cdot Q_{PHA}$) was determined using the calibration curve

obtained by pyrolysing standard polymers of known monomer composition, where X_{HA} is the mass fraction of the monomer in the polymer. The standard PHAs used in this study gave a X_{HA} of 1, 0.75 and 0.90 for the 2-hydroxybutanoate from PHB, P(3HB-co-3HV) and P(3HB-co-3HX) respectively.

3. RESULTS AND DISCUSSION

3.1 RESULTS AND DISCUSSION OF SURFACTANTS' SYNTHESIS

An interesting group of surfactants presented in the last years contain some examples derived from polycarboxylic acids like furmaric acid, IA and aconitic acid [94]. In this group, IA has not been fully exploited as building block in surfactant synthesis [95].

Our group recently reported the physicochemical properties and rheological behaviours of compounds **1**-**3** as exploitable pool of surface active compounds [6]. Moreover, these surfactants act as micellar catalysts in water phase for performing cross-coupling and condensation reactions. This study is aimed at evaluating the biodegradability of these compounds, in addition to a newly synthesized compound, **4**.

3.1.1 SYNTHESIS

Synthetic routes used for compounds **1**–**4** are represented in ***Scheme 2.1***. The compounds prepared are characterized by a 3-substituted pyrrolidine ring with an attached hydrophobic C12 alkyl chain, and can be divided into two groups that belong to two classes of surfactants: ionisable in water solution (**1,3**) and non-ionic (**2**,**4**). Compound **1** bears a 2-pyrrolidone ring and a 3-carboxylic acid functionality, compound **2** has a 2-pyrrolidone ring with a methyl ester group, compound **3** has pyrrolidine ring and an hydroxymethyl moiety on position three of the ring, while compound **4** bears a 2-pyrrolidone ring and an oxoethoxylated chain (diethylene glycol mono methyl ether) on position three of the ring.

The pyrrolidone scaffold was obtained by conjugate addition of an amine to the methylidene group of IA followed by intramolecular spontaneous lactamization between the carboxylic acid in β-position and the secondary amine. Paytash et al. studied the conjugate Michael addition reactions of primary amines to the double bond of IA in solvents like toluene and water [96].

The synthesis of **1** proceeded smoothly under a solventless condition at a temperature of 120 ^{o}C. Methylation of carboxylic acid functionality of compound **1** under acid catalysis afforded the carboxylic acid methyl ester (**2)**. Compound **3** has been obtained by reducing the lactam functionality of **1**. Compound **4** has been synthesized by the ethoxylation of the carboxylic acid functionality of compound **1** with DGME.

3.1.2 BIODEGRADABILITY

Two of the main problems of most surfactants lie in their non-biodegradability and the fact that they are low-dose permanent toxicants in the water ecosystem (e.g. in pharmaceuticals and personal care products). Hence, studies of surfactant toxicities and biodegradation are necessary to withdraw highly toxic and non-biodegradable compounds from commercial use and replace them with more environmentally friendly ones [97]. Surfactants in the environment are primarily degraded through microbial activity, and in sewage treatment plants. Surfactant's chemical structure and physicochemical conditions of its environmental medium are the main determining factors of their biodegradation [97]. Percentage biodegradability of surfactants **1-4** determined in this study are shown in ***Fig. 3.1***.

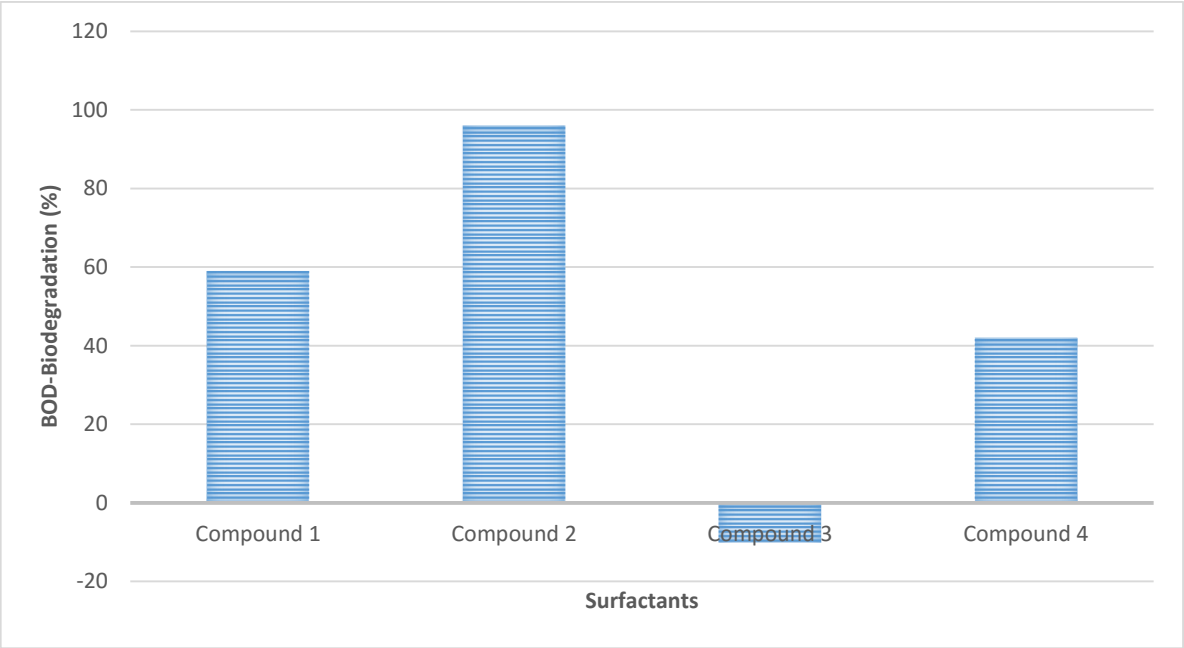


Fig. 3.1: BOD-Biodegradability of compounds **1** – **4** (28 days)

The OECD guidelines state that a compound can be defined as "readily biodegradable" if its level of biodegradation reaches 60% of ThOD, achieved within 10 days after starting the degradation [85]. Thus, of the four surfactant types synthesized, only one is readily biodegradable, two are less biodegradable while one is non-biodegradable.

Compounds **1** and **3** though ionisable in water were not readily biodegradable. While the former was very close to ready biodegradability (59%), the latter was not only non-biodegradable but

resulted in cell lysis (-10%) – noticed by the lower amount of bacteria in the sample than in the blank. On the other hand, compound **2** was found to be readily biodegraded (96%) by activated sludge and perhaps due to the presence of the methyl ester on the 2-pyrrolidone ring. Compound **4** though has an ethoxylated chain on the head is even far less biodegradable (42%) than compound **2** but has a BOD-biodegradation value closer to that of compound **1**. The explanation of Stolte et al. perhaps holds on this case: the effect on the biodegradability that the introduction of oxygen atoms in the alkyl chain can have, depends on the position. The presence of a carboxylic acid moiety facilitates the β-oxidation pathway (for compound **1**) and the two released carbon fragments can enter the tricarboxylic acid cycle as acetylCo-A [98]. On the other hand, the presence of one or more alkoxy units does not significantly improve the primary biodegradation. This finding has already been observed for ethoxylated imidazolium ionic liquids, for which the biodegradation in soil was lower than that of the alkyl counterparts [99].

In comparing the biodegradation of non-ionic compounds **2** and **4** with the traditional alkylphenol ethoxylate (APE) nonionic surfactant (***Fig. 3.2***): APE has a biodegrability of 96% at 25ºC [97] which is similar to the biodegradability of compound **2** at 20±2°C. However, biodegradation of APE results in the shortening of the ethoxylated chains to alkyl phenol carboxylates, ultimately leading to octylphenols (OP) and nonylphenols (NP), both of which have low water solubility and adsorb to suspended solids and sediments [100]. NP is not only approximately 10 times more toxic than its ethoxylate precursor, but also less biodegradable than APE [100,101]. NP has a well-known estrogenic activity and accumulate in aquatic organisms [97,100]. Sub-lethal toxic effects of NP on zooplankton in natural waters interferes with their sex determination and development [102]. These problems are leading to bans and restrictions on the use of APE for household and industrial cleaning applications in Europe [101].

As a result, a further study on the ecotoxicity of compound **2** will be imperative in the quest for environmentally friendly surfactant.

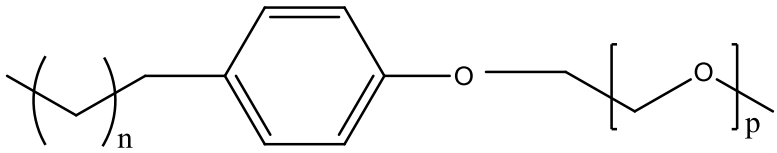


Fig. 3.2: Struture of alkylphenol ethoxylate (APE)

Juxtaposing the biodegradation of ionisable compounds **1** and **3** with widely commercial linear alkylbenzene sulphonic acid (LAS) anionic surfactant (***Fig. 3.3***): LAS are generally regarded as biodegradable surfactants, with very high levels of biodegradation (97 – 99%) under aerobic bacterial process. On the otherhand, compound **1**, which bears a 2-pyrrolidone ring and a 3-carboxylic acid functionality was close to ready biodegradability (59% in 28 days), which in principle can be considered biodegradable.

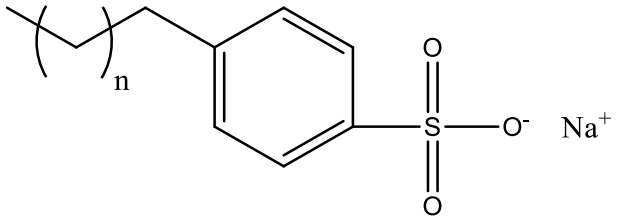


Fig. 3.3: Struture of linear alkylbenzene sulphonic acid (LAS)

3.2 RESULTS AND DISCUSSION OF PHA PRODUCTION

3.2.1 PAPER PYROLYSIS OIL COMPOSITION

Pyrolysis is one of the fastest ways to depolymerise lignocellulosic biomass, which are considered the most ubiquitous and inexpensive sustainable carbon sources [72,73], into smaller fragments that can be anaerobically fermented by bacteria. With the much abundance of waste paper in our environment, converting them to high-value chemicals and materials will be attractive for both economic and environmental concerns. Typical paper which is composed of lignin, cellulose and hemi-cellulose can be pyrolysed to obtain the oil phase (pyrolysis oil/ bio-oil) that can be anaerobically fermented to produce VFAs for PHA production. To the best of our knowledge, this is the first time paper pyrolysis oil is exploited for PHA production, and here lies the novelty of this study.

The amount of bio-oil was calculated as the difference between the weight of the trap system before and after the pyrolysis run. The pyrolysis of 44.2g of paper biomass yielded 16.7g of solid residue (bio-char) and 14.5 g of bio-oil (***Fig. 3.4***).

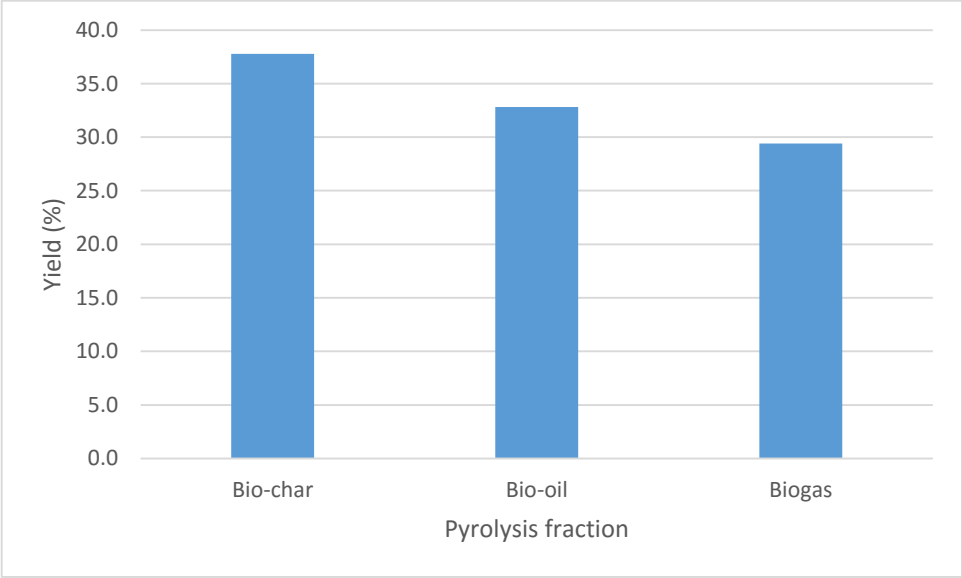


Fig. 3.4: Paper biomass pyrolysis fractions and yield

The ThOD, according to OECD guideline 301F was calculated from elemental analysis using the following formula [85]:

$$ThOD = 16 \cdot (2 \cdot C/12 + H/2 + 2 \cdot S/32 - O/16 - 3 \cdot N/28)$$

where C, H. S, N and O are the % w/w of carbon, hydrogen, sulphur, nitrogen and oxygen respectively. The BOD was determined by a ready biodegradability test (***Fig. 3.5***) in an aerobic aqueous medium according to the OECD guideline 301F, ‘‘Manometric respirometry’’ for 28 days at 20 ± 2 ºC, using bacterial inoculum from an activated sludge taken from a treatment plant receiving domestic sewage (located in Ravenna, Italy).

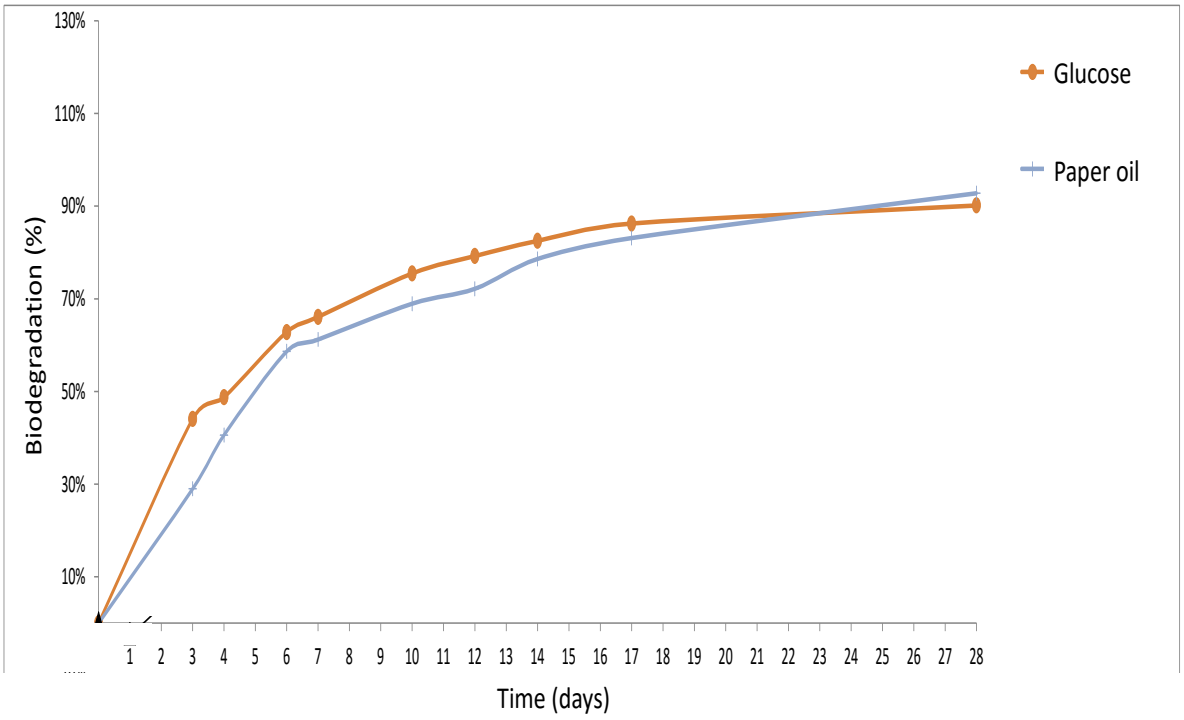


Fig. 3.5: BOD-Biodegradability of paper bio-oil and glucose as reference (28 days)

As shown in ***Table 3.1***, paper intermediate pyrolysis oil contains a relatively high amount of water (43.3%), a high amount of water soluble constituents (40.4%) with 82% if it being sugar derivatives (majorly levoglucasan), and 18% composed of other aldehydes, ketones, acids, phenols and furans.

Table 3.1: Chemical characterization of paper bio-oil in terms of yield (% w/w_{paper}) and elemental analysis (% w/w), oxygen demands (g g^{-1}), main fractions from solvent fractionation (% g g^{-1}), GC-MS detectable compounds (% g g^{-1}) and VFAs (% g g^{-1}). Values are reported as mean ± standard deviation.

	Bio-oil
Yield	32.8
C	29.0
H	5.5
N	0.1
S	0.0
O	64.0
Ash	1.0
ThOD (gCOD g^{-1})	0.60
COD (gCOD g^{-1})	0.57
BOD (gBOD g^{-1})	0.03
Water	43.3 ± 0.3
WS-EI	33.1 ± 1.0
WS-ES	7.3 ± 1.8
WI	17.1 ± 0.6
Levoglucosan	27.8 ± 0.6
Mannosan	1.5 ± 0.1
Other anhydrosugars	0.5 ± 0.0
Hydroxyacetaldehyde	5.8 ± 0.3
Levolinic acid	2.2 ± 1.1
Phenols	1.4 ± 0.0
Furans	1.1 0.0
Hydroxyacids	0.3 ± 0.1
Small oxygenated compounds (aldehydes, ketones, alcohols)	4.5 ± 0.2
Acetic acid	0.6 ± 0.0
Propionic acid	0.2 ± 0.0
Butyric acid	0.1 ± 0.0
Sum GC-MS detected	46.0
Sum VFAs	0.9

37.8% of the paper pyrolysed gave the solid residue (bio-char), 32.8% condensed in the traps as liquid (bio-oil) and the remaining 29.4% constituted the non-condensable gases (***Fig. 3.4***). From ***Table 3.1***, the paper bio-oil has a high amount of oxygen content (64% w/w) and high water content (43.3±0.3), which makes it a low-quality fuel that cannot be used in conventional gasoline and diesel fuels engines, and any further upgrading to suit this application makes it uneconomical when compared to petroleum-based fuels. However, being a polar bio-oil with

high concentrations of aldehydes, ketones, alcohols, carboxylic acids and other polar components, it is an interesting substrate for microbial fermentations.

3.2.2 MICROALGAE AQUEOUS PHASE CHARACTERISATION

Microalgae in the past few years as gained much attention as a good potential feedstock for liquid fuel production. Reasons discussed in **section 1.8**. Being a wet biomass, thermochemical valorisation leads to high energy costs as a result of drying the biomass before the conversion step [75]. Hence, the need to use a wet biomass-handling process, such as HTL is preferred and attractive for the production of liquid fuels from microalgae, as it avoids associated high energy cost for thermal drying.

Not until recent years, the oil fraction of microalgae valorisation by HTL has been the main fraction of interest, while the aqueous phase (AP) discarded as 'waste'. Meanwhile, some of the most productive microalgae strains in terms of biomass production are lower in lipid content but contain larger amounts of proteins and carbohydrates, hence are not valuable for biodiesel production. The concept in this thesis was to use the 'waste' aqueous phase obtained after HTL (HTL-AP) of the microalgae as substrate for VFA production via fermentation.

In this experiment, two species of commercially exploited microalgae [103], having quite similar HTL-AP composition, were used.

Table 3.2: Aqueous phase obtained from hydrothermal liquefaction of microalgae (HTL-AP)

Strain	TC ($g\ L^{-1}$)	TIC ($g\ L^{-1}$)	TOC ($g\ L^{-1}$)	TN ($g\ L^{-1}$)
Scenedesmus almeriensis (18.2%)	27.534	3.028	24.506	7.967
Nannochloropsis gaditana (18.2%)	28.108	2.800	25.308	8.504

TC: total carbon, TIC: total inorganic carbon, TOC: total organic carbon, TN: total nitrogen

Table 3.2 summarises the composition of the HTL-AP (obtained at 350 °C and 15 min, 18.2% concentration) from *Scenedesmus almeriensis* (fresh water) and *Nannochloropsis gaditana* (marine). The former was employed for acidogenesis for the first 50 days while the latter was employed for the remaining 14 days of fermentation.

From ***Table 3.2***, 89% of the total carbon content of *S. almeriensis* is the organic fraction capable of being fermented by acidogenic bacteria, while *N. gaditana* has 90% organic fraction available for fermentation. Moreover, both species have being reported by Barreiro et al. to have ammonium concentrations of 66% (for *S. almeriensis*) and 92% (for *N. gaditana*) of the total nitrogen content [90]. These correspond to 5.2 and 7.8 g L^{-1} ammonium concentrations respectively.

3.2.3 ACIDOGENIC FERMENTATION RESULTS

The intrinsic advantage of biological processing is that bacteria are able to convert, at low temperature, a large array of compounds with different chemical features to few or single product(s) which can be obtained in high purity form. Anaerobic fermentation/digestion is a complex biochemical process of biologically mediated reactions by a consortium of microorganisms to convert organic compounds into methane and carbon dioxide, as final products. It typically involves four stages: hydrolysis – chemical reaction that involves solubilisation of particulates and breakdown of large polymers into simple monomers; acidogenesis – biological reaction where simple monomers are converted into VFAs; acetogenesis – biological reaction where VFAs are converted into acetic acid, carbon dioxide and hydrogen; methanogenesis – the final biological stage where acetates are converted into methane and carbon dioxide, while hydrogen is consumed.

PAPER BIO-OIL FERMENTATION

Fermentative pathways are able to partially deoxygenate sugars with high selectivity, in comparison to chemical pathways [104]. On the other hand, pyrolysis oil contains several compounds at percent level which are toxic to microorganism, hence process optimisation is important to exploit biological transformation, overcoming the toxic effect of pyrolysis derived chemicals [74].

A UASB reactor (33mL working volume) was inoculated with mixed bacterial sludge and acclimatised to the bio-oil feed for a period of 61 days. The sludge was retained in the reactor throughout the fermentation period, while the bio-oil had a HRT of 2 days.

Fig. 3.6 shows the trend in the conversion of paper bio-oil substrate into VFA intermediate and triethylcitrate (TEC). Data reported are the mean value of duplicate, which showed an acceptable relative standard deviation not more than 21% and were explicated as $gCODL^{-1}$ in

order to compare directly the various products in term of chemical energy. The first 18 days of acidogenesis produced exclusively VFAs, mainly acetic and propionic acids, with as high as 1.6 $gCODL^{-1}$ VFA (***Fig. 3.6***). Subsequently, VFA production gradually decreased to zero, while the formation of ethylcitrates (EC: TEC and DEC) was observed from the 19th day until the end of the fermentation process (***Fig. 3.7***). A sharp increase in ethylcitrates content was observed at the start of the citrate production with a peak value of 2.8 ± 0.1 $gCODL^{-1}$, after which it gradually decreased to peak values of roughly 1.0 ± 0.2 $gCODL^{-1}$.

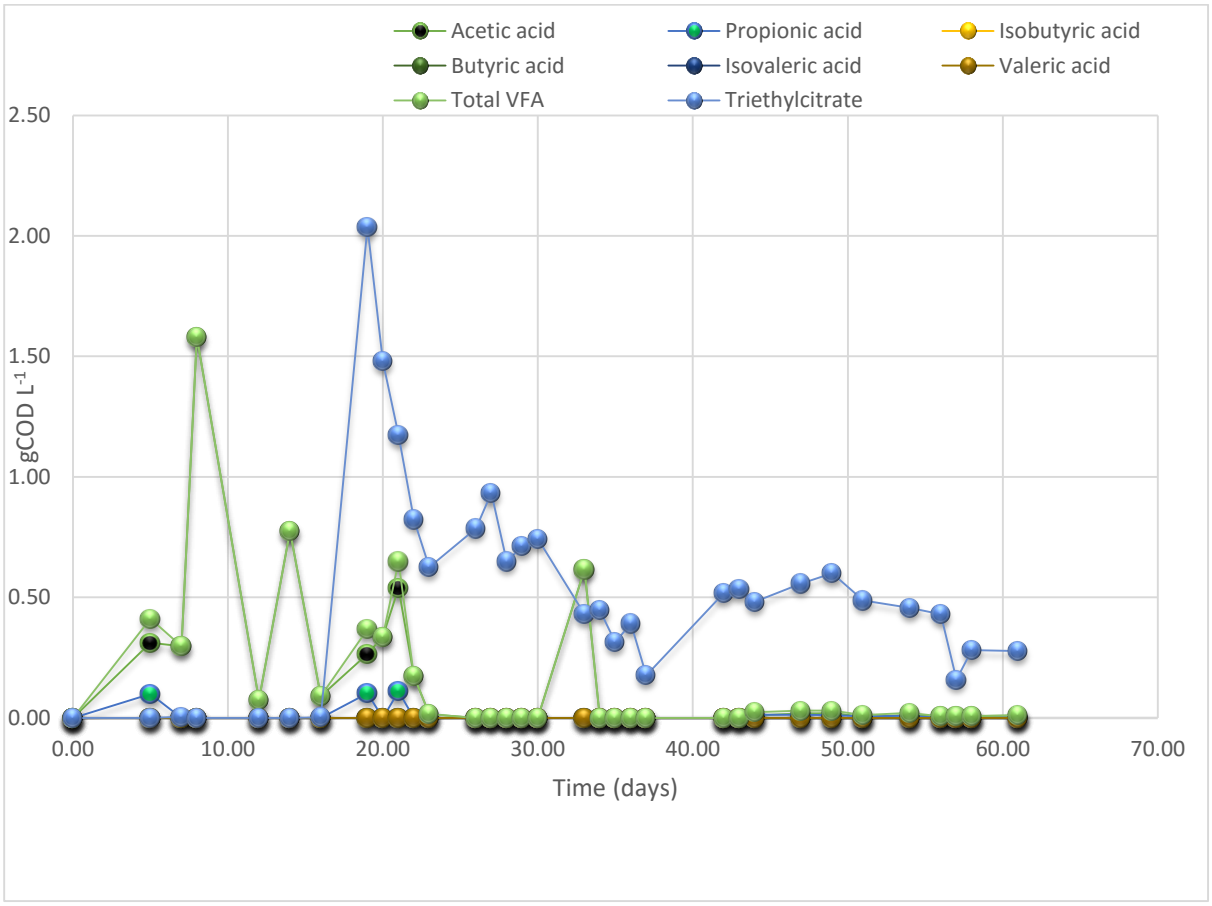


Fig. 3.6: Trend of VFA and triethylcitrate generated during acidogenic fermentation of paper bio-oil

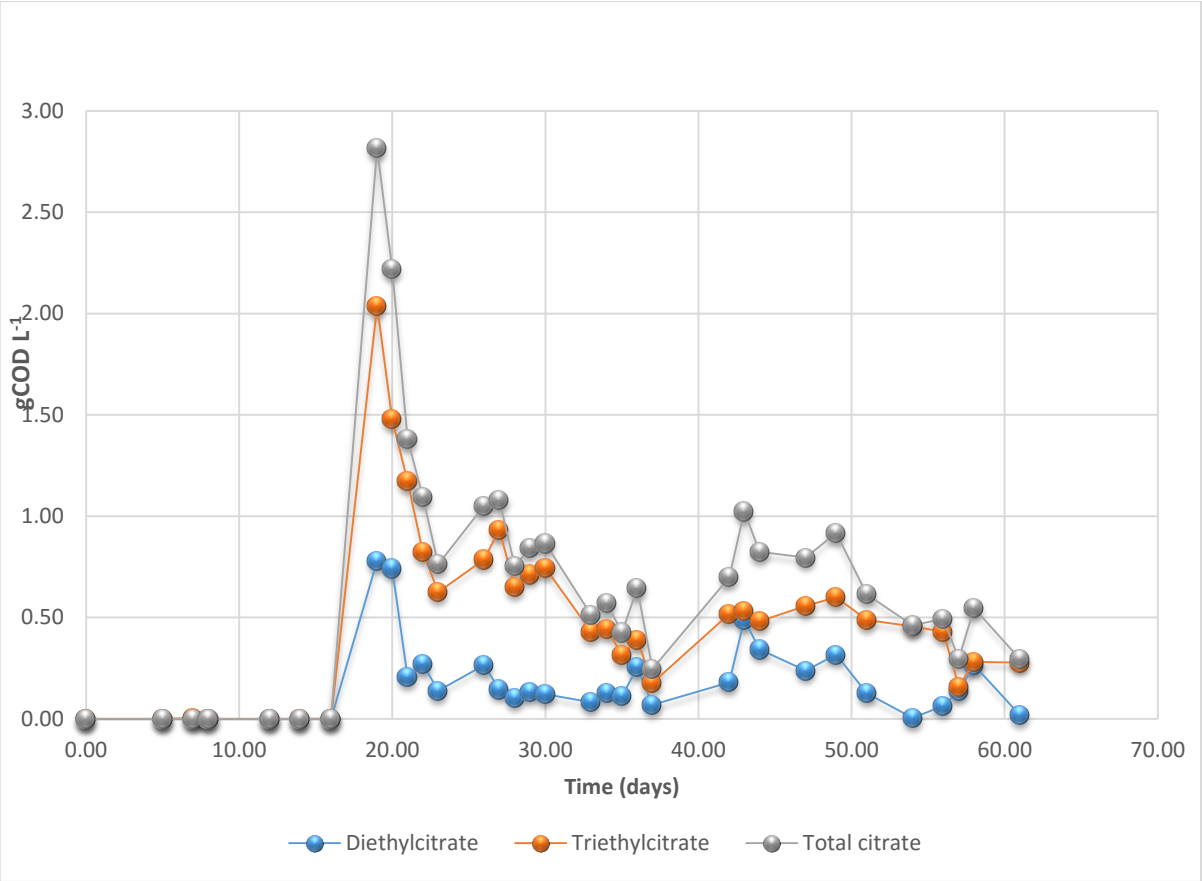


Fig. 3.7: Trend of DEC, TEC, total citrate (DEC and TEC) generated during acidogenic fermentation of paper bio-oil

Looking at the product yield (***Fig. 3.6*** and ***3.7***), with an inlet COD of 5.7g L^{-1}, the maximum VFA peak produced corresponded to 28% while that of EC corresponded to to 49 ± 2 %. The former is comparable with the VFA production result reported by Torri et al. who used corn stalk pellets pyrolysis oil as substrate for VFA intermediate production [74]. In totality, TEC were in much higher yield than DEC, with at least two third of the EC produced being TEC (***Fig. 3.7***). It is also worth mentioning that the undulating trend of VFA and EC produced by the mixed culture is consistent with literature [74] and is partly a function of the response of bacteria to use carbon substrate and convert per time.

The acidogenic fermentation which lasted 61 days was sucessful in the conversion of anhydrosugars in the bio-oil (mainly levoglucosan, see ***Table 3.1***) to several fermentation products. The MMC was able to convert the pyrolysis products into among several products, VFAs, our original compound of interest, and ethylcitrates – first time this is reported as a product of acidogenic fermentation. While VFAs have been considered the best substrates for

PHA accumulation by MMC [73], it was thought interesting to investigate if our MMC can also accumulate PHA from TEC (a triester of ethanol and citric acid, and the major fermentation product), to know the possibility of using directly the fermentation products for PHA accumulation. Hence, the reason for the three SBRs selection in **section 2.2.6** (**R1** – TEC as substrate, **R2** – VFA as substrate, **R3** – Ethanol as substrate).

MICROALGAE AQUEOUS PHASE ACIDOGENESIS

A UASB reactor (33mL working volume) was inoculated with mixed bacterial sludge and acclimatised to the microalgae HTL-AP feed for a period of 64 days. The sludge was retained in the reactor throughout the fermentation period, while the substrate had a HRT of 2 days.

Fig. 3.8 and ***3.9*** show the trend in the conversion of microalgae HTL-AP substrate into VFA intermediate and EC. The presence of VFAs can be observed at the beginning of the fermentation (day zero, 0.5gCODL^{-1}) and gradually decreased until the 22nd day to 0.04gCODL^{-1}, after which it increased with a peak value of 1.0gCODL^{-1}. Meanwhile, the period of low VFA production afforded EC yield, with a maximum yield of 0.7gCODL^{-1} on the 22nd day, and then decreased gradually to 0.05gCODL^{-1}.

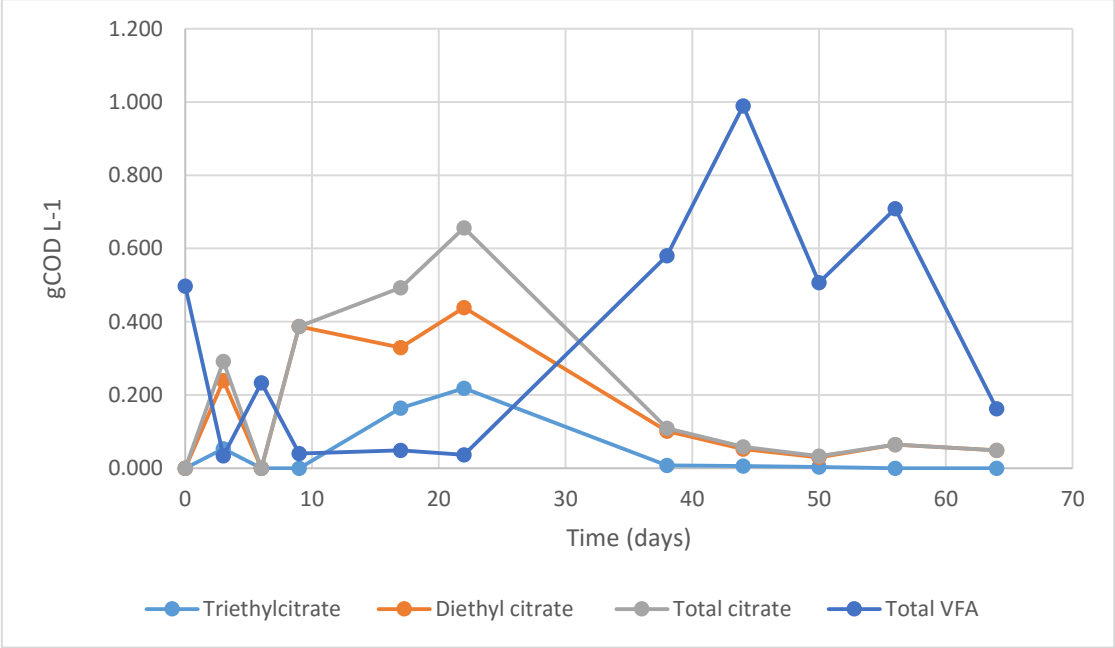


Fig. 3.8: Trend of DEC, TEC, total citrate (DEC and TEC) generated during acidogenic fermentation of microalgae HTL-AP

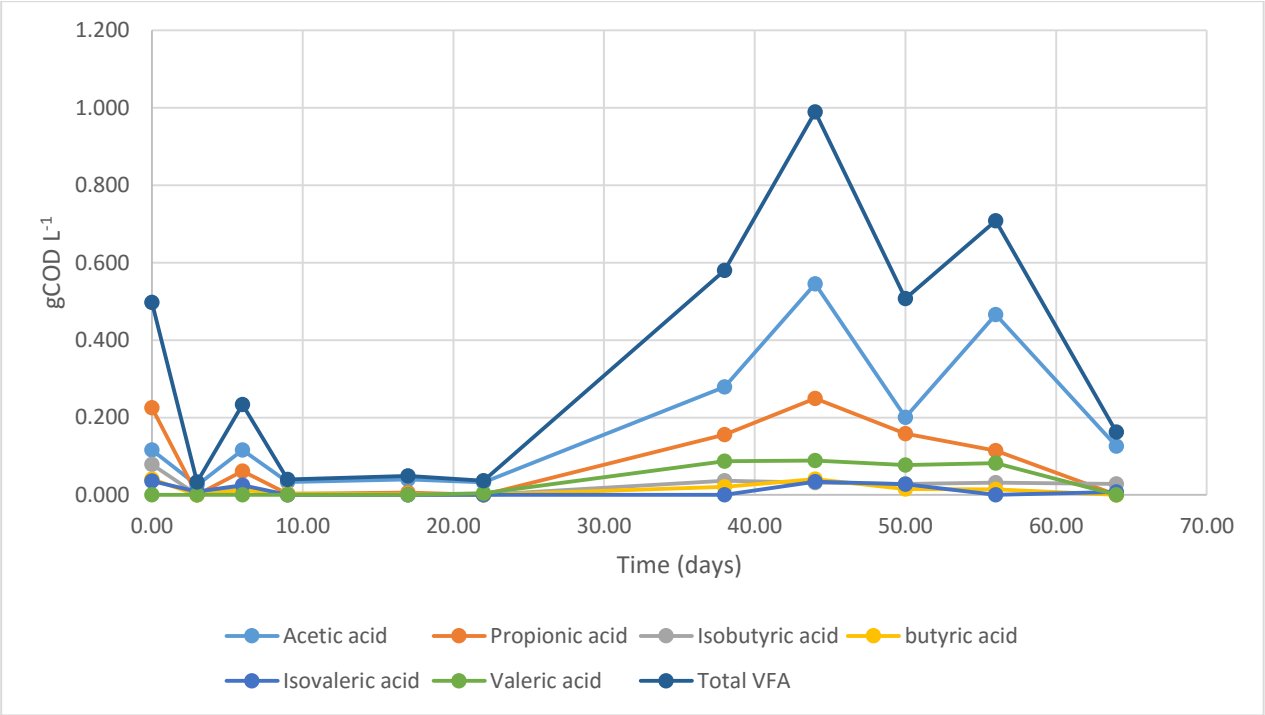


Fig. 3.9: Trend of VFA and triethylcitrate generated during acidogenic fermentation of microalgae HTL-AP

Form the product yield in ***Fig. 3.8*** and ***3.9***, an inlet substrate COD of 13.1g L^{-1} afforded peak values that correspond to 7.6% VFA and 5.3% EC yields. DEC was in a higher yield than TEC and contributed to more than two third of the total citrates produced. This is hypothesized to be a result of selective inhibitors in the metabolic pathway that hampers the further esterification to DEC. The presence of VFAs observed at the beginning of the fermentation is quite in accordance with the report of Zhou et al. [84] on the presence of some VFAs such as acetic acid in the AP of some algae species. Furthermore, the low product yield observed was due to the formation of ammonia (formed as a result of the high nitrogen content in the substrate), which inhibited the acidogenic process, and in turn, evolved methane. Therefore, it can be hypothesized that microalgae AP could act as a strong inhibitor for acidogens, consequently leading to poor acidogenic fermentation products yield.

By juxtaposing the paper oil and microalgae AP fermentation processes, the following were observed: First, at each point of analysis, the production of VFA is inversely proportional to EC production, during the microalgae AP fermentation process (***Fig. 3.8***). Second, unlike paper oil acidogenesis, DEC produced with microalgae HTL-AP fermentation were in a higher yield

than TEC. Thirdly, the microalgae AP acidigenesis produced a wider variety of VFAs though in lower concentration when compared to paper pyrolysis oil fermentation.

3.2.4 EXTRACTION OF FERMENTATION PRODUCTS

Extractive recovery of carboxylic acids from dilute, aqueous solutions such as fermentation broth and waste water, which have low acid concentrations (typically lower than 10% w/w), has received increasing attention. In this study, it was not only important to use an efficient extractive to 'capture' the interesting fermentation products (carboxylic acids: VFAs, di and triethylcitrates) from the acidogenic broth, but to also 'release' them as substrates for the next stage of the process (aerobic fermentation).

ETHYLCITRATE EXTRACTION AND RECOVERY

In the quest to optimise the extraction and recovery efficiencies of the extractants used, some adjustments were made to the procedure described in **section 2.2.5**. First, fermentation products extraction using *HLB* cartridge was carried out once [reported as "*HLB* (once)" in ***Fig. 3.10*** and ***3.11***] before the second extraction (reported as "*HLB*" in ***Fig. 3.10*** and ***3.11***); subsequent extraction with the other resins was done twice. Second, after the use of 3mL broth sample for the separating funnel extraction experiment, a higher volume of 20mL was also used (reported with the suffix "bis" in ***Fig. 3.10*** and ***3.11***) to compare results based on difference in volume.

As shown in ***Fig. 3.10***, all the resins used, except *XAD-2* showed excellent extraction of triethylcitrates (TEC, the most abundant fermentation product) from the acidogenic broth under the conditions described in **section 2.2.5**. *Purolite A103*$^{+}$ and *HLB* resins afforded the best extraction (99% and 96/92% respectively); at the same time, *lewatit*, *C18* and *purolite A847* gave good extraction of TEC (87, 78, 72 % respectively). The *purolite* resins exhibited good diethylcitrate (DEC) extraction (*A103*$^{+}$ – 86% and *A847* – 91%), while *C18* and *lewatit* performed poorly for DEC extraction (25% and 0% respectively). Meanwhile, the first extraction of the fermentation products with *HLB* cartridge afforded 92% extraction of TEC and no extraction of DEC, however, a second extraction slightly increased the capturing of TEC to 96% and more importantly gave a 67% extraction of DEC. This suggested that a second SPE experiment was necessary for maximum capture of the citrate derivatives. Hence, the extraction experiments were carried out twice.

With respect to the total ethylcitrate (EC, i.e. DEC and TEC) extracted by the resins (***Fig. 3.11***), *lewatit* showed the lowest capture performance (44%) in comparison to others. Next in performance are the strongly hydrophobic resins (*C18* and *XAD-2*), affording 52% and 64% extraction respectively. *HLB* and the two *purolite* resins were the highest performing extractants for citrates: while *purolite A847* showed a good citrate capture performance (82%), *purolite A103*$^{+}$ exhibited a more excellent performance (93%).

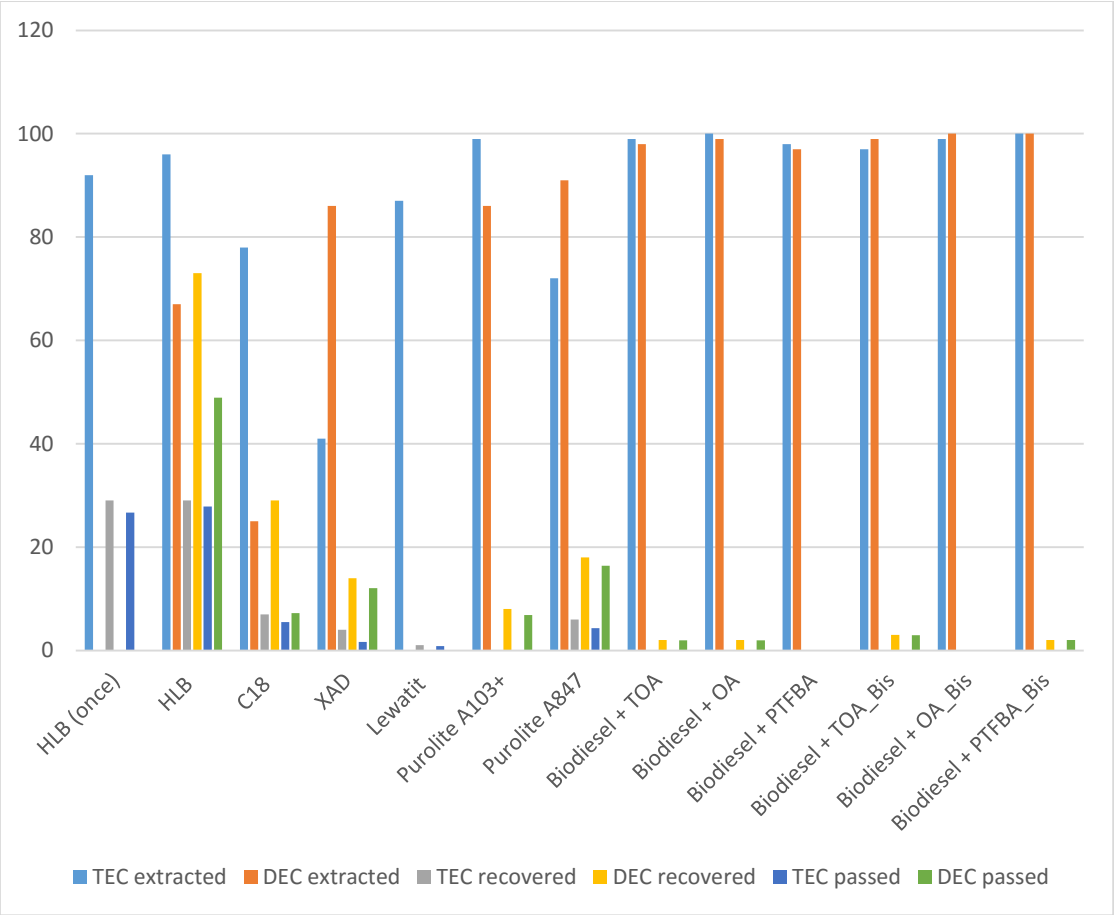


Fig 3.10: Extraction of TEC & DEC from acidogenic fermentation broth and their recovery (with respect to the amount extracted) with ADSS, using various resins and amine-biodiesel mixtures

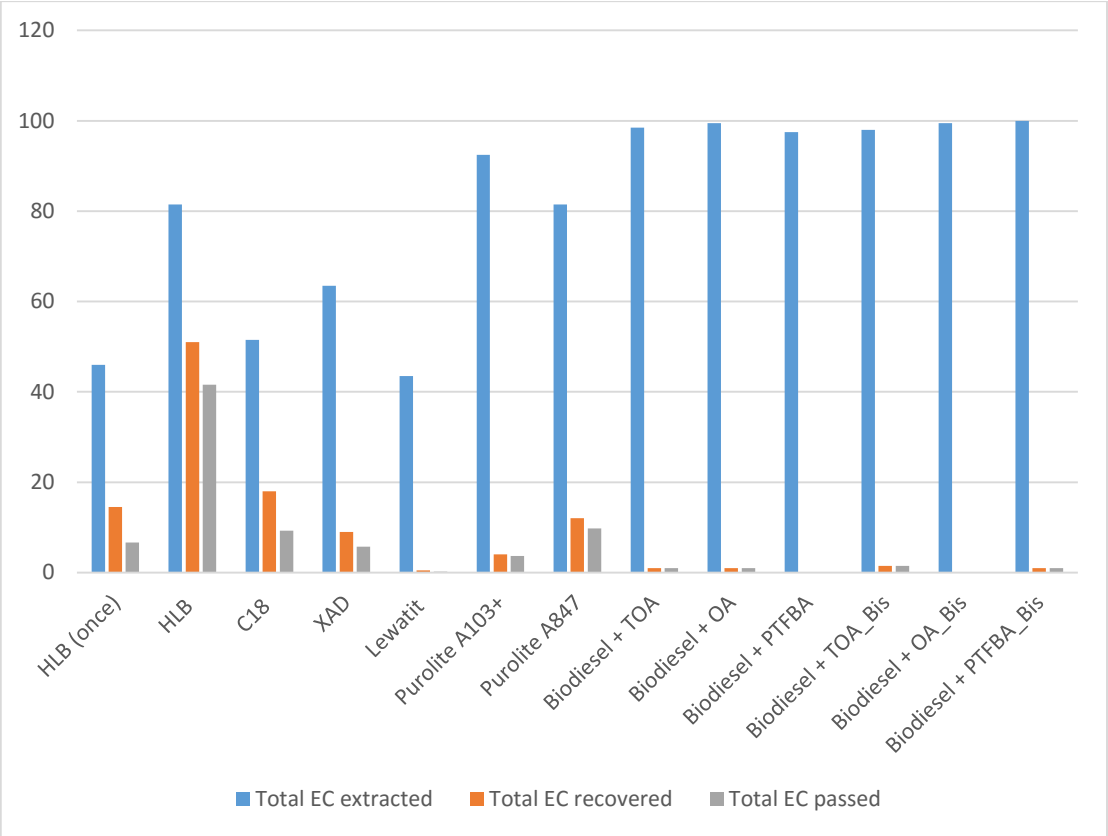


Fig 3.11: Extraction of total EC (DEC and TEC combined) from acidogenic fermentation broth and their recovery (with respect to the amount extracted) with ADSS, using various resins and amine-biodiesel mixtures

The explanation for resin performances can be related to their functionality. The two purolite resins showed good to excellent extraction of DEC and TEC due to the tertiary amine functionality on both resins. The strong amine interactions with the acid allow for formation of acid-amine complexes and thus provide for high distribution coefficients [105]. However, the slight difference in their extraction efficiency can be attributed to their different polymer structure. On the other hand, *lewatit* resin exhibited the lowest extraction performance. *HLB* cartridge performed as good as the *purolite A847* due to its excellent and unique hydrophilic-lipophilic balance, capable of extracting a wide range of acidic, basic and neutral compounds.

Meanwhile, the second set of extraction experiments involved the use of palm oil methyl ester (POME, biodiesel) as a diluent in amine. Low volatility, high biodegradability, low toxicity, insolubility in water, derivation from renewable resource and safety prompted the use of biodiesel. Recently, biodiesel has been used in a wide range of food, pharmaceutical and

cosmetic industries [115-117], and more importantly, as a replacement or additive to mineral diesel [118]. It can be produced from any product containing fatty acids, such as vegetable oil or animal fats. Biodiesel produced by the transesterification of palm oil and methanol (in the presence of alkaline catalyst) is better known as palm oil methyl ester (POME).

From ***Fig 3.10*** and ***Fig 3.11***, the amine-biodiesel mixtures all showed excellent DEC and TEC extraction (97-100% extraction) at amine concentration of 10 g L^{-1}. In addition to the aforementioned strong amine interactions with the acid which provide high distribution coefficients, the high affinity of the organic base for the acid gives selectivity for the acid over the nonacidic components in the broth sample [105].

However, the recovery of the EC from the amine-biodiesel complex mixture were poor using the ADSS (pH 9), with less than 2% recovery, perhaps due to the very strong amine interactions with the acid. On the other hand, the resins showed better recovery performance in comparison to the amine-biodiesel mixtures, with *HLB* having the highest recovery of 51%, and a total passage of 42%. Thus, 42% of the citrate produced during the acidogenic fermentation can be successfully passed to the aerobic fermentation stage for PHA accumulation.

VOLATILE FATTY ACIDS EXTRACTION AND RECOVERY

The first set of VFA extraction experiments (centrifugal method) using pure tertiary amines (TOA and PTFBA) and TOA-biodiesel mixture, showed zero VFA extraction performance. The change in method to the use of separating funnel instead of centrifugation gave better results.

In addition to the procedure described in **section 2.2.5**, an additional experiment of 2% OA in biodiesel was performed (40mg OA in 2mL biodiesel), reported in ***Fig 3.12*** as “Biodiesel + OA 2%”.

Basically, two kinds of resins and three amine-biodiesel mixtures were used for VFA (mainly acetic and propanoic acids) extraction. *Purolite A103*$^{+}$ extracted 56% VFA from the fermentation broth with 73% of it recoverable, while *A847* afforded a lower extraction performance of 28% VFA and was able to release all. TOA-biodiesel, OA-biodiesel and PTFBA-biodiesel showed 8, 22 and 3% VFA extraction performance respectively, when 1% amine concentration was used. However, on increasing the OA concentration to 2%, 94% VFA extraction was observed.

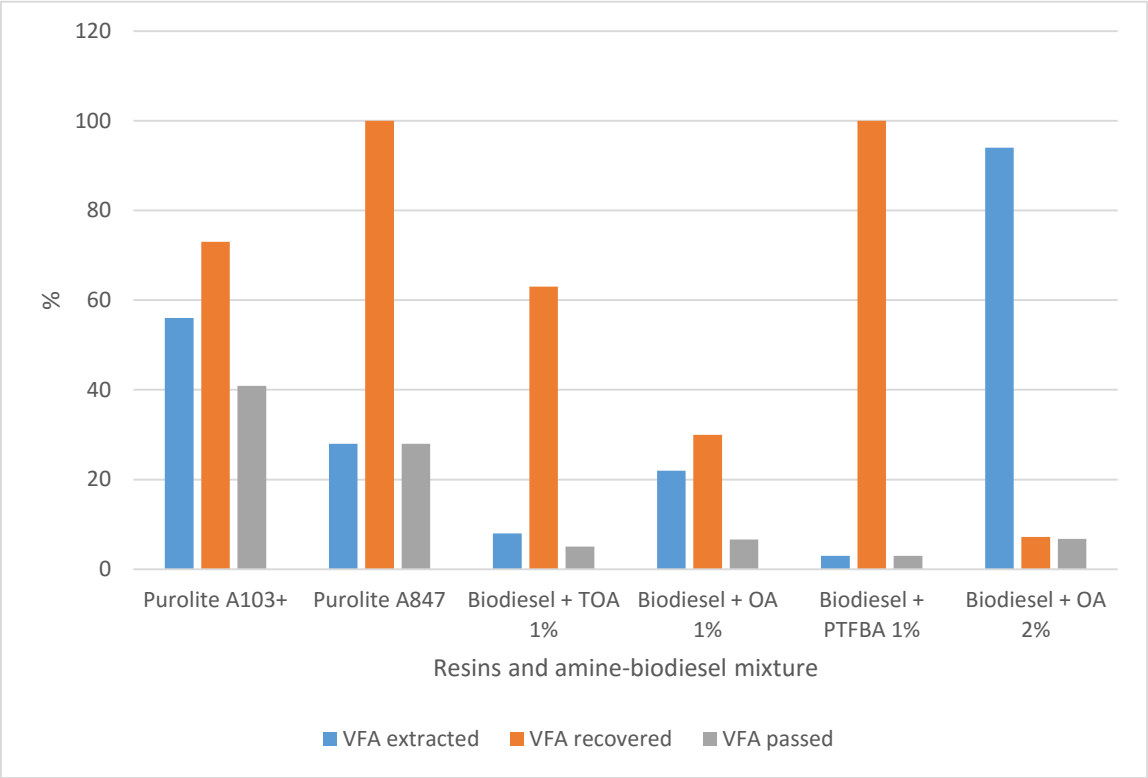


Fig 3.12: Extraction of VFAs (mainly acetic and propionoic acids) from acidogenic fermentation broth and their recovery (with respect to the amount extracted) with ADSS, using resins and amine-biodiesel mixture

It was observed that the extraction efficiency of the extractants for VFAs is not as high as for the citrates, but increasing slightly the concentration of the best forming amine (OA) from 1% to 2% afforded a more efficient extraction performance (22% to 94%). Thus a higher concentration of the amine was needed to extract VFAs unlike citrate derivatives that needed 1% amine concentration.

On the other hand, the recovery of these extractants varied in performance. TOA-biodiesel and PTFBA-biodiesel mixtures with very low extraction performance were able to release the small captured VFA very easily using ADSS. However, due to the strong interaction between the much extracted VFA on the 2% OA-biodiesel mixture, recovery performance with ADSS was low (7%). In general, *purolite A103*$^{+}$ resin afforded a total passage of 41% VFA from the acidogenic fermentation to the aerobic fermentation stage.

In summary, the *HLB* cartridge was the best performing for recovering di- and triethylcitrates extraction and recovery (42% passage), while the *purolite A103*$^{+}$ resin was best for VFA extraction and recovery (41% passage).

3.2.5 MMC: GROWTH CONDITIONS AND PHA ACCUMULATION

The SBR operation period lasted 26 days with a clear F & F regime. This condition allowed the selection of an adapted MMC, specialised on intracellular PHA storage, but was not further exploited for setting up the accumulation batch based on the results obtained. ***Fig. 3.13*** shows the trend of the control SBR experiment fed with synthetic VFA (**R2**). The PHA growth had a maximum yield of 22.2% g/g dry cell weight (with 3HB and 3HV percentages of 87 and 13 respectively) which is quite consistent with literature [53, 59]. However, the low yield at some point is perhaps due to some nutrient imbalance.

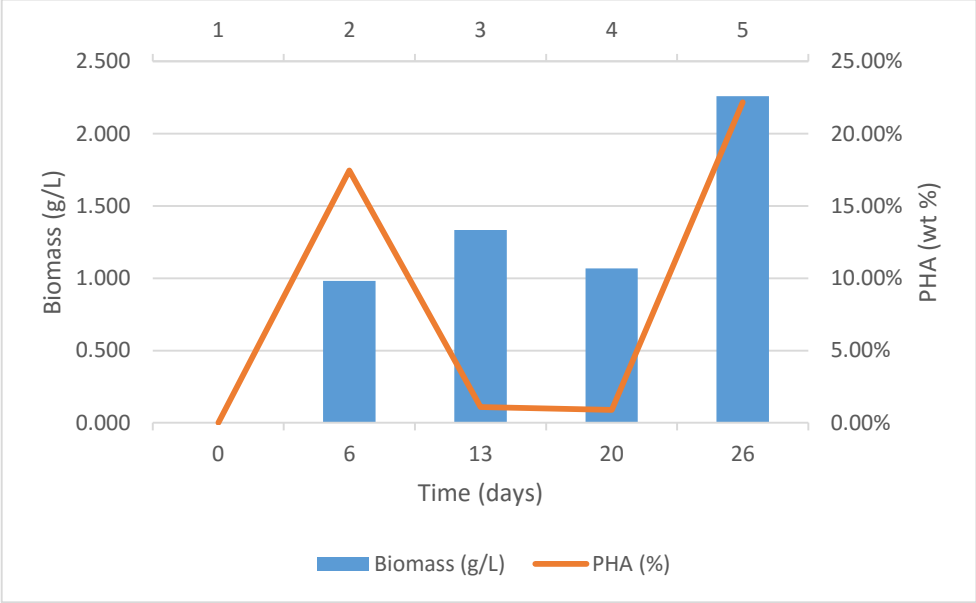


Fig. 3.13: Trend of biomass growth and PHA amount in microbial cells (wt %) in **R2**

Meanwhile, the test experiments, **R1** (SBR fed with triethylcitrates) and **R3** (SBR fed with ethanol) though produced quite a lot of biomass, but very little amount of PHA (***Fig. 3.14*** and ***3.15*** respectively). 4.5% and 4.7% g/g dry cell weight were the peak PHA values of **R1** and **R3** respectively.

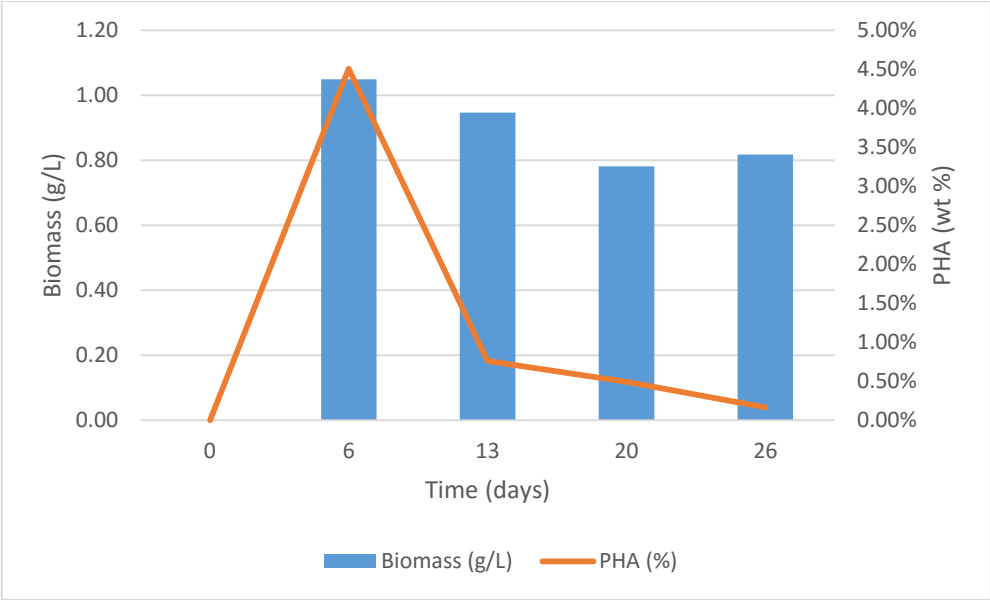


Fig. 3.14: Trend of biomass growth and PHA amount in microbial cells (wt %) in **R1**

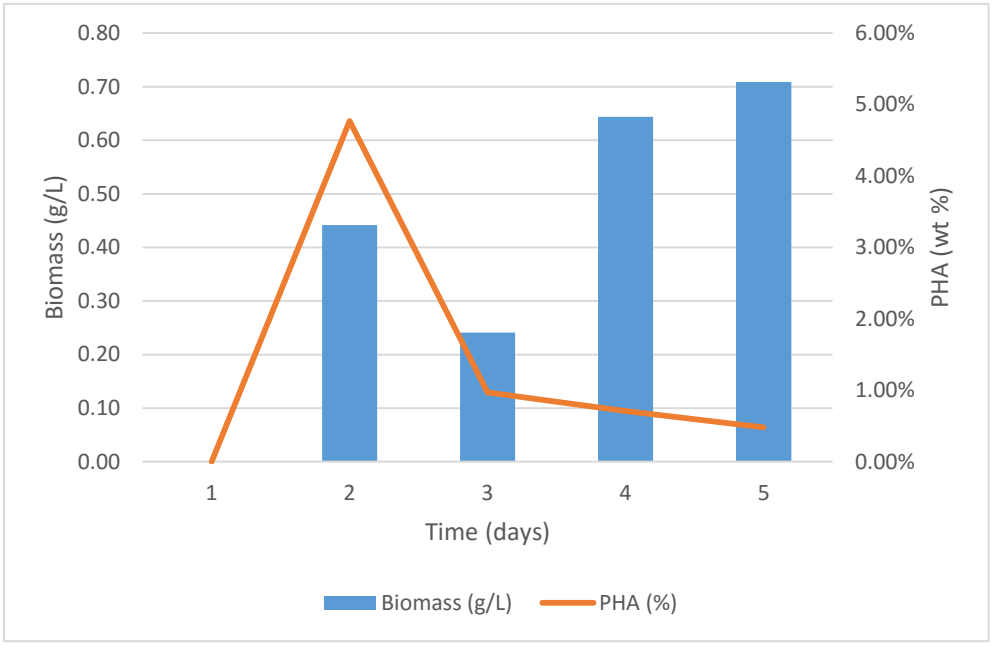


Fig. 3.15: Trend of biomass growth and PHA amount in microbial cells (wt %) in **R3**

This suggests that both TEC and ethanol are not good substrates for adapting our MMC to PHA accumulation. As a result, a further accumulation batch reactor was not set up. Furthermore, it will be safe to assert that: of the acidogenic fermentation products, only VFAs produced are

excellent substrates for PHA accumulation, and will need to be selectively extracted from the fermentation broth for aerobic fermentation.

3.2.6 MMC: QUALITATIVE CHARACTERISATION

With the aid of PCR-DGGE and gene sequencing, it was revealed that the microbial community for **R2** was mainly constituted by α- and β-Proteobacteria, gram-negative microorganisms. A dominance of genera *Azoarcus* and *Amaricoccus* was observed, in addition to the presence of genera *Thauera* (***Table 3.3***).

Table 3.3: Comparison between microbial community analyses of the mixed sample used for PHA recovery and a feast phase sample of the **R2**. +: present; -: not present.

Bacteria strain	Feast phase sample	Mixed sample for extraction
Azoarcus sp KH32C	+	-
Amaricoccus sp WTFO4	-	+
Uncultured *Azoarcus* sp	+	+
Azoarcus sp BH72 strain BH72	+	+
Azoarcus sp NSC3	+	+
Thauera terpenica 165 rRNA gene, strain 21 Mol	-	+

All these genera have already been found in activated sludge environments and their PHA storage have been reported [106,107]. Several studies demonstrated that the use of acetate as carbon source favours *Thauera* and *Azoarcus* spp selection, while propionate tends to favour *Amaricoccus* spp growth [107-110].

From the comparison in ***Table 3.3***, *Azoarcus* was the predominant genus in the microbial community analysis of a feast phase sample, while *Amaricoccus* and *Thauera* genera were not present. This suggests that process parameters may influence the presence and abundance of different microorganisms, as already reported in some studies [107, 109, 110].

Work is presently ongoing on the qualitative characterisation of **R1** and **R3** microbial communities.

4. CONCLUSIONS

A two-step synthesis of four new types of surfactants derived from itaconic acid and fatty amine has been reported and an evaluation of their aerobic biodegradability has been accomplished. The compounds prepared represent different types of biobased surface active molecules, all characterized by a common structural motif: a polar pyrrolidine head bearing a lipophilic alkyl chain. The novelty of the structural feature in the field of surfactants, the high yields, robustness and sustainability of the synthetic procedures, together with their renewable source origin, make these compounds very attractive for possible exploitation in many industrially relevant fields. Different structural modification can be introduced in either the polar head or the chain (length, unsaturation, etc.); from this point of view, each of the four compounds studied in this work can be seen as the archetype of molecular libraries in which a structural modification provides a desired change of some properties. The building of such libraries will be pursued in the future, using the present assessment as starting point.

Of the four surfactant types synthesized, one has proved to be readily biodegradable, two were less ready biodegradable, while one was non-biodegradable. The ready biodegradable nonionic surfactant **2** (1-dodecyl-5-oxopyrrolidine-3-carboxylic acid methyl ester, with a biodegradability of 96%) has a potential as a candidate to substitute the commercially used nonionic APE surfactant - really biodegradable but precursor of toxic and hazardous compounds. We are confident to find out the molecular libraries based on **1** and **4** compounds possessing good biodegradability, besides other attractive properties. Compound **3** is toxic to microorganisms and this rules out its potential application in many fields; however, the biocide features manifested in this study could be exploited for sanitization and sterilization purposes, provided they will be effective in suitable application environments.

In the second study, it has been demonstrated that both carbon sources – paper residues and algal biomass, are susceptible to acidogenic fermentation to produce VFAs and citric acid derivatives. While pyrolysis oil from paper was a better substrate for the production of these fermentation products, the HTL-AP of *Scenedesmus almeriensis* (fresh water microalgae) and *Nannochloropsis gaditana* (marine microalgae) were not very selective in the conversion to VFAs and ethylcitrates, and in turn, produced methane. VFAs produced from the fermentation stage were best extracted and recovered using a tertiary amine-based resin while a hydrophilic-lipophilic resin was best for DEC and TEC.

Since VFAs are considered the best substrates for PHA accumulation by mixed cultures, the higher PHA content (22.2% g/g dry cell weight) achieved with VFAs as substrate in the feast and famine regime was expected. Meanwhile, triethylcitrate and ethanol were poor substrates for PHA production (peak PHA content of 4.5 and 4.7 % g/g dry cell weight, respectively). Hence, it will be necessary to selectively extract VFAs from the fermentation broth and use for PHA production, instead of the direct use of the fermentation products (VFAs and ethylcitrates, since the latter are not good substrates for PHA accumulation).

Moreover, the production of citric acid derivatives through a completely novel anaerobic fermentation has never been described before and this opens the possibility to the production of these value-added chemicals via this biorefinery pathway. Specifically, TEC is an important added value chemical used as a plasticizer for resins, inks, polishes, cosmetics and personal care products [111], polyvinylchloride and similar plastics [112]; as a food additive (E number: E1505) to stabilize foams, especially as whipping aid for egg white [113]; and in pharmaceutical coatings [114].

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