Universidade de Aveiro Departamento de Química
2017

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Separação e purificação de bioplásticos microbianos

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo de Biotecnologia Industrial e Ambiental, realizada sob a orientação da Doutora Luísa Seuanes Serafim, professora auxiliar e da Doutora Sónia Ventura, investigadora auxiliar com agregação ao Departamento de Química da Universidade de Aveiro.

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Agradecimentos

Agradeço à minha orientadora, a Doutora Luísa Seuanes Serafim, por me ter dado esta oportunidade e por todo o apoio e ensinamentos, não só durante este ano de tese, mas também os restantes anos universitários em que fui seu aluno.

À minha coorientadora, Doutora Sónia Ventura, pela sua ajuda, coorientação e simpatia para com todos os problemas que encontrei durante o desenvolvimento deste trabalho.

À professora Ana Xavier pela companhia, ensinamentos e ajuda perante todos os percalços em meio laboratório.

À Margarida Martins por todo o tempo e paciência gastos comigo, perante todos e mais alguns problemas, bem como pela sua constante vontade de ajudar e ensinar.

À Daniela por toda a sua ajuda e à Dona Graça pela sua boa disposição e pontuais conversas, tornando o laboratório um local menos isolado.

A todos os meus amigos (Les Memes) com os quais evoluí, aprendi e cresci como pessoa. Pela vossa constante companhia, inesquecíveis momentos e animação. Espero poder continuar convosco nos próximos anos vindouros.

Agradeço também aquelas amizades antigas, mas intemporais, principalmente a Catarina e o Diogo, mas também Filipa e Lima. Que os regulares jantares continuem a juntar-nos durante muitos anos.

Aos meus 2 irmãos, Ana e José, as minhas duas maiores forças de vida. Não consigo descrever toda a vossa importância na minha vida nos últimos anos, mas espero que continuemos unidos contra todos os obstáculos que encontrarmos.

À minha família, tios e primos, não só em terra continental, como também na nossa eterna ilha, por todos os passeios e bons momentos passados juntos.

Finalmente, e principalmente, aos meus pais pelo seu apoio incondicional, por acreditarem em mim durante todo este percurso e por terem ajudado a criar a pessoa que sou hoje.

Palavras-chave Polihidroxialcanoatos, culturas mistas, bioplásticos, extração, separação, surfactantes, líquidos iónicos.

Resumo

Os polihidroxialcanoatos são poliésteres biodegradáveis produzidos por inúmeros organismos. Possuem características muito semelhantes às dos plásticos, apresentando-se como um possível substituto dos mesmos. Os PHAs são produzidos na forma de corpos de inclusão, sendo necessária a sua extração. Os surfactantes são compostos tensioativos capazes de extrair os PHAs, através da sua incorporação, e consequente saturação, nas membranas bacterianas, auxiliando na sua lise. Neste trabalho começou-se por testar a eficácia de uma variedade de surfactantes, e alguns líquidos iónicos, na extração de PHA de culturas mistas. O Tween 20 destacou-se dos restantes visto praticamente não apresentar acumulação de surfactante na amostra de PHA, tendo igualmente sido responsável pelo isolamento e análise do mesmo, de forma eficiente. Com este composto realizaram-se testes onde se variaram a quantidade de biomassa, a concentração de surfactante e o tempo de digestão. Quanto às concentrações de surfactante utilizadas, 50mM, 150mM, 250mM, 400mM e 500mM, os melhores rendimentos de extração (56-61%) foram conseguidos com menores concentrações de surfactante (50mM e 150mM). Com os tempos de digestão (2h, 4h, 6h, 8h e 14h) e com a quantidade de biomassa (0.3g e 0.8g), verificou-se que com quantidades e tempos mais reduzidos (4h e 0.3g) foi possível obter melhores ou semelhantes resultados aos obtidos com valores superiores de tempo e biomassa. O clorofórmio foi substituído pelo dimetil carbonato no passo de purificação, reduzindo bastante a toxicidade do processo. Foi também elaborada uma extração utilizando apenas digestão com surfactante, sem purificação, atingindo-se um rendimento de 16.17%. Para além dos semelhantes resultados obtidos neste trabalho, um novo processo foi testado e redesenhado para a extração de PHAs em culturas mistas, verificando-se até aqui uma redução dos custos e da toxidade do processo, com a possibilidade de remover totalmente o passo de solubilização do polímero num solvente orgânico.

Key words Polyhydroxyalkanoates, mixed cultures, bioplastics, extraction, separation, surfactants, ionic liquids.

Abstract Polyhydroxyalkanoates (PHA) are biodegradable polyesters produced by a variety of organisms. Their characteristics are very similar to those found on plastics, making them a viable replacement for this important product. Inside cells, PHA are produced under the form of inclusion bodies, and its extraction is required. Surfactants are compounds able to carry out PHA extraction by incorporating, and consequently saturating, the bacterial membrane, facilitating the lysis process. In this thesis, we begun by testing the extraction efficiency of a screening of surfactants, and some ionic liquids, on mixed cultures. Tween 20 stood out from the rest, as the PHA biofilm obtained was free from any surfactant and was possible to isolate and analyse the polymer efficiently. Using Tween 20 as extractive agent, more tests were carried out, where the amount of biomass, concentration of surfactant, and time of digestion were studied. As for the surfactant concentrations 50mM, 150mM, 250mM, 400mM e 500Mm, better extraction yields (56-61%) were achieved with lower concentrations (50mM and 150mM). As for digestion times (2h, 4h, 6h, 8h and 14h) and biomass quantity (0.3g and 0.8g), it was verified that with lower values and times (4h and 0.3g), results obtained were very similar, or better, than those obtained with overnight digestion and 0.8g of biomass. Chloroform was replaced by dimethyl carbonate, greatly reducing the process toxicity in the purification step. An extraction using solely surfactant digestion, without a purification step, was also performed, achieving an extraction yield of 16.17%. In addition to the similar yields obtained in this work, a new process was tested, and later redesigned, for PHA extraction in mixed cultures, with a reduction of costs and toxicity of the process, and with the future possibility of completely removing the step based on the solubilization and isolation of the polymer in an organic solvent.

Abbreviations

ABS	Aqueous biphasic system
CMC	Critical micelle concentration
DMC	Dimethyl carbonate
DTAB	Dodecyltrimethylammonium bromide
DTPA	Diethylenetriaminepentaacetic acid
EDTA	Ethylenediamine tetraacetic acid
FTIR	Fourier-transform infrared spectroscopy
GAO	Glycogen-accumulating organisms
GC	Gas chromatography
IL's	Ionic liquids
LC	Liquid chromatography
mcl-PHAs	Medium chain length polyhydroxyalkanoates
MMC	Mixed microbial cultures
NPCM	Non-PHA cell mass
NTA	Nitrilotriacetic acid
P(3HB)	Poly(3-hydroxybutyrate)
P(3HB3HV)	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
P(3HV)	Poly(3-hydroxyvalerate)
PAO	Polyphosphate-accumulating organisms
PCR	Polymerase chain reaction
PHAs	Polyhydroxyalkanoates
SAS	Switchable anionic surfactant
SBR	Sequenced batch reactor
scl-PHAs	Short chain length polyhydroxyalkanoates
SDS	Sodium dodecyl sulfate
TEM	Transmission electron microscopy

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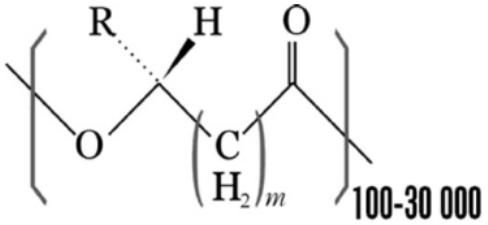
1. Introduction

Plastics play a key role in nowadays society and represent an essential part of the industry, for example, by replacing paper and glass in packaging processes (1). Their annual production rounds the 300 million tons, with an increase of 5% *per* year (2). The various properties of support, brightness, durability and resistance to degradation made plastics essential goods to improve comfort and quality of life. However, they present several disadvantages (1). Due to their massive importance in the society of today, plastics are mass-produced, leading to an excessive petroleum consumption (2) which represents a dramatic increase on demand of this non-renewable resource. Being a non-biodegradable material, an excessive accumulation of plastics in the environment (25 million tons *per* year) results from this (3) which desperately requires a more viable and biodegradable alternative for the planet. A potential alternative is the replacement of petroleum-based plastics for biodegradable polymers (4), as polyhydroxyalkanoates (PHAs).

PHAs are polyesters consisting of hydroxyalkanoates, with the general structure shown in Figure 1. There is a huge variety of different PHAs which results from the different combinations of the *R* group and the number of repeats of the *n* group (1). PHAs are optically active and 100% degradable biopolymers which exhibit similar characteristics to several synthetic polymers (5), thus being a possible alternative to plastics. There are many microorganisms capable of producing and storage these biopolymers, namely *Cupriavidus necator*, *Haloferax mediterranei* or *Bacillus megaterium* (6). This last one was the first bacterium to ever be found to produce Poly(3-hydroxybutyrate), P(3HB), in 1926. Since then, PHAs have been studied, both in terms of their structure and their biosynthesis.

Nowadays, PHAs face the harsh disadvantage of having a huge production cost (7), mainly due to the fermentation (energy and substrate cost) and the downstream processes (extraction and purification) (8). In order to obtain a viable process of production, it is necessary to improve, to its maximum, the efficiency and yield of downstream process of the final product (5). It is estimated that these final steps exceed 50% of the total production price of P(3HB) (4).



m=1	R=H	Poly(3-hydroxypropionate)	P(3HP)
	$R=CH_3$	Poly(3-hydroxybutyrate)	P(3HB)
	$R=C_2H_5$	Poly(3-hydroxyvalerate)	P(3HV)
	$R=C_3H_7$	Poly(3-hydroxyhexanoate)	P(3HX)
m=2	R=H	Poly(4-hydroxybutyrate)	P(4HB)
m=3	R=H	Poly(5-hydroxyvalerate)	P(5HV)

Figure 1 – PHA general estruture

2. State of art: Polyhydroxyalkanoates

2.1 Polyhydroxyalkanoates

PHAs are intracellular products, being stored as inclusion bodies with 0.2 – 0.5 μm of diameter. The inclusion bodies are located in the cell cytoplasm and can be easily seen with a phase contrast microscope due to PHA high refractivity (5). PHAs production occurs under bacterial stress conditions, during which microorganisms are submitted to some particular nutrient limitations with an excess of carbon source (9). The nutrient deprivation activates a metabolic pathway that diverges acetyl groups from Krebs cycle to PHAs production (10).

There is a diverse number of bacterial strains capable of producing PHAs, like *Aeromonas hidrophila*, *Bacillus flexus, Pseudomonas stutzeri*, *C. necator* and even recombinant *E. coli.* Pure cultures are vastly utilized for PHA production, namely at an industrial level, since they are able to achieve high production rates (Table 1). However, the costs associated with production control, sterilization equipment and substrates make this process less competitive when compared to conventional plastics. *C. necator* is a *Gram-negative* bacteria and is the most widely studied (4) due to its capacity to produce and store larger quantities of PHA while consuming simple carbon sources like glucose, fructose or acetic acid (1). Furthermore, PHA production by *C. necator* from a variety of waste materials, such as lignocellulosic materials (11) and oil-containing substrates, like

cooking oil, was also reported (12). It is known that *C. necator* can store PHAs up to 80% of its dry weight when exposed to limited concentrations of nitrogen and phosphorus (1). PHAs biosynthesis occurs in aqueous medium, preferentially at 30-37ºC (13) and can be produced through fed-batch or continuous fermentation, being the last the most used with *C. necator* cells (1). These methods aim to reduce the overall production cost and to increase productivity (7).

PHA production can also be performed by mixed cultures, which present some advantages, namely the inoculum being made of activated sludge, while the expensive carbon sources used with pure cultures are switched with industrial sub products. It also is a more economic process since sterilization is not performed and the overall fermentation and production equipment are cheaper (14). An example of bacteria groups responsible for this type of production are glycogen-accumulating organisms (GAO's) and polyphosphate accumulating organisms (PAO's) (15). This production was initially tested in wastewater treatment plants to perform biological phosphorus removal (16), and is based on alternating anaerobic with aerobic cycles (15). In the absence of oxygen, PAO's and GAO's both produce PHA by using energy, gained by releasing phosphate by the first microorganisms (17), while the second's is produced solely by glycolysis of the glycogen (18). Once the conditions turn aerobic, PHA is used for microorganism growth, while the glycogen levels rise (17). PHA production by microbial mixed cultures (MMC) can also be achieved by a feast and famine process (19). This was observed in aerobic wastewater treatment plants where periods of excess carbon (feast) and substrate limitation (famine) were alternated. After testing this procedure on lab-scale reactors, it was discovered that, during the famine period, a decrease on the amount of intracellular components was verified, caused by the absence of substrate. Once the feast period starts, the difference between the high substrate concentration and the amount of intracellular compounds during the famine period (namely enzymes), greatly decreases the growth rate (slow growth response), being instead promoted the PHA accumulation process (fast storage response) (20). Despite PHA storage occurring at the same conditions, by growth prevention, for both PAOSs/GAOs microorganisms and those based on the aerobic feast and famine process, PAOs and GAOs storage is promoted by an external growth limitation caused by the absence of and electron donor, while in the feast and famine process both electrons (donor and acceptor) are present in the feast period, being the storage caused by an internal growth limitation in the famine period, due to the lack of intracellular component like RNA and enzymes (8).

There is a variety of methods that can be used in order to detect the PHA presence and to calculate its production capacity. A simple laboratory method involves the usage of Nile red and Nile blue A and its direct addition to the liquid growth medium, causing PHA coloration and easy detection under UV light (21). The most used process for PHA analysis and determination the polymer methanolysis followed by gas chromatography-mass spectrometry (GC-MS) and analysis of the methylated monomers. Despite the highly sensitive and effective process, this procedure requires the usage of toxic chlorinated solvents, such as chloroform, and additional harmful reagents like sulfuric acid (22). Polymerase chain reaction (PCR) gene detection and transmission electron microscopy (TEM) are two advanced methods widely used for high throughput screening and PHA detection, thanks to the ease of sample preparation and short analysis time (22). Polymer chain reaction is often used to identify new and potential microorganisms that can produce PHA, since it specifically amplifies the *pha*C gene, present only on PHA-accumulating organisms. This gene encodes the PHA synthase, responsible for the synthesis of PHA (23). TEM is often used to directly visualize and measure intracellular granules of PHA through high magnification. However, despite being able to provide reliable information related to PHA production or PHA production capacity, hazards are used during TEM sample preparation, destroying cells and being time consuming (22).

Table 1 – PHA production by pure cultures (adapted from (22))

Microorganism	Strain	Carbon source	PHA monomer/polymer	PHA content /%)	Average PHA productivity ($gL^{-1}h^{-1}$)	REF
***Gram-negative* bacteria**						
Alcaligenes latus	ATCC 29713 DSM 1124	Malt waste	P(3HB)	70.1	0.445	(24)
Cupriavidus necator	DSM 428 ATCC17699	Fructose, lactose Acetate, butyrate	P(3HB) P(3HB)/P(3HV)	67.0-70.5 3.9-40.7	0.052-0.067 0.001-0.037	(25) (26)
Cupriavidus necator	DSM 545	Molasses Glucose, propionic acid	P(3HB) P(3HB3HV)	31.0-44.0 80.0	0.080-0.120 0.820	(27) (28)
Hlomonas bolivensis LC1	ATCC BAA-759	Hydrolysed starch	P(3HB)	56.0	NG	(29)
Hydrogenophaga pseudoflava	ATCC 33668	Lactose, sucrose	P(3HB3HV)	20.2-62.5	0.018-0.117	(30)
Methylobacterium extorquens	ATCC 55366	Methanol	P(3HB)	40.0-46.0	0.250-0.600	(31)
Paracoccus denitrificans	DSM 413	*n*-Pentanol	P(3HV)	22.0-24.0	NG	(32)
Pseudomonas aeruginosa	NCIM 2948	Fructose, glycerol	P(3HB)	12.4-62.0	0.012-0.110	(33)
Pseudomonas marginalis	DSM 50276	1,3-Butanediol, octanoate	scl-mcl-PHA mcl-PHA	11.9-31.4	NG	(34)

Table 1 (cont.)

Microorganism	Strain	Carbon source	PHA monomer/polymer	PHA content (%)	Average PHA productivity ($gL^{-1}h^{-1}$)	REF
***Gram-negative* bacteria**						
Pseudomonas mendocina	ATCC 25411 DSM 50017	1,3-Butanediol, octanoate	scl-mcl-PHA	13.5-19.3	NG	(34)
Pseudomonas oleovarus	ATCC 8062 DSM 1045	4-Hydroxyhexanoic acid	scl-mcl-PHA	18.6	NG	(35)
Pseudomonas putida CA-3	NCIMB 41162	Styrene	mcl-PHA	31.8	0.063	(36)
Pseudomonas putida GO16	NCIMB 41538	Terephthalic acid	mcl-PHA	27.0	0.005, 0.008	(37)
***Gram-positive* bacteria**						
Bacillus megaterium	DSM 90	Citric acid Glycerol	P(3HB)	9.0-50.0	NG	(38)
Corynebacterium hydrocarboxydans	ATCC 21767	Acetate, glucose	3HB, 3HV	8.0-21.0	NG	(39)
Archea						
Haloferax mediterranei	ATCC 33500 DSM 1411	Vinasse Hydrolyzed whey	P(3HB3HV)	50.0-73.0 72.8	0.050-0.210 0.090	(40) (41)

2.2 Separation processes

2.2.1 Mechanical and Physical methods

Mechanical methods involve physical processes to disrupt cells and recover intracellular products or proteins. They can be divided in two types, namely the solid shear and the liquid shear techniques (13).

A. Temperature

The application of high temperatures to PHA-containing cells can cause the denaturation of DNA and proteins, as well as the cell wall destabilization. The weakness of the overall resistance of the membrane, results in an easier breaking and releasing of PHA granules. The effect of temperature and time of exposure varies with the bacteria type and strain (3). For example, while the perfect conditions for the disruption of *C. necator* cells were 85ºC for 15 minutes (2), other studies show that *Pseudomonads* only required 1 min of this treatment at 120ºC (2). Despite these differences, the membrane destabilization occurred in both cases. Furthermore, in the *C. necator*, the denaturation of PHB polymerase, an enzyme capable of degrading the biopolymer, was also verified, being this an advantage since the desired product to be separated, the PHA, become protected from degradation (2).

Meanwhile, when cells are exposed to low temperatures, the mechanical disruption of cells is facilitated due to the formation of water crystals during the freezing process. Moreover, the exposure of cells to consequent low temperature cycles helps the extraction of PHA granules, which are then easily hydrolyzed by other solvents, namely the sodium dodecyl sulfate (SDS) and sodium hypochlorite (NaClO) (42). Usually, the biomass is free-dried after being washed with deionized water. However, due to the high energy costs, it is not recommended for the industrial scale production of PHA (42).

B. Bead mills

The bead mill is a solid shear technique that consists on the contact of many tiny spheres with biomass in a cylindrical chamber. The cell slurry is inserted in the base of

the chamber, through an annular gab and exits in the top (43). By rotating the cylinder, to promote the contact between cells and spheres, the resulted friction causes the disruption of biomass. Heat can be generated during the process, which is removed by cooling water that circulates on the external side of the chamber. The method application and efficiency do not depend on the biomass concentration. It also avoids the use of chemicals that could potentially react with the intracellular products to be recovered or contaminate the samples. However, the method is slow, requiring a large number of steps and cycles until an efficient result is achieved (13).

Usually, once the PHA production process is finished, the wet biomass is harvested by centrifugation, followed by one or more pre-treatments in order to facilitate the cell disruption and further extraction processes, namely by the exposure of the cells to high or low temperatures and to acidic, alkaline or saline pre-treatments (43).

C. High pressure homogenization (HPH)

Another used process for cell disruption is the high pressure homogenization (HPH) (3). With this process, samples are forced, by a high pressure bomb, to pass through two small parallel slots (100 μm), resulting in two parallel fluid streams, consisted of disrupted cells. Unlike of what happens with the bead mill, the HPH efficiency varies with the biomass concentration. This method becomes more effective than the bead mill at cell dry weight concentrations above 45kgm^{-3}, showing low efficiencies with low cell concentrations. Despite of not requiring the addition of chemicals, the exposure to high pressure may degrade the biopolymer (13) and create fine debris that will negatively interfere with later downstream processes (44). Tests combining high pressure homogenization with a solvent-assisted method, using the surfactant sodium dodecyl (SDS) (See section “Chemical methods”) were developed to recover P(3HB) from *Methylobacterium*. The maximum yield achieved was 98% with a purity of about 95%, after applying an operating pressure of 400 kgcm^{-2} and a two cycle process using 5% (w/v) of SDS solution (43).

D. Sonication

Sonication is a process that involves the application of sonic waves to biomass. The sound waves propagate into the medium resulting in alternating moments of high pressure (compression) and low-pressure (rarefaction). These events lead to a high cell membrane destabilization and disruption, releasing the cell material to the surrounding liquid. This method was used for PHA extraction from *Bacillus flexus* cells. Biomass was sonicated for 10 minutes, throughout 5 cycles, after being suspended in 50 mL of water. The separation and purification steps were then performed using an aqueous biphasic system (ABS). Up to 20.3% of PHA was isolated, with a purity of about 92%. These values were low when compared to other processes of cell hydrolysis, mainly due to an incomplete cell lysis (10).

E. Osmotic pressure

The use of saline conditions is other method employed, usually as an initial step, for the bacterial cell disruption, aiming at the recovery of PHA by applying an osmotic pressure. During this process, cells are subjected to a very high osmotic pressure resulting from the hypertonic medium created by the highly concentrated saline solution. Consequently, cells release water by diffusion in order to stabilize this difference, causing their plasmolysis and disruption (3). Osmotic pressure alone is considered a bad disruptor. As a result, it should be combined with other pretreatments, like the alkaline one (13). By combining saline, alkaline and heat treatments, a decrease in the number of steps needed to fully disrupt cells and release the stored material can be observed. *Alcaligenes latus* biomass was treated with sodium chloride ($8 kgm^{-3}$) at 60ºC for 1h, followed by a temperature decrease to 4ºC, while the pH was adjusted to 11.5 by saturating the sample with sodium hydroxide. Results show that the number of steps needed to achieve a complete disruption decreased from 8 to 4, when compared to the use of the bead mills process alone (13).

F. Air classification

This method uses an air classifier (Figure 2), which separates diverse materials and substances of different sizes, shapes and densities. First the sample is injected (Inlet scroll) into a chamber that contains a column of rising air. Inside this chamber, the components of the samples to be separated are lifted up (through the gas outlet tube) by an upward force that counteracts the force of gravity. Due to the dependence of air drag on object size and shape, the components in the moving air column are sorted vertically and can be separated. This process was combined with ultrasonication and used to recover P(3HB) from *C. necator* biomass (45). The first step involved a treatment with sonication, creating a suspension of PHA granules and cellular debris. The suspension was then freeze dried and pulverized. The resultant sample suffered an air classification and PHA (38%) was separated from cell debris and other non-PHA material (62%). Chloroform was then used to extract PHA from the fine fraction, followed by methanol precipitation, reaching purity values of 95% and yields higher than 85% (45). Even though smaller volumes of chloroform are used when compared to the chloroform conventional method, the usage of this solvent still represents risks to the health and food industries, making questionable this process viability.

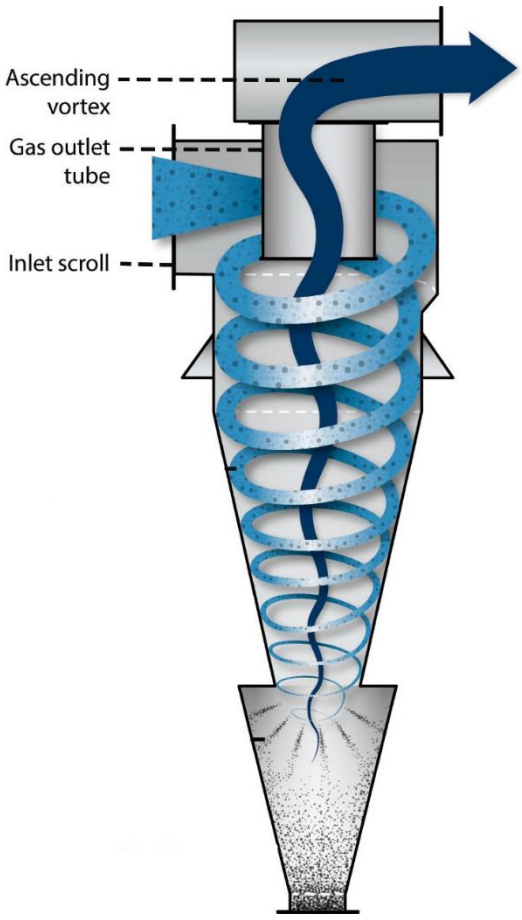


Figure 2 – Air classifier

G. Gamma irradiation

This method involves irradiation of the bacterial wet cells with gamma waves to promote cell disruption. Wet biomass of *Bacillus flexus* was treated using this process, with PHA recovery yield values of 54% when the irradiated cells, 10 kGy, were subjected to chloroform extraction during a short period of time. The use of gamma irradiation meant and increased the recovery yields since the unirradiated samples allowed for only 18% to 20% of recovery (46). The gamma irradiation process offers optimal cell wall disruption with a low dosage of irradiation and lower volumes of chloroform, while it is independent of any chemicals that can contaminate the extraction process. However, this mechanism has an initial investment cost that hinders large scale applications (47), while still requires a final use of organic solvents.

2.2.2 Chemical methods

A. Solvent extraction

This is the most traditional and, probably, applied method to disrupt cells and, consequently, release PHA (2). In this process, solvents can be divided in two types of action, as (i) cell disruption agents and as (ii) PHA solvents. The biopolymer recovery is then easily achieved by solvent evaporation since they usually present high volatility (48). Despite all these advantages, some of the most used organic solvents, namely chloroform or dichloromethane (Figure 3), represent a huge risk to human health and the environment. For those reasons, alternatives to these solvents were studied (2). Vanlautem and Gilain (49) studied PHA extraction from *C. necator* using another class of liquid chlorinated solvents, like chloropropanes or chloroethanes. Results varied according to solvents structure, being solvents with at least one chlorine atom those that allowed for the best results (49). Tests performed to extract a co-polymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-co-HV)) from *C. necator* with dichloromethane resulted on

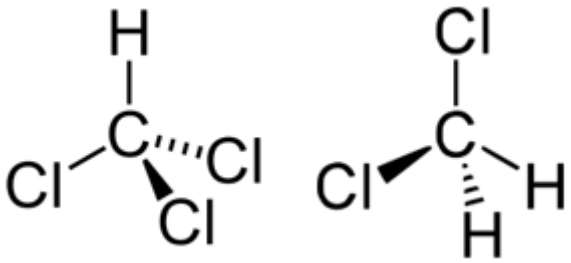


Figure 3 – Chloroform and dichloromethane structures.

purity values above 98%, after concentration by distillation, precipitation on ice-cold methanol and recrystallization (50). Besides chlorinated solvents, other classes of compounds were also studied to extract PHAs. Diols, like 1,2-propandiol, were also tested to extract PHAs from *C. necator* resulting in a recovery of 79% and a purity of 99.1% at 140ºC (49). Also with the same biomass, acetalized triols, as glicerol formal, resulted in 85% of recovery yield, and in a purity of 99.7% at 120ºC. A di-carboxilic acid ester, as diethyl succinate, led to 90 % of recovery yield and a purity of 100% at 110ºC (49), and butyrolactone allowed for a recovery yield of 90% and a purity of 99,5% at 110ºC (49). When using solvent extraction, the biopolymer solutions obtained, could present more than 5% (w/v) of P(3HB), with high viscosities. However, the removal of cell debris proves to be difficult (2). In order to overcome this problem, Vanlautem and Gilain et all (1982) (49) proposed a process were the P(3HB) was solubilized in a solvent immiscible with water, at temperatures above 120ºC (49). The recovery of PHA was then made by simply adding cool water, leading to the separation of the complex solvent and PHA. Rosengart et al. (51) also tested other non-chlorinated solvents, namely anisole and cyclohexanone (Figure 4) for PHA separation from *Burkholderia sacchari.* To a mass of lyophilized cells, 0.6g, were added to 40 mL of solvent (anisole and cyclohexanone), in a glass balloon, and the solutions were left to rest in an oil bath, at 120ºC, for 1h. A temperature of 60ºC for 2h was also tested, but the results were unsatisfactory. Cell debris

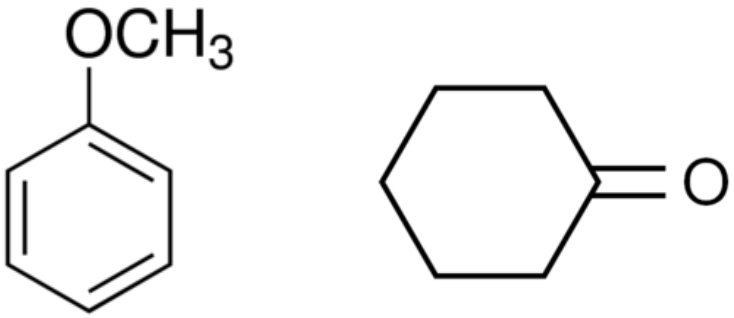


Figure 4 – Anisole and Cyclohexanone

were then removed by filtration and the solutions were mixed with 100 mL of ethanol at room temperature. The precipitated polymer was then recovered using a pre-weighed filter and by solvent evaporation. Anisole allowed for a recovery yield of 97% with a purity of 98.3% while cyclohexane reached a yield of 93% and a purity of 98.2% (51).

A process with PHAs solvent and non-solvent was proposed by Noda (1998) (45). First the biomass was mixed with the solutions of solvent and non-solvents, then the insoluble biomass was separated and, in the final step, the solvent was removed promoting PHAs precipitation in the non-solvent (45).

The reuse of solvents was tested several times before being distilled, lowering the overall extraction process costs. Furthermore, solvent usage often leads to alterations of the natural morphology of PHA granules, which can be useful in certain applications like in the production of strong fibers (52). Solvent extraction processes, unlike some other separation methods, do not degrade and allow the purification of the biopolymer, which can be useful in some applications, namely in the medical field. Many *Gram-negative* bacteria were used for PHA production and this type of bacteria possesses endotoxins in its membranes. These endotoxins represent a health risk, so their removal or, at least, concentration reduction to safe levels, is needed for biopolymers used in medical or food purposes. The use of chloroform to extract P(3HB) from *E. coli,* lead to a decrease in the endotoxins levels lower than the allowed limit of pyrogen level, 5.0 Endotoxin Units kg (53).

However, the organic solvent-assisted extraction still presents some serious problems, mainly because many solvents are considered toxic for humans and the environment. This is one of the main reasons why solvent extraction is used widely and successfully at laboratory scale but not at industrial one (54). To overcome these problems, the extraction processes using non-halogenated solvents have been tested, by using long chain alcohols, esters, amides, and ketones (both cyclic and acyclic compounds). Some examples of these solvents are butyl propionate, toluene, heptanol, ethyl benzene, ethylene glycol diethyl ether and methyl ethyl ketone. Some tests also

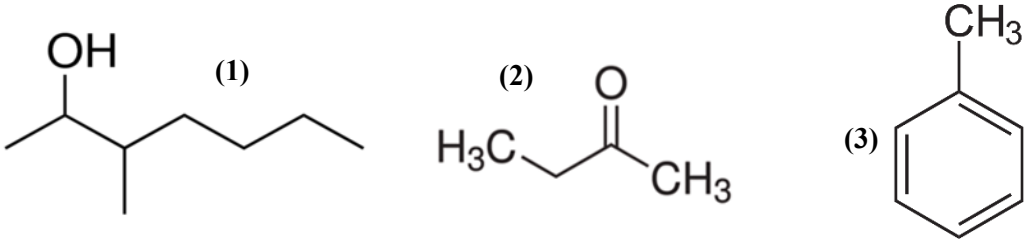


Figure 5 – Non-halogenated solvents: heptanol (1), methyl ethyl ketone (2) and toluene (3)

involve the use of these solvents at high temperatures, above 80ºC, being the polymer recovery performed by cooling the mixture (55). The manipulation of pressure and temperature was performed to improve the extraction. In these methodologies, firstly the biomass is solubilized at high temperatures, near the PHA melting point, during a certain period, normally between 1s to 15 min. The temperature is then reduced while the pressure increases, around 1-10 bar, stimulating the PHA extraction (56).

Dimethyl carbonate (DMC) is another example of a considered green solvent used for PHA extraction from *C. necator* freeze-dried biomass (74 wt% in P(3HB)) (57). After adding the solvent to the biomass, being the biomass to solvent ratio 2.5% (w/v) (50 mg of biomass extracted with 2 mL of DMC), the sample was kept at 90ºC for 4h. Finished that time, PHA was then easily recovered by filtration and solvent evaporation, leading this method to a recovery yield value of 88% and purity of 95% (57). Other improvements could also be done, like performing the organic solvent extraction directly on wet biomass, avoiding a lyophilization step to remove the water. To test this possibility, Samori et all (2014) also tested DMC extraction capacity directly on microbial slurries. The process followed started by adding 1 mL of microbial slurry with 50 gL^{-1} with 2 mL of DMC, for 1h at 90ºC. After centrifugation, the residual biomass was separated, alongside with the water phase. The PHA was then recovered by filtration and solvent evaporation, leading to a recovery yield of 94% and a purity of 93%. These values proved that similar results were obtained with both freeze-dried biomass and microbial slurries, making the liophilization step avoidable. However, this can only be applied to low concentrated slurries (<250 gL^{-1}), as with higher concentrations DMC formed a gel with the biopolymer, making the separation in that case impossible (57).

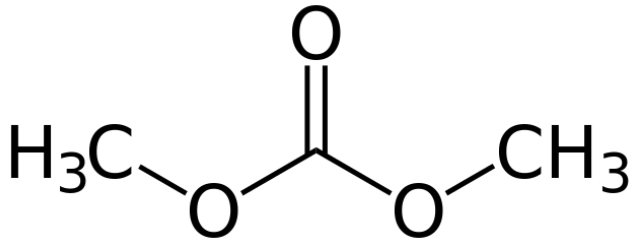


Figure 6 – Dimethyl Carbonate

B. Alkaline and acidic solutions

Regarding the alkaline method, alkaline solutions are used to destabilize the cell membrane thus facilitating its disruption. One of the most used alkaline solutions is sodium hydroxide. The effect of its alkalinity results in a reduction of time and steps used in further treatments or pretreatments, however, it could cause some polymer degradation.

In PHA extraction from *Alcaligenes latus*, a concentration of 0,8 kg kg^{-1} of sodium hydroxide was enough to reduce, from 6 to 2, the number of steps needed in the bead mill (see section "Separation processes: Mechanical and Physical methods") (2). Mohammadi et all (58) tested the alkali method against the water for PHA recovery from recombinant *C. necator* cells. The water method occurred at 30ºC, 0 rpm for 1h, while the NaOH method, at similar conditions, occurred at a concentration of base of 0.01M, 0 rpm, at 30ºC for 1h. Purification was performed by precipitating the biopolymer in ethanol. When comparing these 2 different methods, higher values of both recovery yield (96.1%) and purity (80.6%) were obtained with water, rather than with NaOH (recovery of 74.9% and purity of 48.4%). However, by using NaOH favorable conditions (0.05M, 3h, 0rpm, 4ºC), instead of similar conditions to water, both the recovery yield (96.9%) and purity (96.6%) greatly increased (58).

Another known method involves the use of acids, instead of alkaline solutions. Acid solutions can degrade cells with minimal hydrolysis of the biopolymer stored, contrarily to what happens in the alkaline treatment. This occurs because, while acid protons work for both hydrolysis and esterification, hydroxyl anions remove protons from the acid produced during the cleavage of ester bonds, thus resulting in polymer degradation. Sulfuric acid is one of the acids already tested (3). A suspension of 5% (w/v) was initially digested with 0.64M of H_2SO_4, during 6h, at 80ºC, followed by a hypochlorite treatment. Recovery yield values of 80% and purity of 95% were achieved (3). The high purity percentages may be due to selective degradation of molecules, namely peptidoglycan that forms a protective membrane that helps stabilizing the biopolymer granules (59).

B.1 Selective dissolution of non-PHA cell mass by protons.

This method was introduced by Yu and Chen (59) and uses protons to dissolve non-PHA cell mass/material (NPCM) in an aqueous solution, while inducing the PHA crystallization. The process begun with the heating of *C. necator* cell slurries in sealed glass tubes. Then, the temperature of the suspension was decreased with water and the pH adjusted to 10 by adding NaOH 5M. The supernatant was discarded after centrifugation (5.000rpm for 15 minutes) and the pellets washed with an equal volume of water. A bleaching solution, to discolor the pellets, was added (Clorox containing 6% NaOH), followed by the addition of sodium hypochlorite to obtain a ratio of 0.5-1:1 with

initial biomass weight, at room temperature for 1-2 h. Finally, the solid phase consisting of PHA granules was recovered by centrifugation and after drying in an oven. While extracting P(3HB), recovery yields and purity reached 98,7%, and 97.9% respectively, purity values of 98.5% and recovery yields of 95.4% were obtained when recovering PHBV. This method can be considered environmental friendly and much cheaper than conventional recovery, since it was estimated to decrease the overall recovery process cost by up to 90% (59).

C. Digestion by surfactants.

Aiming to decrease the environmental impact of the processes that use chlorinated solvents, some new ideas came out, namely the application of aqueous solutions of surfactants (2). Surfactants are amphiphilic compounds, composed by a charged hydrophilic "head" and a long alkyl hydrophobic chain "tail", Surfactants can be cationic ("head" positively charged), anionic ("head" negatively charged), non-ionic ("head" without charge) and zwitterionic ("head" positively and negatively charged). Examples of surfactants belonging to some of these categories are SDS (anionic), the hexadecyltrimethylammonium (cationic), the Tween 20 and Triton X-100 (both non-ionic) (2) and phosphatidylethanolamine (zwitterionic). The mode of action of surfactants is based on their incorporation in the lipid bilayer of the cell membranes, which leads to a volume increase. After the constant accumulation of the surfactant, the membrane saturation occurs leading to its disruption. After the membrane collapse, micelles consisting of surfactant and phospholipids are formed, while the PHA granules are released to the external medium (60). Surfactants are also able to solubilize some cellular debris, proteins and other non-PHA material, without degrading the biopolymer granules (61). SDS, with a surfactant/biomass solid liquid ratio higher than 0.4 (v/m), was already successfully applied without any other extraction agent, resulting in a purity around 95% and a P(3HB) recovery yield above 90%, when applied to *C. necator* cells. However, high purity values, above 97%, are only achievable using high amounts of surfactant (> 5 wt%), which increases the overall extraction cost, and makes wastewater treatment and reuse more difficult. Other solutions can be used, like hypochlorite and sodium hydroxide, to reduce the amount of surfactant needed to reach higher purities. Furthermore, to obtain

larger amounts of PHA, a combination of surfactants with chelating agents can also be used (2).

Recent tests tried to improve the surfactant extraction process by using ammonium dodecanoate (laurate), a switchable anionic surfactant (SAS) (57). SASs are a group of surfactants that can reverse their forms and change their properties. Before a pH shift, usually achieved by CO_2 removal or addition, these surfactants can turn from water-insoluble neutral forms into anionic water-soluble compounds. The first step is to treat the microbial slurry directly with ammonium laurate solution, leading to the dissolution of the non-PHA cellular material in the surfactant. This makes PHA recovery easily achieved by centrifugation. Once the polymer is recovered, the addition of CO_2 increases the solution pH, converting the water soluble SAS into a neutral lauric acid and ammonium hydrogen carbonate. Another centrifugation separates the lauric acid while the NH_4OH-solution can be added to carboxylic acid to recover the ammonium laurate to be reused in subsequent extraction procedures. *C. necator* biomass was combined with ammonium laurate with four different solid-liquid ratios of 2:1; 1:1; 0.5:1; and 0.2:1. With solid-liquid ratio of 2:1, fully recover of PHA was achieved while lower amounts resulted in less satisfactory results (70% - 90%). The same was verified for purity, where the 2:1 led to highest purity, 98%. In addition to the high recovery yields obtained, SAS usage has other advantages like the skipping of the biomass drying process, their excellent recyclability and the low price, being cheaper than other common surfactants (e.g. SDS). However, it presents two major drawbacks: unlike the organic solvent treatment, SAS treatment does not guarantee the reduction of endotoxins which can prove to be a problem in certain applications and causes a polymer molecular reduction (57).

D. Ionic liquids

Ionic liquids (ILs) are a promising and viable alternative to organic solvents. These compounds are defined as organic compounds with melting points lower than 100ºC (62). They are collectively known as organic salts and, because of their low fusion temperature, unlike common electrolytes, some of them are liquids at room temperature. Thanks to their ionic structure, these salts present low or null volatility, non-flammability, varied viscosities, thermal stability, high solvent power and highly tunable nature (62). Furthermore, their hydrophobicity, solvent miscibility and polarity can be fine-tuned by tailoring the cation/anion combination, allowing ILs to meet the requirements for their

many applications. This is a huge advantage since the separation systems based on polymers, which had the disadvantaged of having short polarity gaps, can be improved by the addition of ILs to the systems. It is known that their good purification performance is justified by the specific and different interactions that distinct ILs can establish with different solutes/molecules. Furthermore, these new systems present better results than the conventional aqueous biphasic systems (ABS) with higher extraction efficiencies in the separation of bio compounds like testosterone, epitestosterone and BSA (bovine serum albumin) (63), among other (64). An example of a good ionic liquid is composed by cholinium ([Ch], the cation component of the IL), which presents low toxicity, and good biodegradability (65). The usage of ionic liquids in PHA extraction is still low, but has been continuously studied in order to improve current and develop new extraction processes.

E. Chelate complexes.

A chelate can be described as a heterocyclic compound with a central metallic ion attached by covalent bonds to two or more nonmetallic atoms in the same molecule (Figure 7). Examples of this compounds are ethylenediamine tetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA) and nitrilotriacetic acid (NTA). They have the capacity to form complexes with divalent cations, like Ca^{2+} or Mg^{2+} present in the outer membrane of *Gram-negative* bacteria, increasing the destabilization of both outer and inner membranes, which leads to an easier cell disruption and increase the amount of PHA released (61). By mixing a chelating agent as EDTA disodium salt with a surfactant (betaine), obtaining a ratio chelate:surfactant-biomass of 0.08:0.12:1, at pH 13 and 50ºC degrees for 10 minutes, purities of 98,7% and recovery yield values of 93,3% were achieved. Despite producing successful results and being environmental friendly, the surfactant-chelate method usually results in large amounts of wastewater during the recovery step. In order to attenuate this disadvantage, a process involving the wastewater recycling was already proposed (61). In the final steps, after the treatment with the complex surfactant-chelate, the wastewater was submitted to 5-cycles

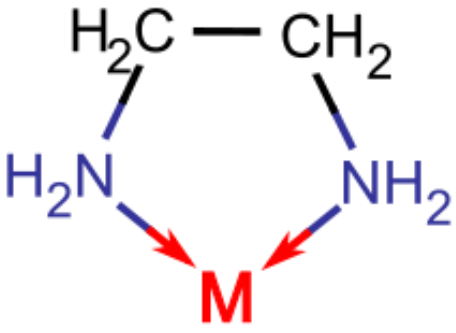


Figure 7 – Chelate complex

of washing with small amounts of hydrochloric acid and activated carbon, enabling its recycling for several rounds. With a 0.0075:0.01 surfactant-chelate/dry biomass ratio, using *C. necator* cells, at 15º C and pH 13, the purity obtained was higher than 96% while recovery yield values rounded the 90% (61). In addition to surfactants, the use of chelate complexes was studied with other compounds as well. A chelate-hydrogen peroxide complex treatment was developed by Liddell et al. (66). This method was applied to PHA-storing *C. necator* cultures in order to extract and recover the PHBV (poly-3-hydroxybutyrate/3-hydroxyvalerate) (66). An initial heat pretreatment was applied, at 150ºC for 80s, followed by the addition of hydrogen peroxide and the chelating agent (diethylenetriamine-pentamethylene phosphonic acid). The process required 10h to be complete, reaching purity values of 99.5% after PHBV recovery by centrifugation (66).

F. Sodium hypochlorite

Sodium hypochlorite works as an oxidant agent and is able to perform a partial digestion of the cell material by degrading and dissolving the non-PHA polymeric constituents (67). This makes PHA granules isolation easier, by simply performing a filtration or centrifugation (6). High purity levels can be achieved using this method, namely 86% for *C. necator* and 93% for the recombinant *E. coli* (68). However, PHA do not totally resist the chemical attack, and a molecular weight reduction, up to 50%, can occur, confirmed by a decrease on the intrinsic viscosity (69). This problem was mainly observed in *C. necator* cultures, in opposition to recombinant *E. coli* cells. The difference on the stability during this treatment was probably due to PHA morphology. While P(3HB) produced by recombinant *E.* coli present a crystalline structure, that produced by *C. necator* is mostly on an amorphous state (70). A step to attenuate this molecular weight reduction, by adding sodium bisulfite as an anti-oxidant agent, was proposed by Roh et all (48). The reduction of PHA molecular weight extracted from *C. taiwanensis 184* cultures, dropped from 30-40% to 14% (71).

A combination of sodium hypochlorite with organic solvents or surfactants was also proposed. For example, during P(3HB) extraction from *C. necator* cultures, sodium hypochlorite was combined with chloroform to avoid PHA degradation during the process. Chloroform acted as a protective agent, since it promptly dissolves the polymer, and the degradation caused by the sodium hypochlorite can be reduced (71). In fact, three

different phases were obtained using this method. The upper phase contained the sodium hypochlorite solution, the middle one the cellular debris and the lower phase contained the biopolymer dissolved in chloroform. Precipitation of PHA with a non-solvent followed by filtration allowed its recovery. Recovery yield values around 91% were obtained while the purity exceeded 97%. The optimal conditions tested for the biopolymer extraction from *C. necator* cultures were 30% (w/v) of hypochlorite, 1:1 chloroform-to-aqueous phase volume ratio, 4% (w/v) of cells in dispersion, at 30 ◦C, and during 90 minutes of treatment (70). However, despite the successful decrease of the PHA degradation, this combined method required large amounts of solvent, thus resulting in an increase of the overall process cost (70).

To further improve the sodium hypochlorite method, the combination with SDS surfactant was developed (72). A freezing step of cells was initially performed to promote their lysis. Then the suspension was mixed with $10g.L^{-1}$ of SDS for 15 min. Finished this step, 30% of NaClO were added at 30ºC. With an initial biomass concentration of 30 gL^{-1}, this method allowed to reach a purity of 98% and a recovery yield of 86.6%. Comparing the costs of this method and the most common method using chloroform, the method using SDS represented lower costs while keeping limited the PHA degradation (72).

G. Supercritical fluids

Every compound possesses a critical point at a specific pressure and temperature. When it is subject to higher values of temperature and pressure of its critical point, it is considered as a "supercritical" fluid. In the supercritical region, fluids express properties and intermediate diffusivities. Supercritical fluids (SC) usually possess liquid-like densities, gas-like viscosities and gas-liquid intermediate diffusivities. All these physicochemical properties make SCs good candidates to be used as extractive solvents. Carbon dioxide (CO_2) is one of the most used for this purpose, mainly because of its moderate critical point (31ºC, 73 atm), low toxicity and low cost (73). The use of supercritical CO_2 to extract P(3HB) from *C. necator* cells was tested to disrupt the microbial cells and other intracellular materials (74). The optimal conditions determined for this process were an exposure time of 100 min under a pressure of 200 atm, at 40ºC. The recovery yield values were around 89%, similar to those reported for other methods (73). However, in order to improve the yields of biopolymer recovery, combinations of this method with NaOH or salt pretreatments were under scrutiny (48). One of these

combined processes used 1% (v/v) of toluene as a modifier while using the conditions of 200 bar, 30ºC and twice the supercritical CO_2 pressure release. The last step involved a purifying treatment with 0,4% (ww) of sodium hydroxide (NaOH). The results showed that up to 81% of (P(3HB)) could be extracted from *C. necator* wet biomass, resulting in a decrease of the overall extraction process, since the freeze-drying step was avoided. Furthermore, this method was proved to be successful when applied to matured cells, which are usually harder to be disrupted (48).

2.2.3 Enzymatic digestion

This process uses enzymes to digest cell membranes. When compared to other processes, namely solvent extraction and chemical digestions, this method requires milder operating conditions while having negligible degradation of the biopolymers, resulting in a much environmental friendly process (2). Proteolytic enzymes, lipases and nucleases are examples of enzymes capable of dissolving proteins present in the membranes without affecting the composition or structure of PHAs (75). Some enzymes already tested are lysozyme, alcalase, pancreatin and bromelain (1). The typical process involves an initial heat pretreatment, followed by the enzymatic hydrolyzation and finally a decolorization step with hydrogen peroxide, to increase purity. Enzymes can fully hydrolyze cells without the need of any mechanical pretreatment. Harrison (1991) reported the complete lysis of *C. necator* cells after using lytic enzymes of *Cytophaga sp* for 60 minutes at 37.5ºC and pH 7.3 (76). Further tests involving different enzymes were also developed for the lysis of *C. necator* biomass. The best results were obtained with (i) 2.0% of bromelain (mass of enzyme/mass of biomass), in which a purity of 88,8% was achieved at 50ºC of temperature and pH 9.0; and (ii) pancreatin, leading to a 90% biopolymer purity, and a cheaper process, since the latter is 3 times cheaper than the former (77). Even though successful results and high yields can be achieved by applying enzymes, their high cost is the major drawback (75). Combined methods were also studied, by often paring the enzymatic treatment with sodium hypochlorite or surfactants extraction (78). By using an alcalaze action with sodium dodecyl sulfate (SDS) surfactant, helped by the addition of Ethylenediaminetetraacetic acid (EDTA), allowed a recovery yield of 90% of PHA from *P. putida*. After the treatment, PHA granules were easily recovered in a water phase by simply ultrafiltrating the solubilized non PHA cell material. Purity was

improved through a continuous diafiltration process, reaching values of 92,6% (78). Martino et al. (12) also tested a similar process for PHA recovery from *C. necator* cells grown on cooking oil. The recovery was carried out in a NA_2HPO_4 buffer and alcalase (0.3 AU g^{-1}) was added for enzymatic digestion, plus a surfactant, SDS (0.3 gg^{-1}) and EDTA (0.01gg^{-1}), a chelate complex. The separation process lasted 1h, at 55ºC. The biopolymer was then recovered by centrifugation, washes and re-suspended 3 times in water, and finally centrifuged again. Purity reached 94% and PHA was recovered in its amorphous state. This proves that the process was mild, since after extraction or damage of PHA, the amorphous coating layer is usually removed (12).

2.2.4 Cell fragility

Cell fragility method is based on modifications made to PHA producing bacteria growth media, to weaken their cell walls and facilitate the biopolymer extraction. Furthermore, this process is not restricted to *Gram-negative* bacteria, as it can also be applied to *Gram-positive* microorganisms (46). Tests performed to *A. vinelandii* UWD, a *Gram-negative* bacteria, involved the addition of fish peptone to the growth medium, resulting in the formation of large and osmotically sensitive cell walls, while improving the P(3HB) accumulation, reaching recovery yield values of 92%. This recovery step can be quickly performed using a 1N aqueous NH_3 solution (pH 11.4) at 45ºC during 10min (79). Similar results were achieved with *Bacillus flexus*, a *Gram-positive* bacteria. Bacteria cells grew on an inorganic salt medium, with sucrose as carbon source, while being deprived from diaminopilemic acid (DAP) and other amino acids. DAP is a vital component to cell walls stability since it is responsible to form cross bridges in the peptidoglycan, increasing the bacterial wall resistance. Due to its absence, results showed that up to 86 – 100% of PHA were easily extracted using lower volumes of chloroform, when compared to normal cells, or mild alkaline hydrolysis. While cultures grown in yeast extract or peptone achieved lower results (32-56%). Despite this, it is necessary to further study and balance the cell integrity in order to enhance microbial growth and PHA accumulation, while still trying to reduce chloroform usage or studying alternative solvents (79).

2.2.5 Spontaneous liberation

Spontaneous liberation is a more advanced method that occurs in genetically modified PHA-producing organisms, since they usually present a fragile cell membrane, like recombinant *E. coli* bacteria, able to automatically secrete the usually intracellular PHA granules, after reaching a determined cell content. Recombinant *E. coli* harboring *Alcaligenes phbCAB* genes, were described to spontaneous liberate PHA granules (80). By using LB medium containing 21% of glucose, cells were able to accumulate P(3HB) with an efficiency up to 99% of the glucose supplied. Furthermore, 80% of the PHA produced was spontaneously secreted, followed only by a simple purification step of centrifugation (80). Further tests combined the *phaCAB* genes with cloned lysis E genes from bacteriophage PhiX174 (plasmidpSH2). The results showed that the PHA granules, which were at a semi-liquid state, were squeezed out of the *E. coli* cells through E-lysis tunnel structures, without suffering any morphology alterations (81).

2.2.6 Predatory bacterium

This process was recently developed by Martínez et al. (82) and consists on using predatory bacterium and their cell-lytic ability to extract valuable intracellular bio-products, namely PHA. *Bdellovibrio bacteriovorus* HD100 is a *Gram-negative* bacteria and an obligate predator. It attacks other *Gram-negative* bacteria, invading their periplasm where, once inside, develops and grows a bdelloplast, eventually leading to the prey cell lysis. Initial tests showed that, despite being extremely effective at disrupting

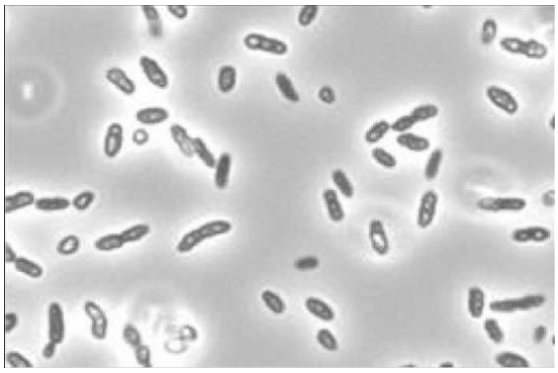


Figure 8 – *P. putida* KT2440 cells

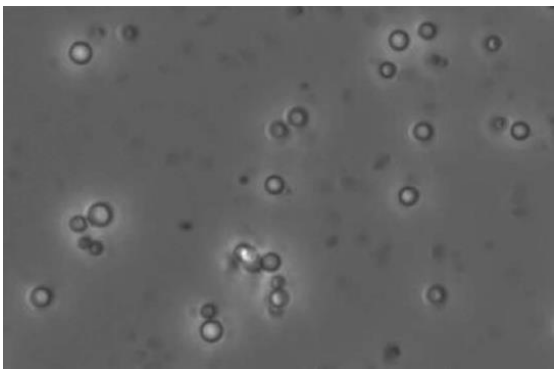


Figure 9 – PHA granules in extracellular medium

Pseudomonas putida KT2440, *B. bacteriovorus* HD100 hydrolyses, not all, but a big part of the PHA released to the surrounding medium, being this done by a PHA depolymerase ($PhaZ_{bd}$) naturally present in *B. bacteriovorus* HD100 cells. To counter PHA hydrolysis, a first step involved the creation of a recombinant version of *B. bacteriovorus* HD100, *B. bacteriovorus* Bd3709, by inserting a kanamycin resistance gene, causing $phaZ_{Bd}$ disruption. After performing another round of tests, results showed that *B. bacteriovorus* HD100 released bigger amounts of HAs while its recombinant counterpart released practically PHAs, proving the PHA degradation absence during predation with *B. bacteriovorus* Bd3709. Since the industrial viability of *B. bacteriovorus* as a living, cell-lytic system is based on its capacity to prey and hydrolyze high density cell cultures, its ability to do so was also tested. 6.3 plaque forming unit (pfu) mL-1 of *B. bacteriovorus* HD100 were mixed with 30.5 gL^{-1} of wet *P. putida* KT2440 biomass (Figure 8). The predation and hydrolysis lasted 4h, time after which a reduction of 1-log of *P. putida* KT2440 cells was observed, while the amount of viable *B. bacteriovorus* HD100 cells increased in the same amount. After phase-contrast microscopy, it was visible that PHA granules were released into the extracellular medium (Figure 9) (82).

2.2.7 Comparison of extraction methods

There are a huge variety of processes that have already been tested for PHA extraction, each having its owns advantages and disadvantages (45,67,70,83). In Table 1 the pros and cons associated to some of the methods are summarized. Organic solvents have been the most used method thanks to its high recovery yields and purity, despite the big volumes of solvent needed and the health and environment risks it represents. To counter these problems, non-chlorinated and non-halogenated solvents have been used, as well as surfactants. With these, higher purity and less polymer degradation can be achieved. Sodium hypochlorite also is a very used extractor, reaching high purities despite the polymer degradation it causes. In order to reduce cons that many of the methods alone provide, while trying to improve results, different methods can be coupled and used together or in succession. It is important to test these combinations since these can greatly increase extraction efficiency. For example, when combining surfactant usage with sodium hypochlorite, the polymer degradation obtained with NaOCl alone can be significantly reduced. Many other methods are also usually combined, mainly

temperature and pH manipulation, which, when used early in the extraction process, can facilitate the membrane disruption, increasing further methods, like surfactants or sodium hypochlorite, efficiency. Other processes like gamma irradiation, cell fragility and air classification, used alongside chloroform, prove to be effective to reduce volume solvent usage, despite still presenting health and environmental risks, reason why their utility needs further studies.

Table 2 – Comparison of some extraction methods (adapted from (2))

Extraction methods	Advantages	Disadvantages
Bead mills	No chemicals used	Requires several steps Long periods of time
High pressure homogenization	No chemicals used	Poor disruption rate for low biomass levels
Solvent extraction	Elimination of endotoxins No polymer degradation	High prices Hazards connected to halogenated solvents
Surfactants	No polymer degradation	Water waste treatment needed
NaOCl	High purities	Polymer degradation
NaOCl + surfactants	Limited degradation Low operating cost	
NaOCl + chloroform	Low polymer degradation High purity	Large quantities of solvent needed
Chelate complexes	High purity Low environmental pollution	Large volumes of waste water
Enzymatic digestion	Good recovery	High enzyme cost
Supercritical fluids (CO_2)	Low cost Low toxicity	
Cell fragility	Use of weak extracting conditions	
Air classification	High purity	Low recovery
Spontaneous liberation	No extracting chemicals needed	Low recovery

2.3 Scale up processes

It is known that the biggest drawbacks for PHA commercialization are the high production cost and expensive separation and purification technologies. Still, many processes have been developed in an attempt to scale-up PHA production (84). *P. putidia* has already suffered scale up tests, to a medium scale. The fermentation device was composed by a vessel, with over 200L and a sterile filter recirculation loop. Initially a heat pretreatment was performed, followed by an enzymatic digestion carried out by alcalase. This process was combined with EDTA and SDS. The final product was a PHA latex, with up a solid fraction of 30% and a purity exceeding 95% (85). A large-scale process was also developed, this time with *Aeromonas hydrophila* 4AK4. A 20 000L processor was filled with 1% of Na_2HPO_4, 1% of $CaCl_2$, and 100ppm of polyacrylamide. The water excess was removed with the help of a filter press while the cell cakes were processed in a rotating vacuum dryer, being later converted to power in a grinder. The extraction part was then performed on a 30 000L tank, with 5000L of ethylacetate stirred at 60 ◦C for 2 h (86), where 200 to 500kg of cell powder were extracted. The solution containing the polymer was centrifuged and the co-polymer PHBV was later recovered by adding hexane or heptane. The last step involved the filter pressing and washing the polymer flocculants with ethanol before being vacuum-dried. With this process, the cost of the recovery process exceeded more than 50% of the total cost, reason why further large scale studies needed to be developed in order to make this process viable (86). Further studies could also involve the usage of new processes, being the microwaves an example. This treatment has been vastly used on algae processing, being a good candidate to be inserted as a cell disruptor in PHA extraction processes. Improving the current extraction methods while introducing new ones can lead to optimal PHA recovery while keeping lows production costs.

3. Objectives of this work

In the past years a lot of improvements have been made on PHA extraction processes. However, the use of organic solvents is still one of the most successful methods, despite its high risks in terms of human health and the environment. Based on the literature revision made, this Master project intended to improve the efficiency of surfactants on PHA extraction, while using benign solvents, like ethanol, to purify the samples. The activity of some specific ionic liquids (ILs) was also studied, and mixed microbial cultures were used.

4. Materials and Methods

4.1 Biomass

The biomass was collected from a sequenced batch reactor (SBR) operated under feast and famine conditions to select a PHA-storing mixed microbial culture in the research group of REQUIMTE at the FCT/UNL. The SBR was operated with cycles of 12h and fed with pine bio-oil as carbon source, supplemented with phosphorus and ammonia salts in order to keep a C:N:P ratio of 100:5:1. The biomass was purged periodically in order to keep a sludge retention time of five days. The biomass was collected after the end of feast phase and stored at 4ºC to obtain a desirable volume to be used for the extraction procedure. Samples were taken periodically and an average PHA content of the collected biomass was determined.

4.2 Biomass treatment

MMC biomass samples were centrifuged at 5000 rpm and 4ºC for 45min. The supernatant was discarded, and the pellet washed three times with 0.9% of NaCl solution, centrifuged each time at 5000 rpm for 45min and 4ºC. The final pellet was freeze-dried (Telstar Lyo Quest) for 72-120h, and kept in a dessicator for determination of polymer content by gas chromatography.

4.3 Extractive agents

In this study the following solvents were used: dodecyltrimethylammonium bromide (DTAB) $[N_{1,1,1,12}]Br$, tetradecyltrymethylammonium bromide (TTAB) $[N_{1,1,1,4}]Br$, cetyltrimethylammonium bromide, $[N_{1,1,1,6}]Br$ (98 wt%), decyltrimethylammonium bromide, $[N_{1,1,1,10}]Cl$ (99 wt%), cetylpyridinium chloride $[C_{16}py]Cl$ (CPC), tetraoctylphosphonium bromide, $[P_{8,8,8,8}]Br$ (95 wt%), tributyltetradecylphosphonium chloride $[P_{4,4,4,14}]Cl$, polyoxyethylene sorbitan monolaurate (Tween 20), polyethylene glycol dodecyl ether (Brij L4), dymethil carbonate (DMC) were supplied by Sigma; sodium dodecylsulfate (SDS) and Triton X-114 were acquired from Acros Organics. The 1-alkyl-3-methylimidazolium chloride ILs series, $[C_n mim]Cl$, such as 1-ethyl-3-methylimidazolium chloride, $[C_2 mim]Cl$ (99 wt%), 1-butyl-3-methylimidazolium chloride, $[C_4 mim]Cl$ (99 wt%), 1-hexyl-3-methylimidazolium chloride, $[C_6 mim]Cl$ (98 wt%), 1-methyl-3-octylimidazolium

chloride, [C_8mim]Cl (99 wt%), 1-decyl-3-methylimidazolium chloride, [C_{10}mim]Cl (98 wt%), 1-dodecyl-3-methylimidazolium chloride, [C_{12}mim]Cl (98 wt%), 1-methyl-3-tetradecylimidazolium chloride, [C_{14}mim]Cl (98 wt%), 1-hexadecyl-3-methylimidazolium chloride, [C_{16}mim]Cl (98 wt%), 1-Ethyl-3-methylimidazolium methylphosphonate, [C_2mim][MP], 1-ethyl-3-methylimidazolium dicyanamide [C_2mim][$N(CN)_2$], 1-Ethyl-3-methylimidazolium thiocyanate, [C_2mim][SCN] were supplied by IoLiTec. All these chemical structures can be seen in Appendix A.

4.4 Solubilization tests

To minimize the risk of losing PHA by solubilization in the surfactant during biomass digestion, some solubilization tests were performed to confirm that PHA would not solubilize in desired extractive agent. So, 1.0g of industrial P(3HB) was mixed with 10mL of Tween 20, both at room and 60ºC temperature, with gentle mixing.

4.5 Extraction procedure

The reagents used were tested at different concentrations: 50mM, 150mM, 250mM, 400mM and 500mM. A pre-calculated mass of each solvent (Equation 1) was initially weighed in an analytical balance, followed by the addition of distilled water till a certain pre-defined weight was reached.

During this study the extraction procedure suffered several optimizations. The starting process involved the overnight (13h-14h) biomass dissolution of 0.800g of biomass with 10mL of solvent at 28ºC with stirring (80rpm). Finished this step, a centrifugation (5500 rpm, 25ºC, 30min) was performed and the supernatant discarded. The pellet was then left to dissolve in 4mL of dimethyl carbonate (DMC) overnight. Finally, the mixture was vacuum filtrated with glass microfiber membranes with 47mm diameter, and the DMC was left to evaporate. The obtained residue was weighed and the PHA content estimated based on the percentage of PHA present in the initial biomass (Equation 2).

The extraction trials started with a screening of solvents to select the most efficient based on the amount of polymer extracted. Then, different parameters were tested with the selected solvent, namely solvent concentration, ratio solvent/biomass, and the digestion duration. Also, the necessity of using chloroform or DMC in a subsequent purification step was assessed. Table 3 resumes the tests performed.

Table 3 – Different parameters tested as the work was developed.

Parameter	Description
Solvent concentration	Further trials tested using surfactant at 50mM, 150mM, 400mM and 500mM.
Solvent/biomass ratio	Biomass quantity decreased from 0.800g to 0.300g.
Digestion duration	Further trials tested different durations for the biomass digestion: 2h, 4h, 6h and 8h.
Organic solvent usage	Trial performed in the absence of this final step (solely with surfactant).

The parameters that led to the best results in terms of amount of polymer extracted were included in the initial extraction procedure and a new protocol is proposed at the end of this work.

4.6 Analytical methods

A. Gas chromatography

Gas chromatography with a coupled flame ionization detector (GC-FID) was used to determine the PHA concentration in biomass samples, using a chromatograph Clarus 480 from Perkin Elmer equipped with a column SGE BP20 (WAX) (length: 60m: inner diameter: 0.32mm; film thickness: 0.5µm) and using a method adapted from Lemos et al (87). The lyophilized biomass was incubated at 100ºC for 3.5h with heptadecane solution as internal standards, dissolved in chlorophorm (1:1) and 20% acidic methanol. After digestion, the organic fraction of each sample was extracted and injected in the GC--FID. The HB and HV monomers concentration were calculated using P(HB-co-HV) (88%-12%) standards. The column temperature started at 50ºC and was firstly ramped up to 100ºC by 16ºC/min and followed by a second ramp of 9ºC/min until reaching 220ºC and kept and this temperature for 3min.

B. Fourier-transform infrared spectroscopy (FT-IR)

The PHA film and other substances extracted were analysed by FT-IR spectroscopy (PerkinElmer) with the conditions: spectral range, 4000-500 cm^{-1} to allow confirmation of the functional groups presented in the polymer. Sample analysis was

performed by scraping the flask walls or bottom in order to isolate some PHA. The biopolymer was then inserted above the crystal, with solid samples being further squeezed with an equipped press.

4.7 Calculations

The equation used to calculate the mass of surfactant needed, according to the final concentration (50mM, 150mM, 250mM and 500mM), for surfactant solutions preparation was:

$$m_s = C_s(M) \times Mw(gmol^{-1}) \times f_w(kg) \qquad \text{Equation 1}$$

where m_s is the mass of surfactant, C_s the desired surfactant concentration, Mw is the molecular weight and f_w the mixture total weight.

The percentage of PHA extracted and the yield of the overall process was calculated using the following equation:

$$\%PHA_{extracted} = \frac{m_{PHA}(g)}{\%PHA_{biomass} * m_{biomass}(g)} \qquad \text{Equation 2}$$

where m_{PHA} is the mass of polymer film obtained at the end of the process, $\%PHA_{biomass}$ is the percentage of PHA found in the lyophilized biomass and $m_{biomass}$ the mass of biomass weighed for the extraction process.

5. Results and Discussion

5.1 PHA production

The biomass used in extraction assays was collected in three different periods of operation of the SBR. Table 4 shows that the biomass used in this work had very low PHA content, lower than 10%. This situation occurred because the system was a selection reactor, where stability of population was more important than the amount of polymer produced. In this case biomass would be available, despite the low value of PHA stored. A desirable situation was the collection of biomass from an accumulation reactor where more PHA is stored. The storage of biomass at 4ºC has probably contributed to the low amount of biomass, since its collection at the end of the feast phase signified the absence of an external carbon source. Even at such low temperature, cells require a carbon source for their maintenance and, consequently, consumed the stored PHA. From the industrial point of view, it is desirable a high PHA content to maximize the amount of polymer extracted. However, the use of such low PHA contents could be positive for the development of a successful extraction procedure. A method that is efficient to extract low amounts of product can mean an even better performance when applying it to biomass with higher storage contents.

Table 4 – PHA content found in different biomasses used in this work.

	Biomass A	**Biomass B**	**Biomass C**
PHA content (%)	9.6	7.1	6.3

5.2 FTIR characterization of PHA

The traditional method used for PHA analysis, also including the characterization after an extraction procedure, was gas-chromatography (GC). This work started with the development of a FTIR (Fourier-transform infrared spectroscopy) analysis to the freeze-dried biomass and industrial PHA (P(3HB)) (Figure 10), to be used as controls on further

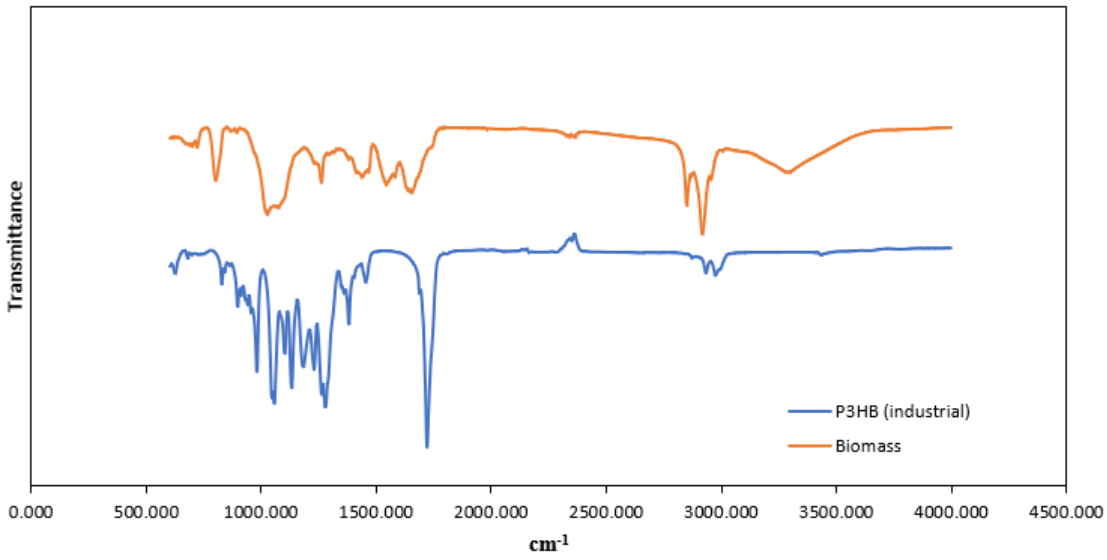


Figure 10 – FTIR spectra of industrial P(3HB) and freeze-dried biomass.

analysis. As FTIR was used only in a few works regarding PHA analysis, in this work it was a useful tool to quickly detect the biopolymer presence in the final samples, by simply comparing the control FTIR spectra characteristics and its peaks, with those obtained for each sample (88). Regarding the industrial P(3HB) FTIR spectra, the main peak for PHA detection can be seen at ~1700cm^{-1}, which represents the ester groups (C=O stretching vibrations). Between ~980cm^{-1} to around ~1350cm^{-1} other peaks can be observed corresponding to C-C bonds (~980cm^{-1}), C-O-C symmetrical and unsymmetrical stretches (~1000cm^{-1}), or CH_3 angular symmetrical deformation (~1350cm^{-1}). The peaks at ~2900cm^{-1} and ~2400cm^{-1} correspond to water and carbon dioxide presence, respectively (88). For the biomass, the characteristic peaks can be seen from ~800cm^{-1} up to ~1600cm^{-1}. These represent a large variety of functional groups like vinyl C-H bonds (~800cm^{-1}), stretching vibrations of -OH groups in polysaccharides (~1040cm^{-1} to 1200cm^{-1}), carboxylic acids, C-O (~1250cm^{-1}-1300cm^{-1}) and multiple different C=O bonds (~1500cm^{-1} to 1750cm^{-1}), as expected to be present in a mixed culture biomass sample (89).

5.3 Screening tests

The main objective of this work was the improvement, and further implementation, of an efficient extraction process of PHA considering more environmental friendly solvents, while trying to reduce the amount of extractive agent used. The first step of the process involved the digestion of freeze-dried biomass samples with the purpose to disrupt cell membranes and release the stored PHA, while partially solubilizing the NPCM. The extractive agents tested were surfactants and IL's (Appendix A) in a concentration of 250mM, which was above the critical micelle concentration (CMC) to all of them (data not shown). The second step aimed to recover and isolate the PHA from the NPCM leftovers. At laboratory level, the extraction of PHA is usually performed using chlorophorm. In this work, DMC was tested as a substitute, for a more viable alternative as a process improvement step (57). The first screening, using biomass A, was performed with a variety of solvents and the final product obtained and visible in each flask can be observed in Appendix B. In Figure 11 it can be observed the final product from an extraction made with DTAB. Despite being possible to observe a small film on the walls, there was also an accumulation of surfactant in the bottom of the flask. This accumulation seemed to be present in practically every sample which reflected the low purity values achieved. To tackle this problem, a purification step was performed by

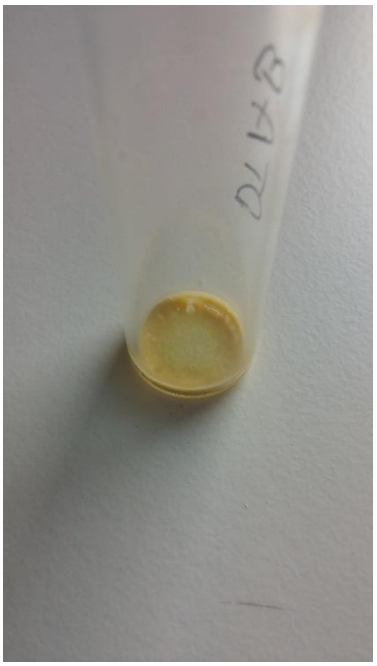


Figure 11 – Extractive agent accumulation in DTAB sample.

adding 4mL of ethanol to the flask and gently mixing. Despite being able to solubilize surfactants, this solubilization was partial, probably due to the absence of proper stirring, to avoid any PHA sample loss, as a stronger mixing would result in the biopolymer detachment from the flask walls, being then poured out alongside with ethanol and surfactant. Consequently, since the complete surfactant removal from the flasks was not possible, these samples were discarded since the weight difference between the final product and the initial biomass was too high. To solve this problem, and increase the overall purity, the washing step performed after centrifugation was improved. Instead of performing one single water washing step, three steps were introduced. First the pellet was washed with 5 mL of water, then with 5 mL of ethanol, and finally again with 5mL of water. Apart from this change, the temperature of the biomass and surfactant mixture was increased to 60ºC, aiming to increase extraction efficiency (90). The results from this extraction can be observed in Figure 12. Regarding the different groups of agents, non tensioactive compounds achieved similar results, while on the tensioactive cationic group was observed a small tendency of extraction yield increase, the bigger the carbonated chains were. As the only anionic agent, SDS, achieved the lowest extraction yield of the screening and the non-ionic tensioactive group showed a big dispersity. Many different yield results were obtained, ranging from lower than 10% to over the 100% limit (Triton X-114 and [$P_{8,8,8,8}$]Br). These last two were automatically discarded since, even if they were able to isolate PHA, the extractive agent accumulation obtained was too high, which

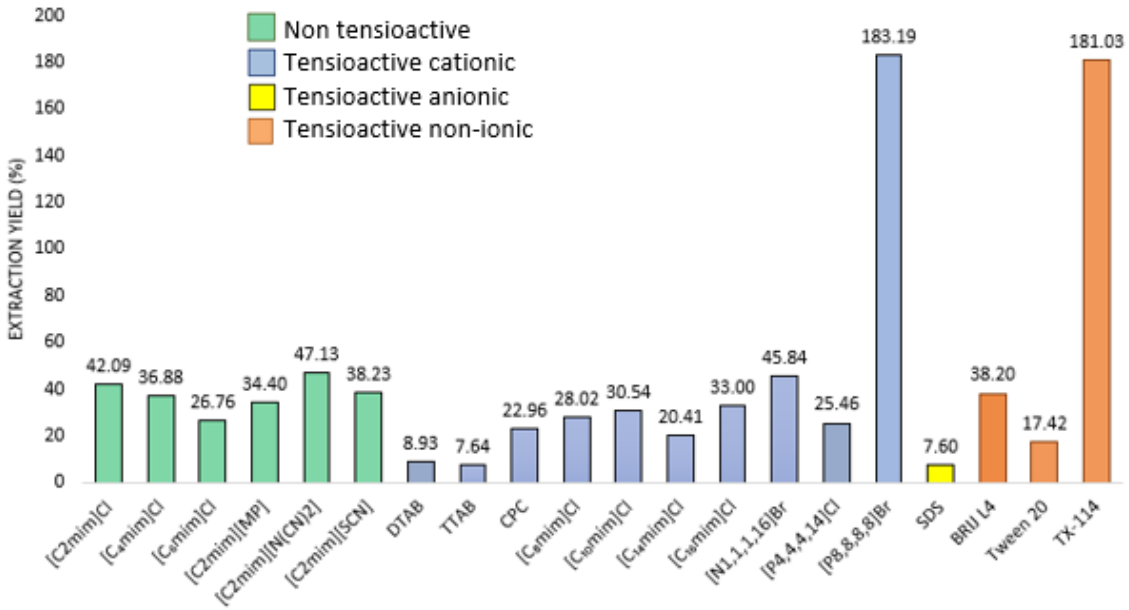


Figure 12 – Results of the initial screening of different extractive agents

actually justifies the much higher values of extraction efficiency, the samples were contaminated with the solvents. Furthermore, despite not trespassing the percentage limit, many other results suffered mild to severe extractive agent contamination. In some of them, this accumulation was able to be partially solubilized with ethanol, while in others practically no removal was observed ([C_2mim][MP], [C_2mim][$N(CN)_2$], [C_2mim][SCN]), probably because these compounds might not be so easily soluble in ethanol (data not shown) and/or other solvents could have been tested for their purification step. Nonetheless, among all these results, Tween 20 seemed to be the only sample without contamination by surfactant accumulation in the flask (Figure 13). In order to better characterize the product of extraction, the FTIR spectra was obtained and compared with the spectra of commercial P(3HB), The characteristic peaks of P(3HB) were clearly seen only on the spectra of the sample obtained with Tween 20 (Figure 14). In some samples, the characteristic peaks appeared but with lower signals and in other the peaks were not observed (Appendix C). A huge similarity between the spectra of P(3HB) obtained with Tween 20 and P(3HB) (industrial) was observed, namely the presence of a strong peak at ~1700cm^{-1}, meaning P(3HB) was extracted and isolated with this procedure using surfactant Tween 20. Furthermore, by comparing P(3HB) (Tween 20) and Tween 20 spectra it can be observed that no surfactant accumulation was found in the final sample. The solubilization tests performed showed that no P(3HB) was solubilized in Tween 20, minimizing the risks of losing polymer during the process. For this reason, further extraction procedures were performed using Tween 20.

Figure 13 – PHA film visible on flask walls, without surfactant accumulation (Tween 20 sample).

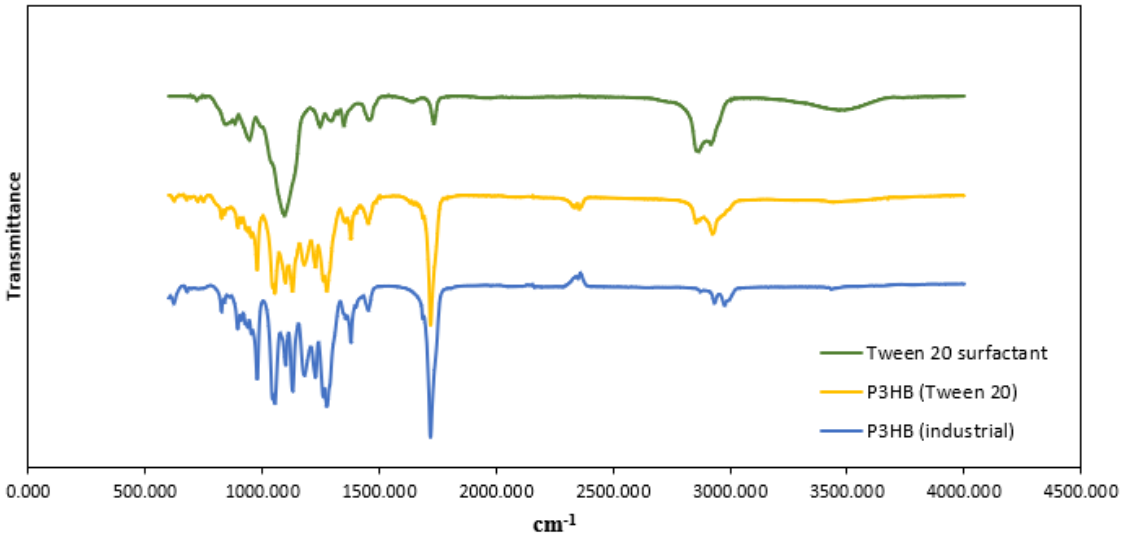


Figure 14 – FTIR analysis comparison of industrial P(3HB), P(3HB) extracted with Tween 20 and Tween 20 surfactant.

5.4 Effect of surfactant concentration

After selection of Tween 20 in the first screening, the effect of this surfactant concentration was evaluated. Different Tween 20 concentrations, all above the CMC point, were tested, namely: 50 mM, 150 mM, 250 mM and 500 mM. Biomass B was used, and the results obtained are shown in Table 5. As it can be observed, higher yield values were achieved using lower surfactant concentrations, namely 50 mM and 150 mM, while these values drastically reduced when using surfactant at 500 mM. Despite the higher volume of surfactant used in this sample, which theoretically should lead to a better cell disruption, NPCM solubilization, and higher yield values, at this concentration the surfactant solution showed a very high viscosity. This property greatly difficulted the stirring procedure, which could have led to a bad mixture and, consequently, low digestion efficiency. Furthermore, the three washing steps performed after centrifugation were not often enough to properly purify the pellet obtained from the high concentrated surfactant. The next step involved the reduction of the amount of biomass used from 0.800g to 0.300g, aiming at to optimize the solid-liquid ratio parameter (Table 6). The use of a lower amount of biomass resulted on the increase of the overall extraction yields, which was expectable, since the same amount of surfactant was used to digest a lower amount of biomass. Furthermore, it can be observed that the extraction yields obtained, more than doubled if half of the previously biomass is used.

Table 5 – Extraction yield variation with different Tween 20 concentrations for 0.800g of biomass.

Tween 20 concentration (mM)	Extraction yield (%)
50	19.69
150	22.46
250	17.42
500	5.18

Table 6 – Extraction yield obtained with low amount of biomass, 0.300g.

Tween 20 concentration (mM)	Extraction yield (%)
50	49.41
150	53.57
250	40.38
400	15.93
500	10.62

In this set of assays, a concentration of 400mM was tested to confirm the result previously obtained with 500mM. Again, a crescent viscosity of these solvent solutions with the increase of concentration was observed, decreasing the overall extraction efficiency. On the other hand, the best results were obtained with lower surfactant concentrations (50, 150 and 250mM) making the process cheaper since similar or better extraction yields can be obtained with smaller amounts of surfactant.

5.5 Effect of digestion time

Previous tests were performed overnight aiming at to maximize the amount of polymer extracted by the extractive agent. The reduction of the digestion time will signify a reduction on the energy costs of this process. Four different digestion times were tested, 2h, 4h, 6h and 8h for four different concentrations of Tween 20, namely 50mM, 150mM, 250mM, 500mM (Figure 15). Despite the overall minimum variation of the extraction yields with the decrease of digestion time, a reduction in extraction yields was observed

for 2h of digestion, especially using the 50mM and 150mM. This probably means that this time was not enough to correctly lyse the biomass considering the low amounts of lysis agent. When comparing the results of the assays at 4h, 6h and 8h, the extraction yields were similar, especially with Tween 20 concentrations at 50mM and 150mM. For 250mM of Tween 20, the values of extraction yields had a high dispersity while for all times tested and 500mM, the extraction yields were the lowest ones. Moreover, the FTIR spectra obtained showed almost no P(3HB) on these samples. Summing up, the obtained results showed that a digestion time of 4h would be preferential and confirmed that lower concentrations of Tween 20 resulted in a more efficient extraction.

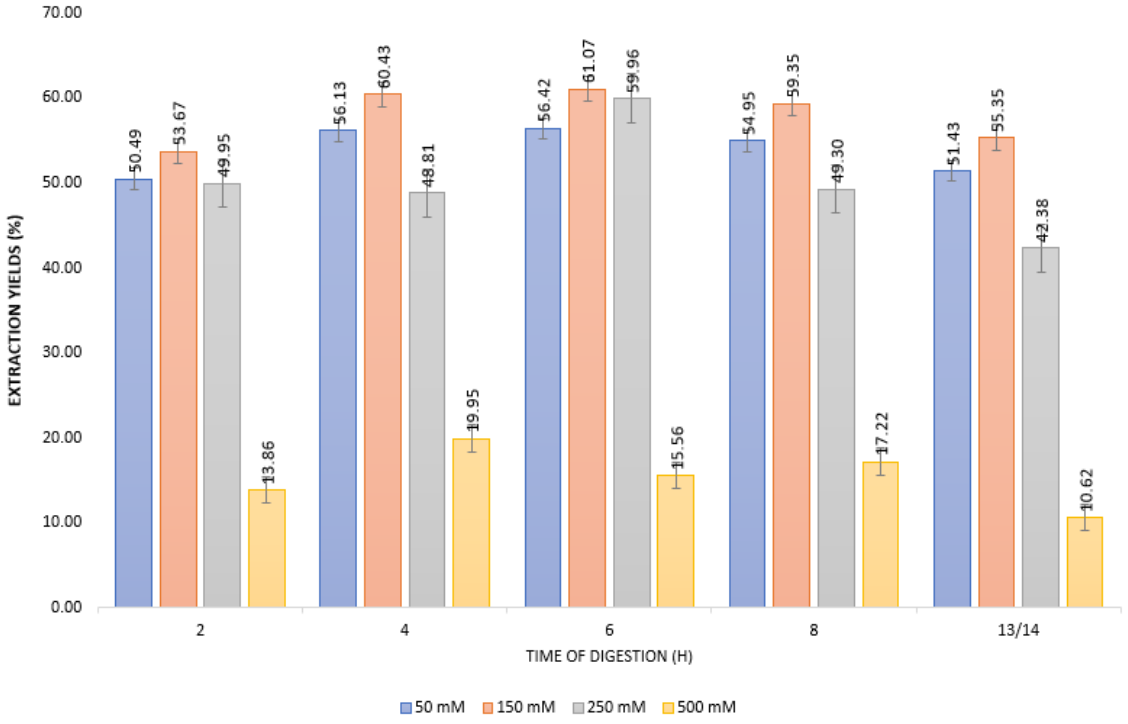


Figure 15 – Comparison of different digestion times on the efficient extraction of PHA.

5.6 The influence of the purification step

Nowadays, one of the main objectives in PHA extraction studies is to completely avoid the use of organic solvents. This has been done by using DMC as an alternative to chloroform or dichloromethane. In this work, the organic solvent DMC was used in a purification step and, from a commercial point of view, due to its lower toxicity, DMC would be the preferred solvent of all three. However, the possibility of eliminating the

purification step would signify a cheaper extraction process. For this reason, an assay using Tween 20 at 50mM and overnight digestion was performed, and the film obtained without the purification step using an organic solvent was analysed by FTIR. Despite the similar results obtained for 50mM and 150mM, the lower Tween 20 concentration was chosen to ensure a reduced presence of surfactant in the extracted polymer. Moreover, the longer digestion time was chosen to maximize the polymer extracted. Biomass digestion was followed by centrifugation and a washing step. After discarding the final supernatant, the sample was analysed by FTIR but, as it can be seen in Figure 16, the water peak (~3400cm^{-1}) obtained was too high. To solve this issue, the sample was then dried at 80ºC for 2h. The dried sample was then analysed by FTIR again (Figure 17). The FTIR spectra of the dried sample showed that, despite of being possible to identify the well-known peak of PHA (~1700cm-1), as well as other peaks from ~900cm^{-1} to ~1200cm^{-1}, there were also peaks from other substances in this last interval. Their presence could either result from the surfactant and/or from the cell debris presence. Actually, the presence of cell debris was clearly confirmed by visualization at naked eye. Because of this, the extraction yield value (16.17%) cannot be properly considered as a final result due to the presence of such contaminations. However, this test confirmed that a purification step not only removes the remaining surfactant, but also the NPCM debris, helping to improve the extraction yield. However, the presence of PHA was still identified despite the process requiring further optimizations in order to find viable alternatives for the polymer purification with organic solvents. Moreover, the inclusion of a purification step should

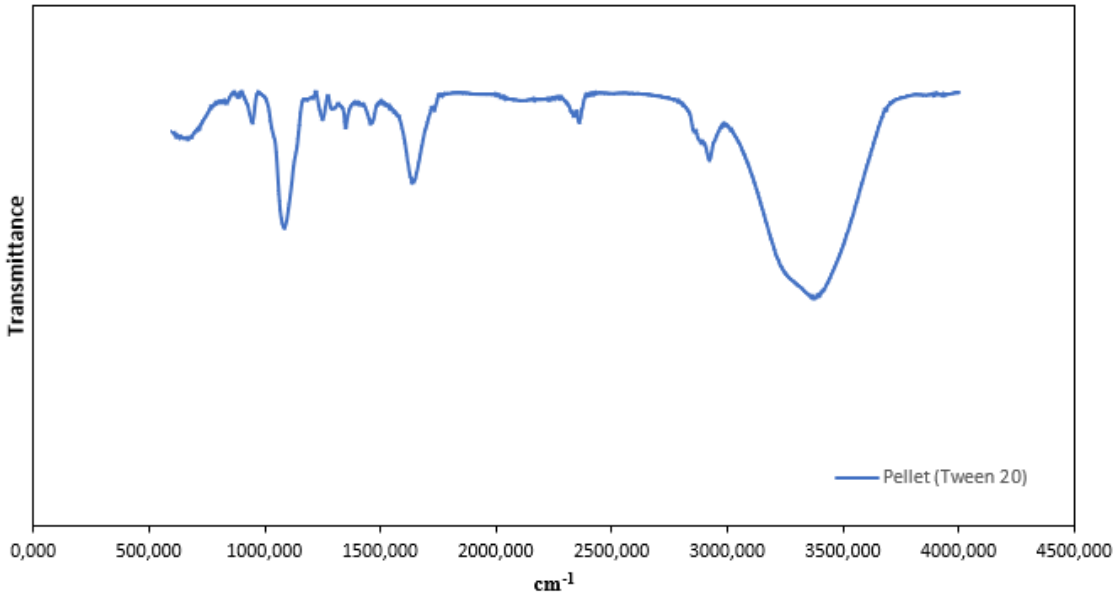


Figure 16 – FTIR analysis of cell pellet after centrifugation rounds.

be assessed considering the final application of the extracted PHA. Less noble applications, as in agriculture, could use less purified PHA, while applications in the medicine field require a very efficient purification step.

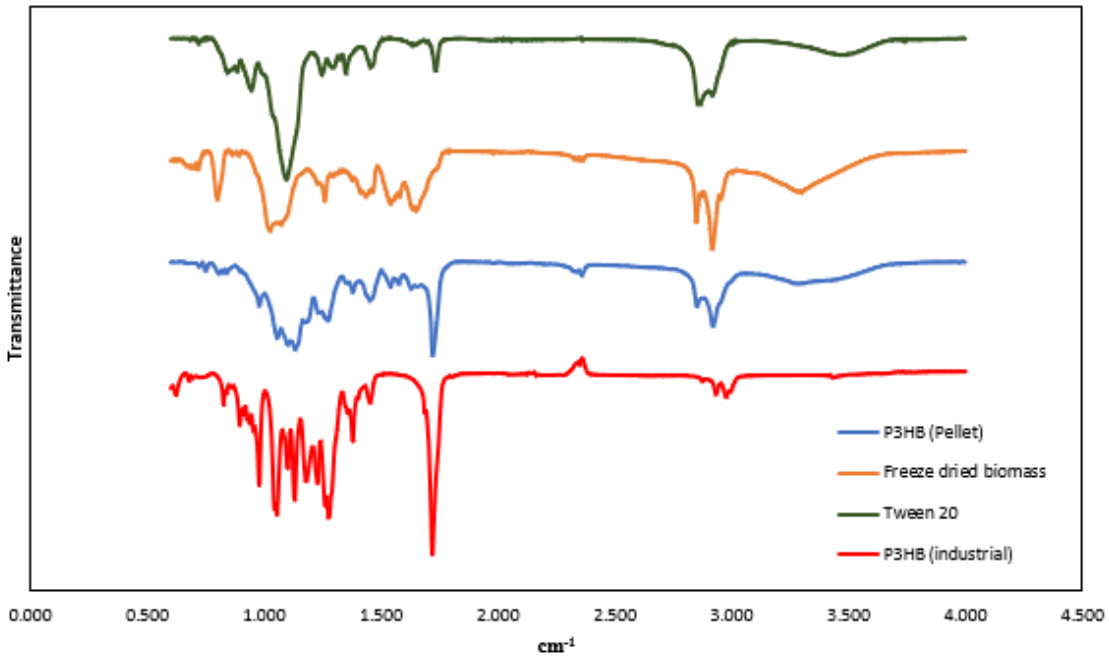


Figure 17 – FTIR analysis of pellet after sample drying and comparison with control samples.

5.7 Redesigning the initial process

Considering the obtained results, the initial extraction process was redesigned (Figure 18). After starting with 0.800g of biomass and 10mL of Tween 20, a successful reduction to 0.300g of biomass was defined. The biomass digestion duration was also reduced, following the last tests performed, proving that 4h are enough to achieve similar results, when compared to yields obtained with overnight digestions. This also reflects on a process efficiency increase, mainly regarding the costs, since less time and energy are required. Despite not being one of the main parameters that were tested, the temperature at which the digestion was performed was also increased to 60ºC following reports that suggested this temperature could be enough to increase, in roughly 20%, the extraction efficiencies (90). Improvements to the purification part were also carried, by turning the single centrifugation washing step into three steps, resulting in much less surfactant accumulation in the resulting samples. The overall process toxicity was greatly reduced since the chlorophorm was completely replaced with DMC, a much less toxic and

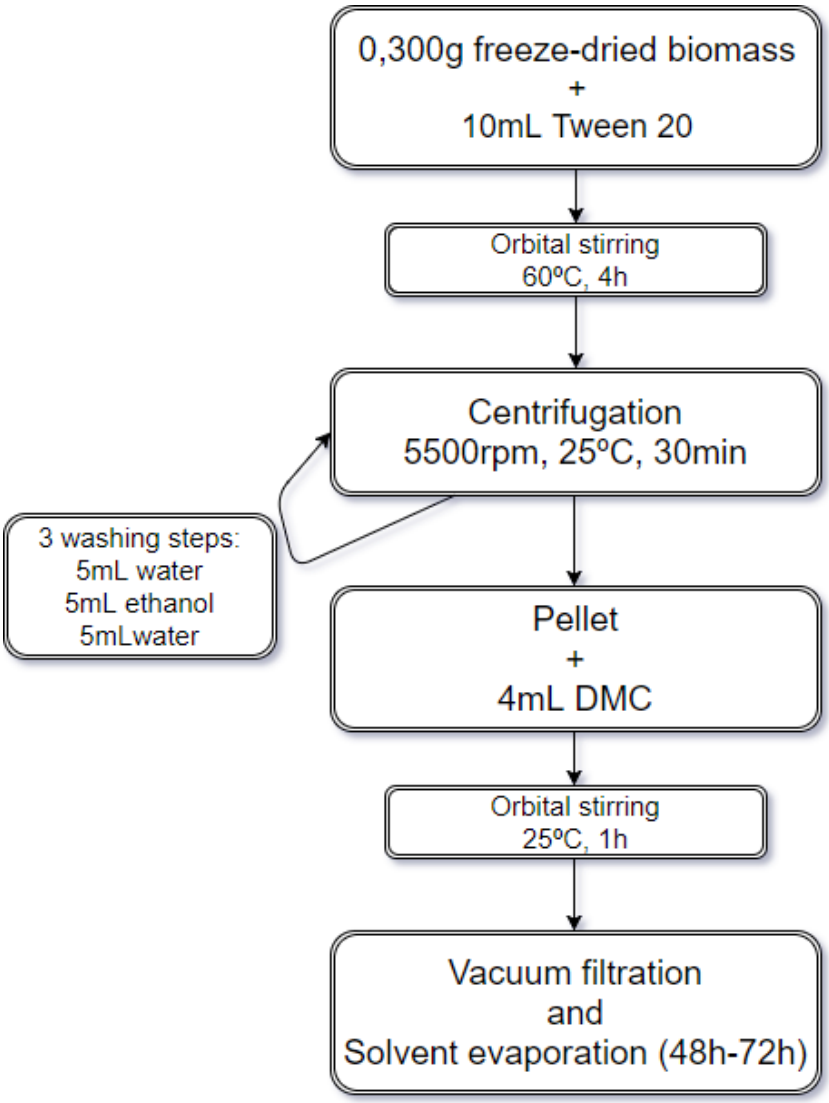


Figure 18 – Final process, with its improved steps, obtained after all tests performed.

biodegradable solvent. Furthermore, the complete removal of this final step is a possibility after a successful isolation and detection of PHA with only surfactant usage. This attempt has already been made, with both pure (90) and mixed (91) cultures, using SDS, although higher recovery yields were achieved with pure cultures (around 90-95%) than with mixed ones (ranging from 49-65%). This difference follows the Patel et al. (2009) findings, that hypothesised a stronger resistance from mixed cultures to chemical treatments, due to the existence of a complex and compact cellular matrix, created between the various bacteria in the mixed culture. PHA extraction is still a poorly studied area, especially when applied to mixed cultures. Results obtained in this work, with surfactant Tween 20 do not differ much from those obtained by Samorì et al (2015) (91) with SDS, despite the differences between both processes, namely surfactant used,

digestion temperature, duration, and PHA percentage found in the biomass. Furthermore, one of the biggest problems when using SDS is the difficulty to remove it from the isolated polymer, which was not a problem with Tween 20, especially when used at lower concentrations. However, several other aspects need to be considered like other solid-liquid ratio, the need of the organic solvent step and the overall process itself, since the number of steps used in this thesis could still be reduced with further investigations.

6. Conclusions

This work allowed the study of the efficiency of surfactants and ionic liquids in the biomass lysis and PHA extraction from mixed cultures. After the initial screening performed with a variety of solvents, Tween 20 was the one chosen due to the lack of surfactant accumulation and the success on the isolation and analysis of PHA film. Further tests were performed with this surfactant to optimize several variables, namely the temperature, biomass quantity, surfactant concentration, digestion duration and organic solvent usage. The best result, 61.07%, of PHA extraction yield was achieved with a surfactant concentration of 150mM and 6h of digestion, with 0.300g of biomass. Extractions performed with higher surfactant concentrations (400mM and 500mM) presented low yields and low PHA isolation, because of the high viscosity of the surfactant solution at this concentration, greatly reducing the efficiency of the stirring step and, consequently, the biomass lysis. Despite the recovery values not changing much with the different biomasses (even reaching higher values with the lower percentage of PHA accumulated), it has been reported that higher polymer accumulation and percentage often leads to higher biopolymer purity (91). However, the extraction procedure developed in this study worked with low amounts of polymer accumulated with quite significant extraction yields. The temperature was set at 60ºC, as reported in literature, and 4h proved to be enough time for the biomass digestion. Chlorophorm was completely replaced with DMC, greatly reducing the toxicity of the overall process. Furthermore, an extraction without the organic solvent usage step was performed and it was possible to isolate PHA with an extraction yield of 16.17%. At the end of this work, an improved and redesigned process for PHA extraction from mixed cultures was obtained (Figure 18), and future work could possibly start from here, aiming to develop new improvements and strategies for PHA extraction.

7. Future perspectives/work

The polyhydroxyalkanoates industry still requires further investigation and studies to make it economically viable in nowadays big industries. One of the major drawbacks, when compared to common plastics, is the increased price, not only of the final biopolymer, but of the entire extraction process as well. First, despite costing less than 50% of the total final cost of the entire process, PHA production needs to be optimized to produce cells with very high PHA percentage accumulation. New types of substrates and feedstocks could be tested, following the already usage of wastewaters and such materials, to further reduce the costs, as well as keeping the process environmentally friendly. Regarding the extraction phase, wet biomass could be applied directly into the process, avoiding a freeze-drying step, as well as eventually keeping the cells complete integrity. However, the water and other substances presence in the fresh biomass could negatively impact the biomass digestion, as well as the stipulated extractive solvents concentration. Nowadays the main step involves the isolation of the biopolymer from the resulting cell debris, usually made by solubilization in an organic solvent. Despite being extremely effective, many other strategies using DMC or water based extractions should be further tested to increase the lower yields, while greatly reducing process's toxicity as well as increasing the range of application areas of the resulting biopolymer. The complete removal of this step is also a possibility, since further studies around NPCM solubilization can develop new strategies that could allow high purity PHA isolation through simple centrifugation, after the main digestion.

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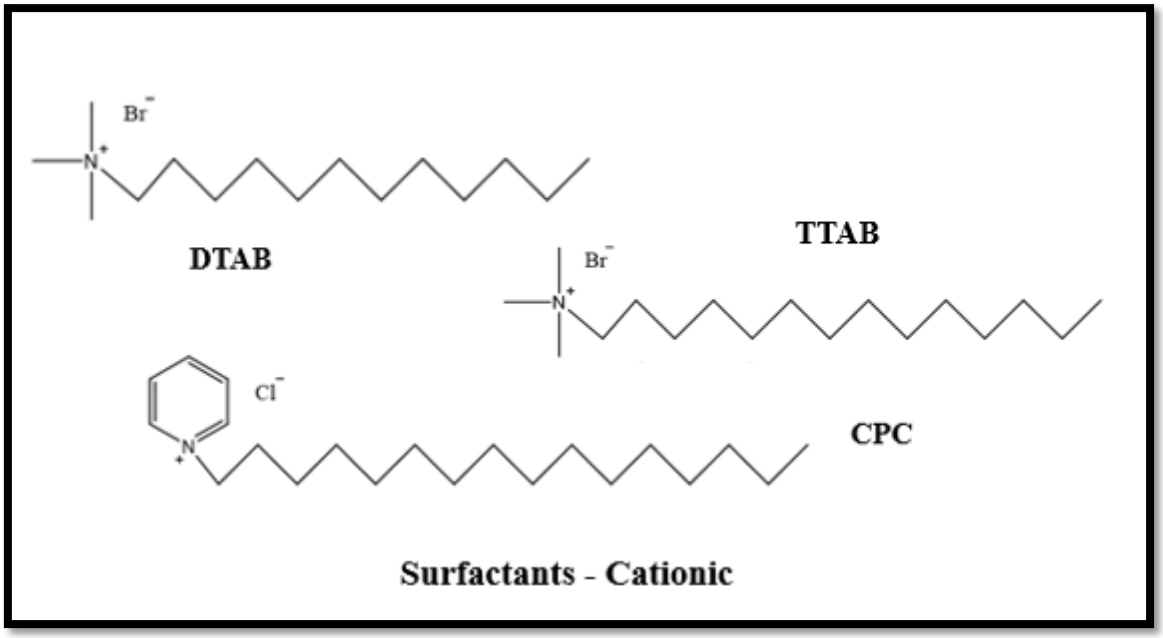
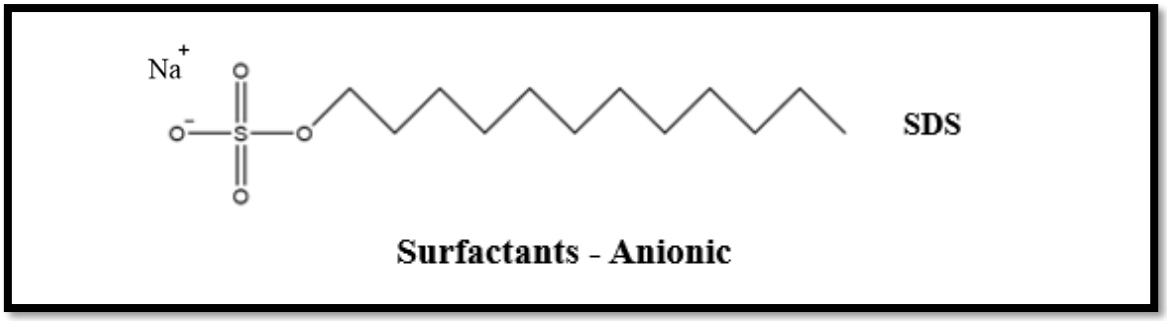
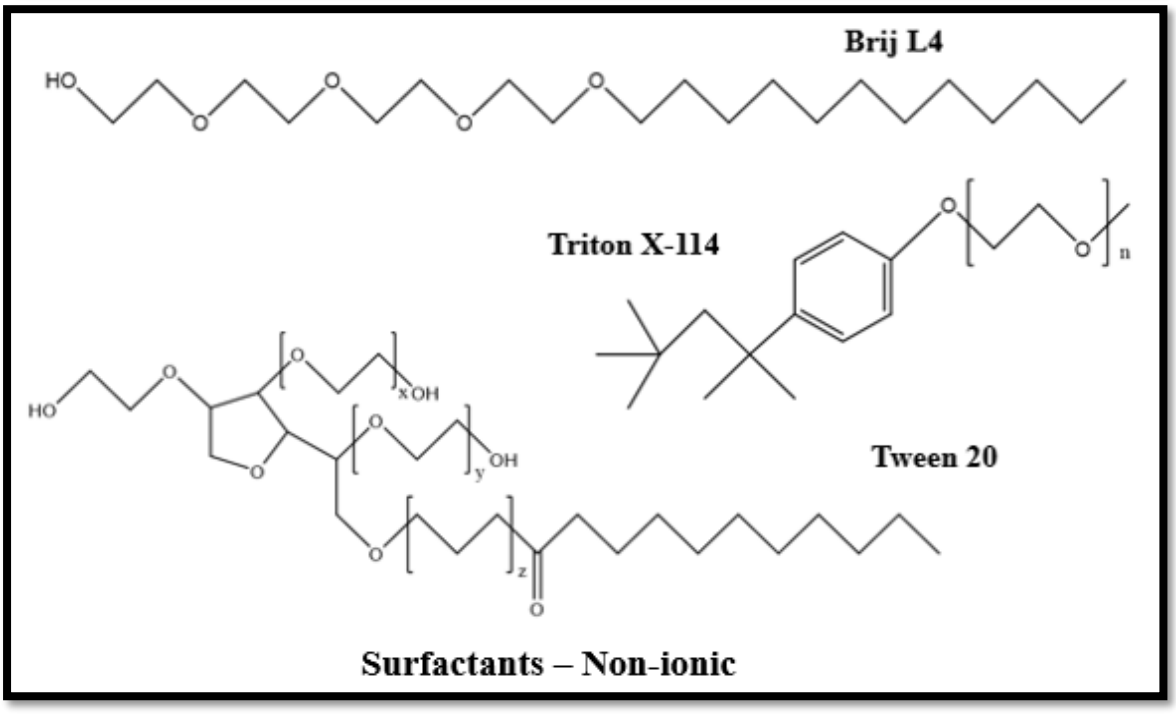
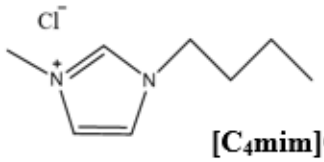
9. Appendix

Appendix A – Extractive agent's structures.









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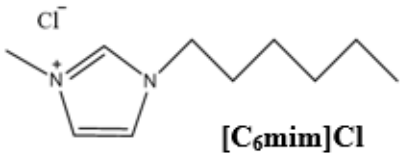

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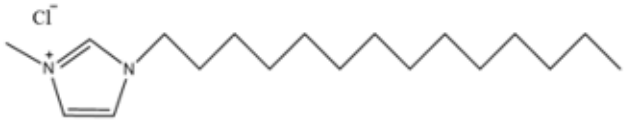

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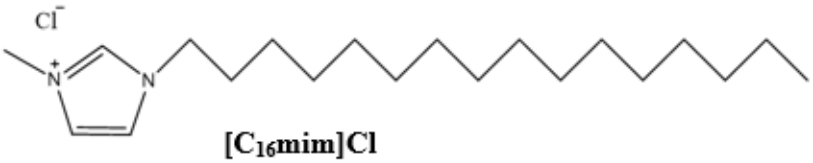

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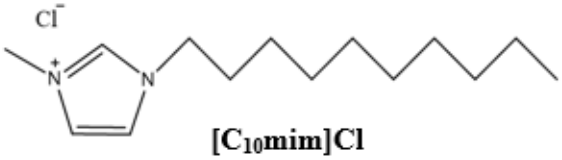

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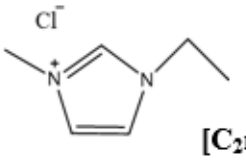

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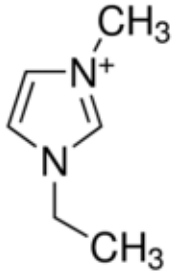

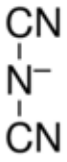

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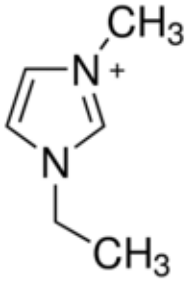

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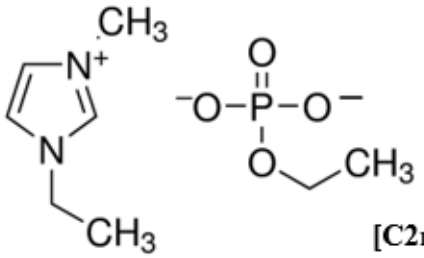
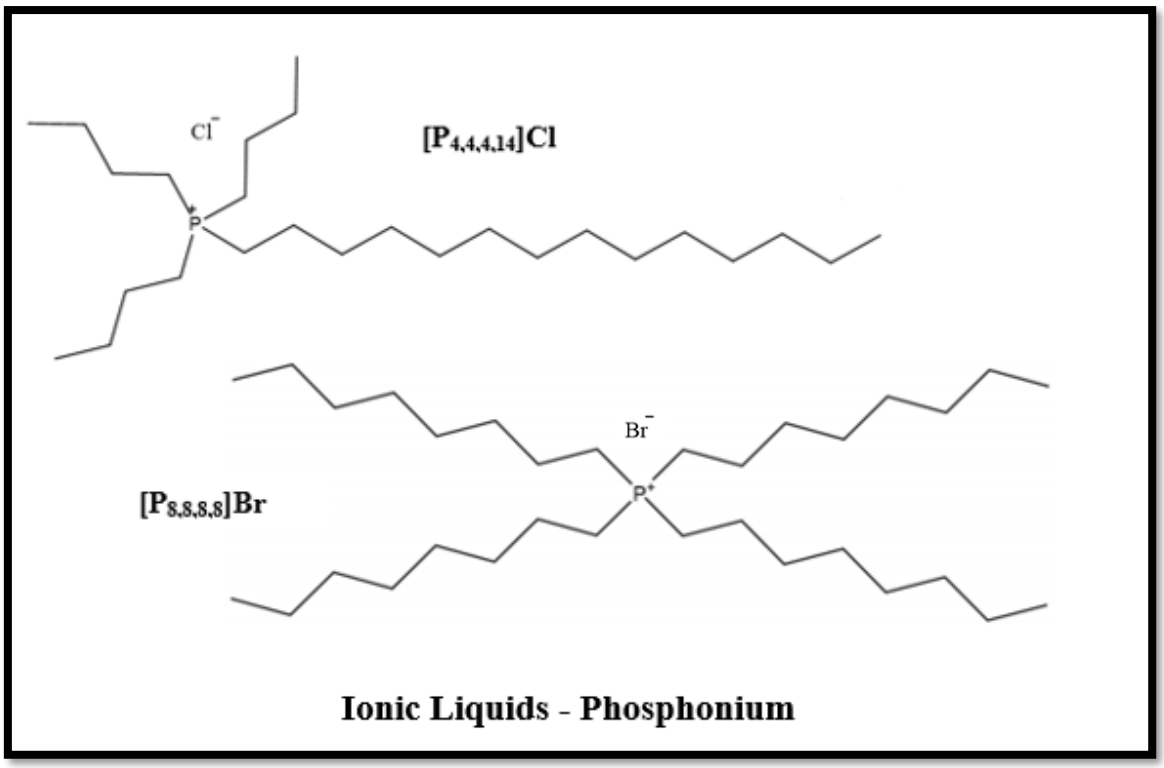
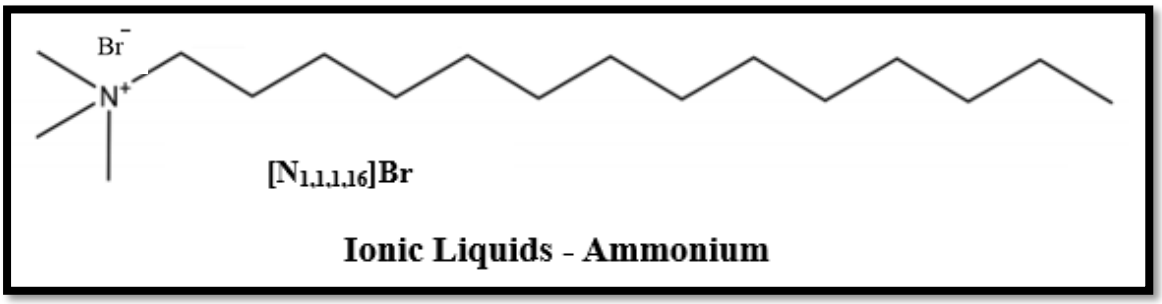


[C2mim][MP]

Ionic Liquids - Imidazolium



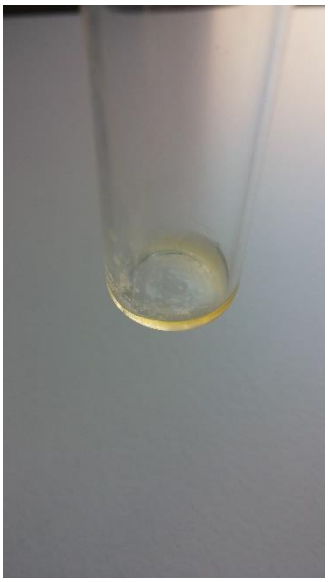
Ionic Liquids - Phosphonium



Ionic Liquids - Ammonium

Appendix B

Results from the first screening (before washing steps improvement)



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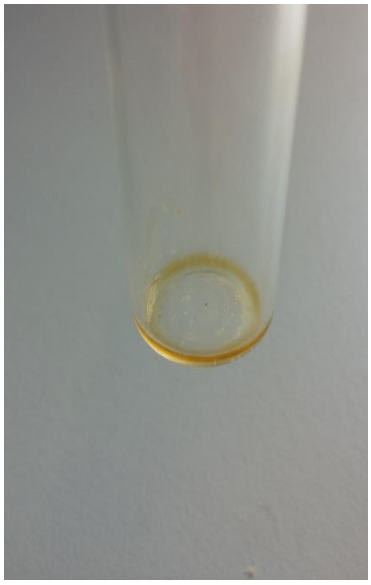

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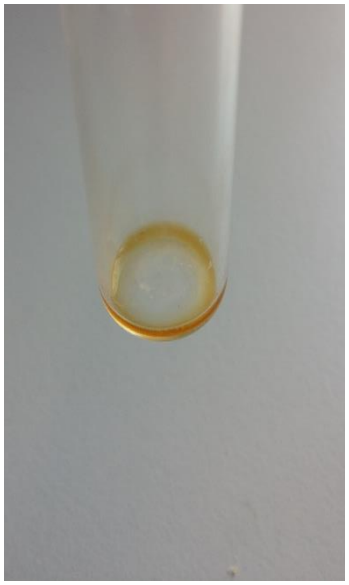

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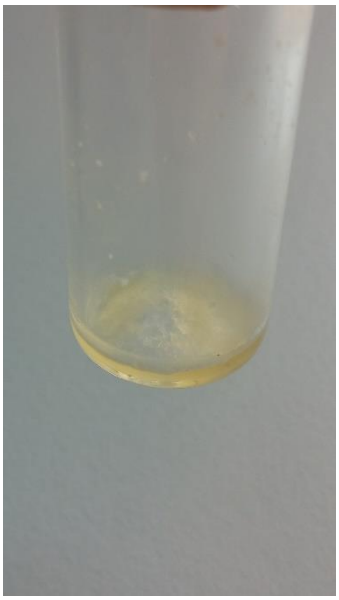

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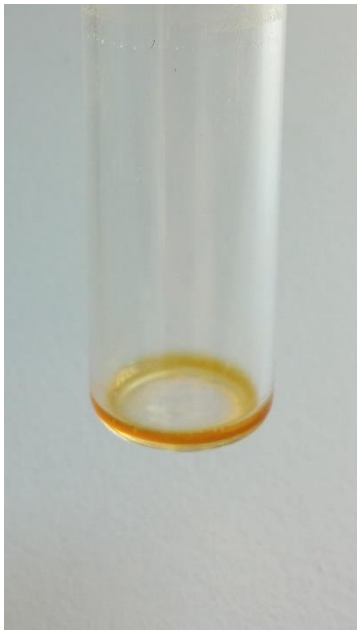

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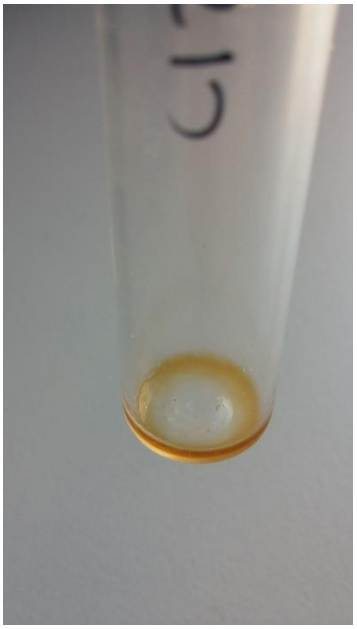
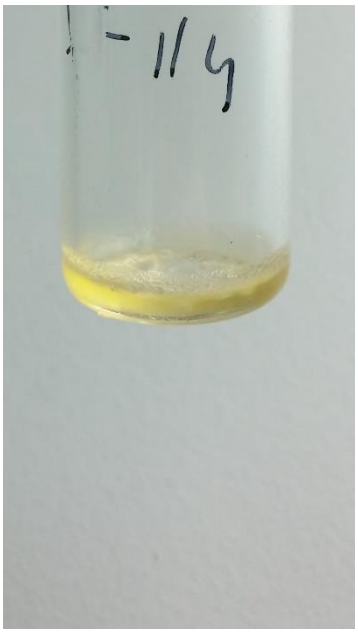
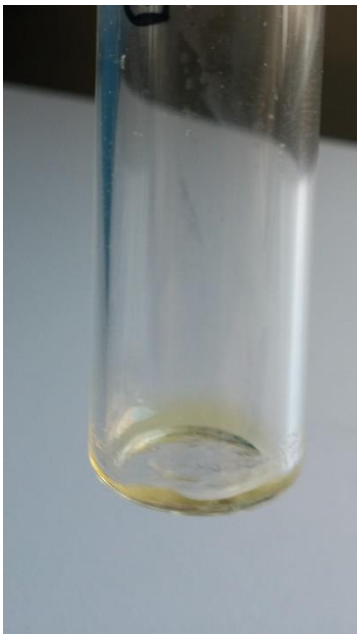


[C_{12}mim]Cl

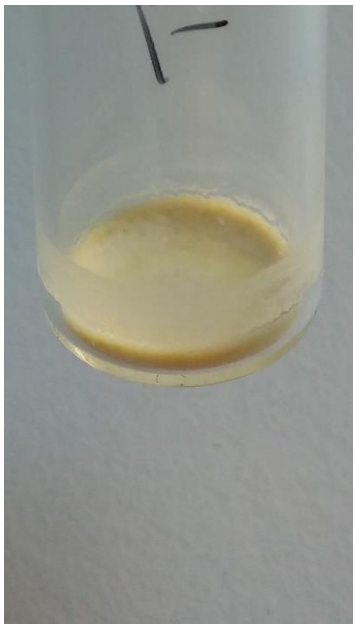
[$P_{8,8,8,8}$]Br



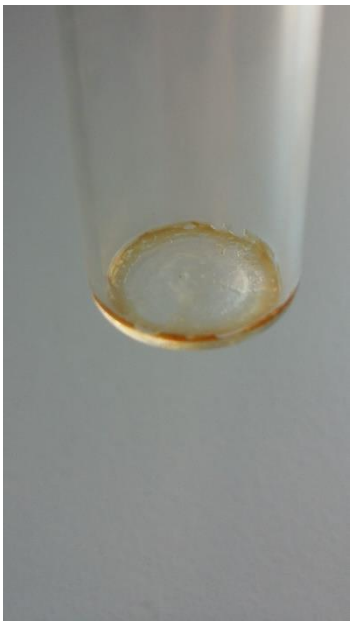
Triton X-114



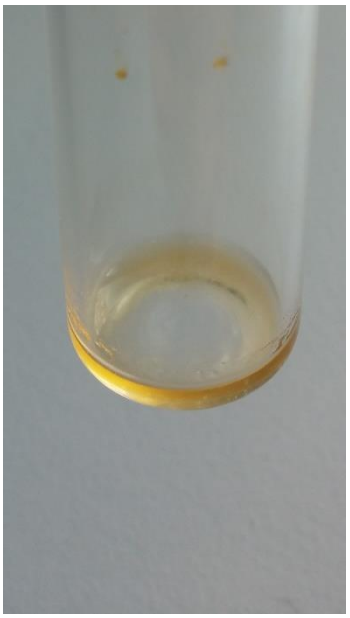
Brij L4



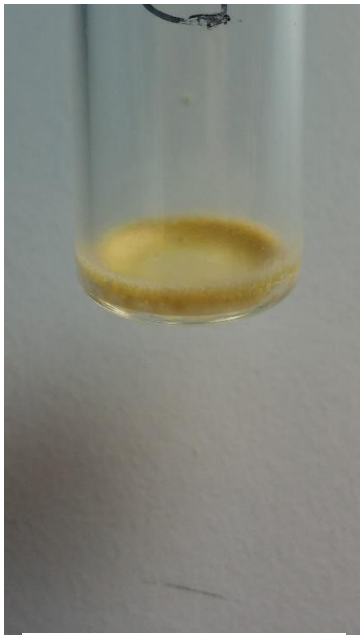
Tween 20



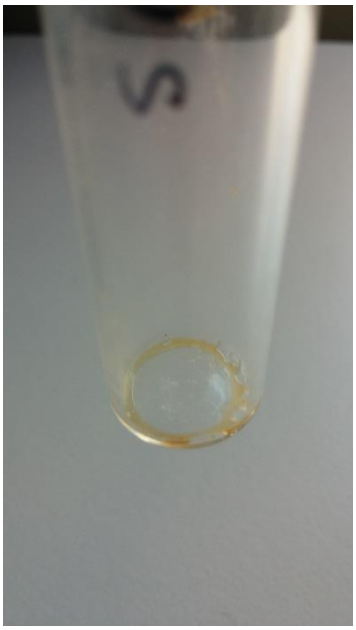
$[N_{1,1,1,16}]Br$



$[P_{4,4,4,14}]Cl$

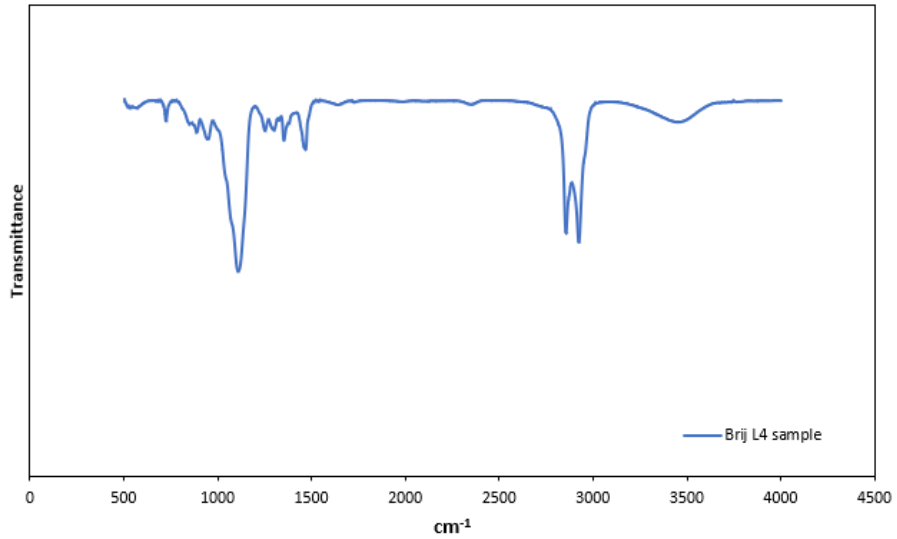


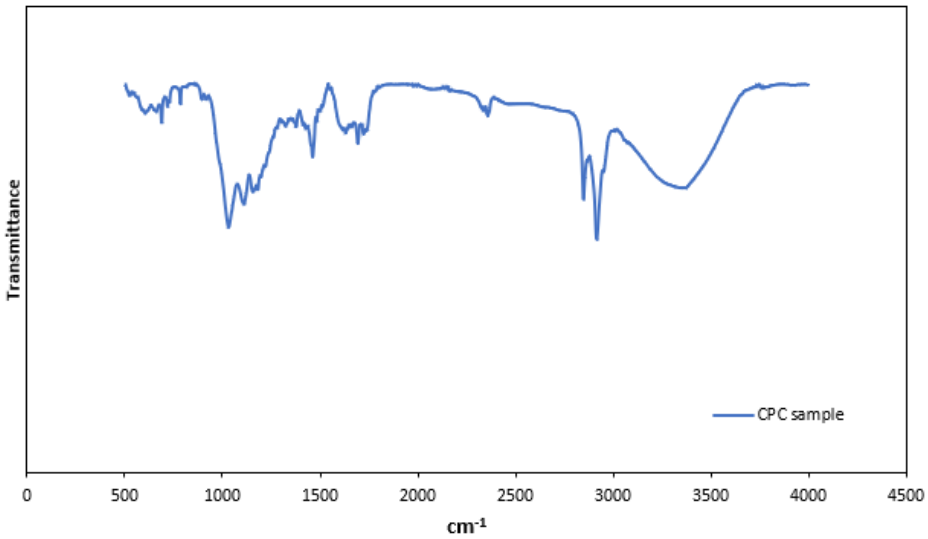
CPC

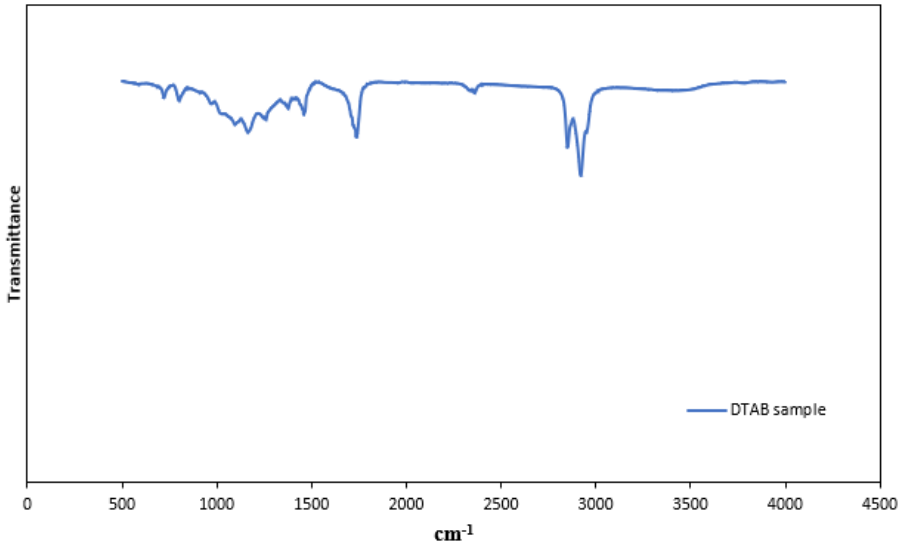


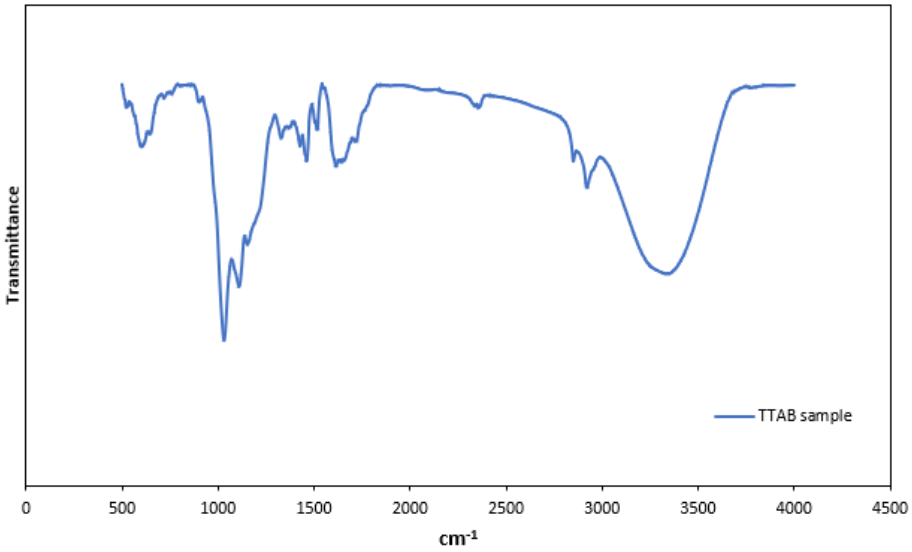
SDS

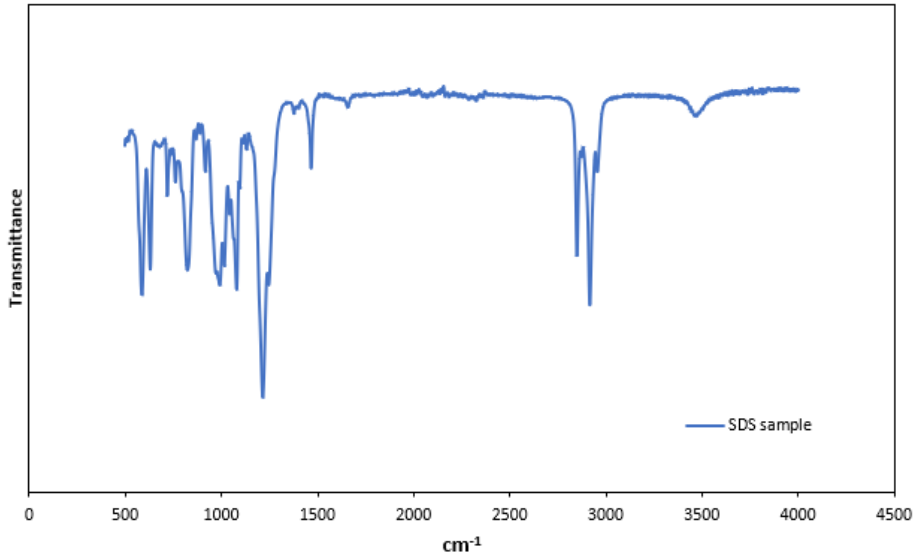
Appendix C – FTIR analysis of the second screening.

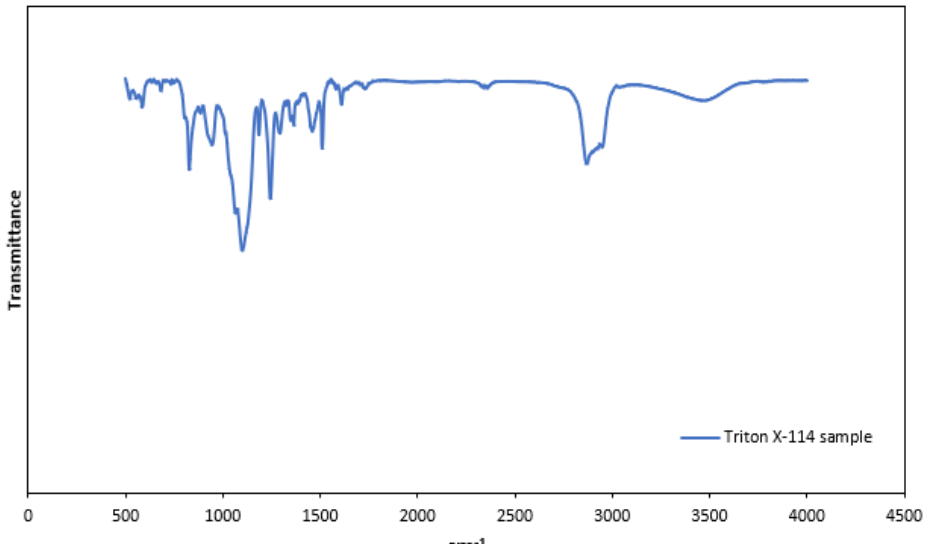









Transmittance
SDS sample
0
500
1000
1500
2000
2500
3000
3500
4000
4500
cm-1


Transmittance
Triton X-114 sample
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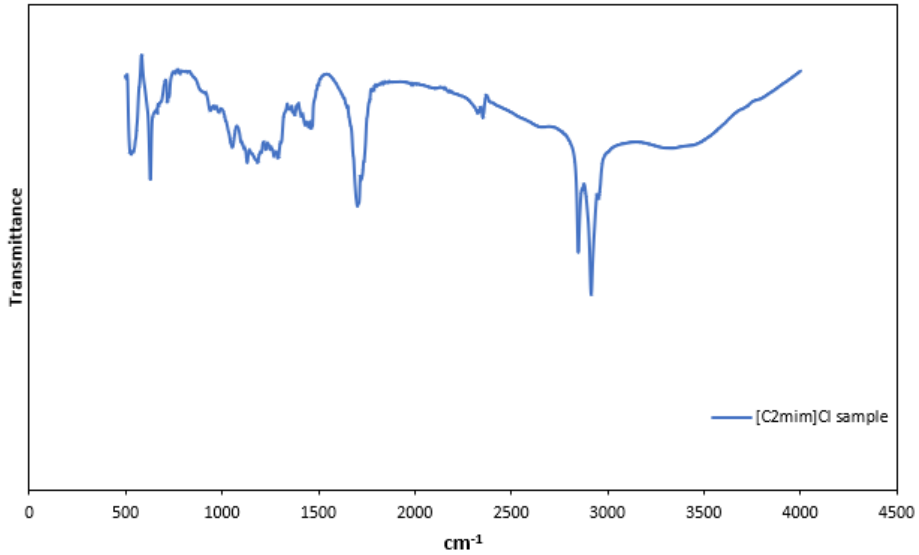
1000
1500
2000
2500
3000
3500
4000
4500


Transmittance
[C2mim]Cl sample
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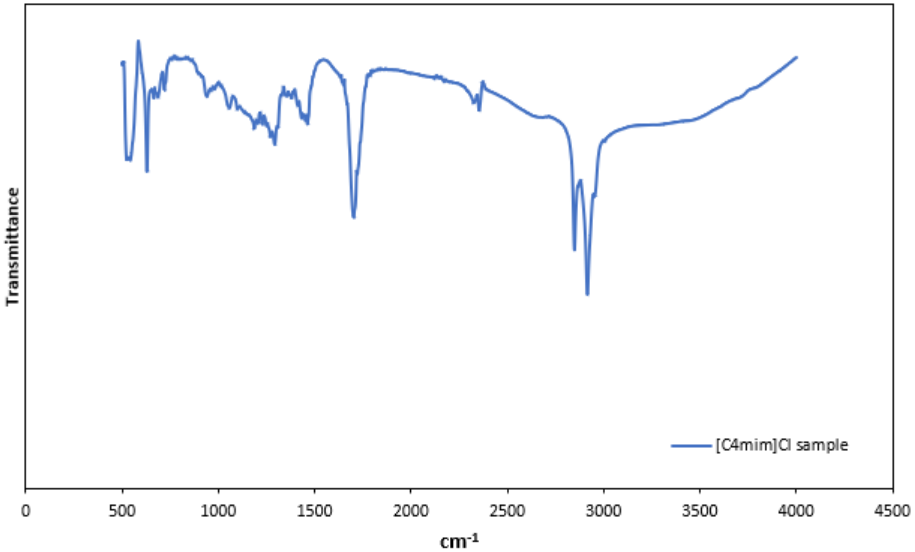
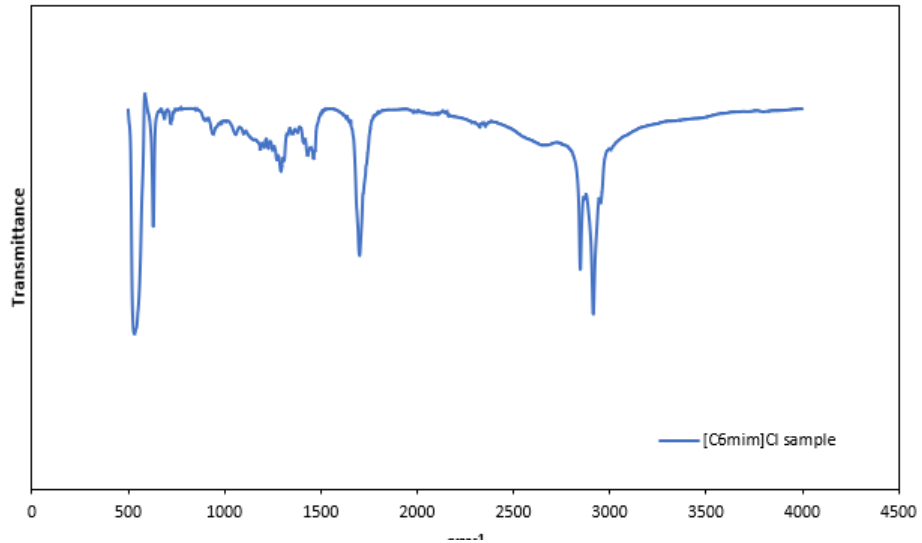
500
1000
1500
2000
2500
3000
3500
4000
4500
cm-1


Transmittance
[C4mim]Cl sample
0
500
1000
1500
2000
2500
3000
3500
4000
4500
cm-1


Transmittance
[C6mim]Cl sample
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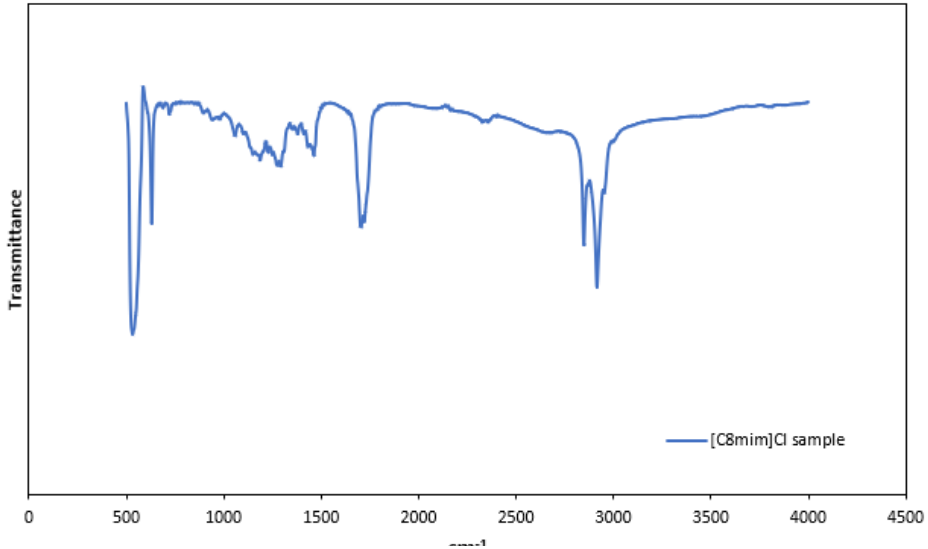
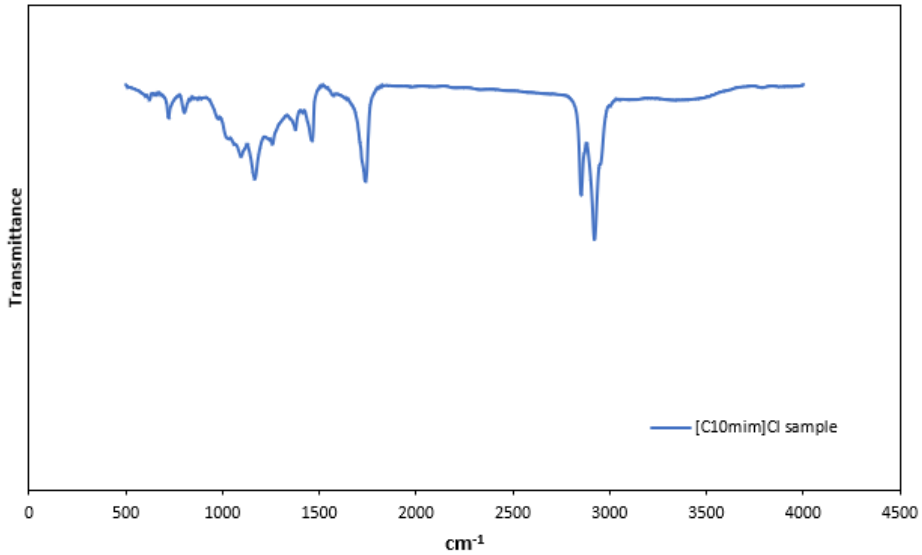
500
1000
1500
2000
2500
3000
3500
4000
4500


Transmittance
[C8mim]Cl sample
0
500
1000
1500
2000
2500
3000
3500
4000
4500


Transmittance
[C10mim]Cl sample
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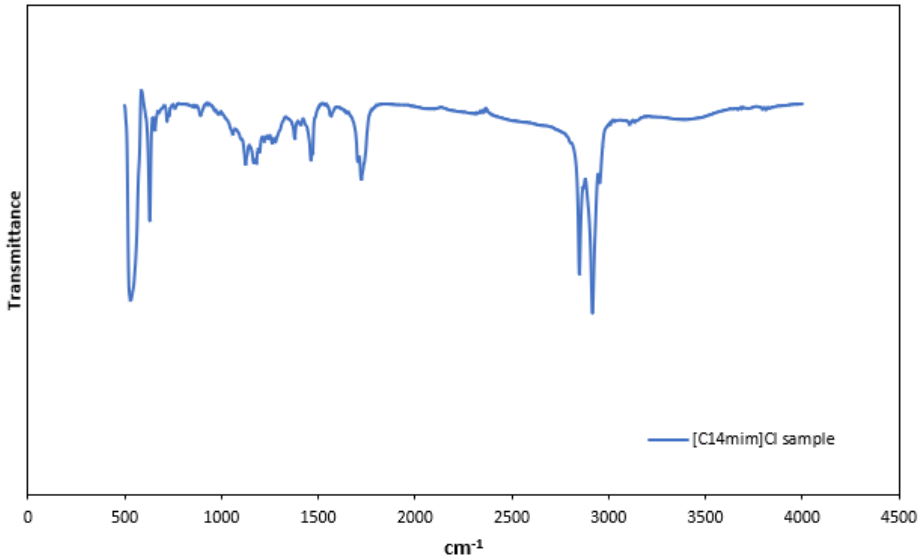
500
1000
1500
2000
2500
3000
3500
4000
4500
cm-1


Transmittance
[C14mim]Cl sample
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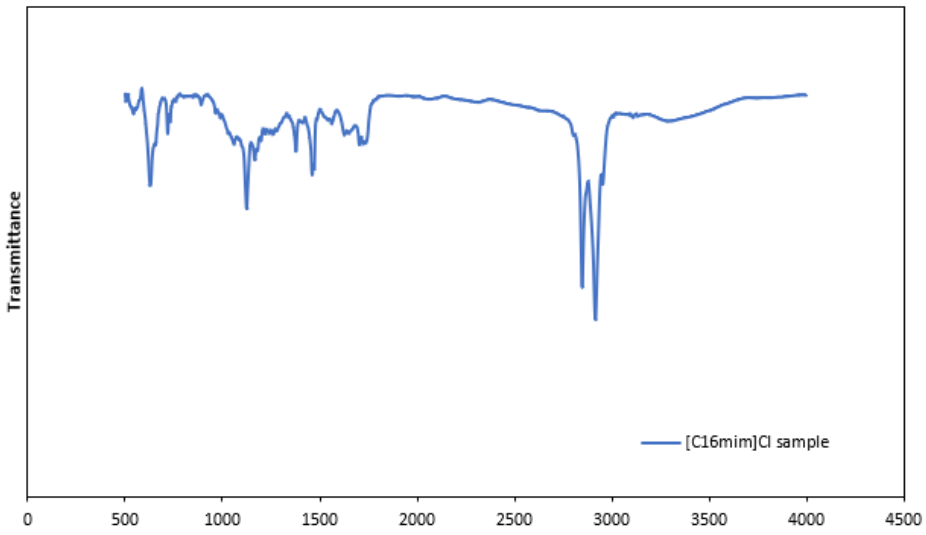
500
1000
1500
2000
2500
3000
3500
4000
4500
cm-1


Transmittance
[C16mim]Cl sample
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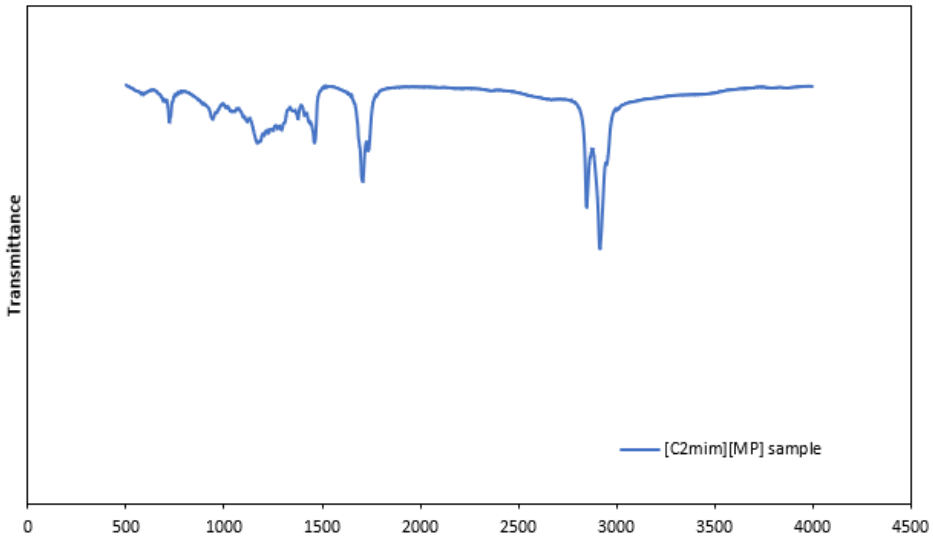
1000
1500
2000
2500
3000
3500
4000
4500


Transmittance
[C2mim][MP] sample
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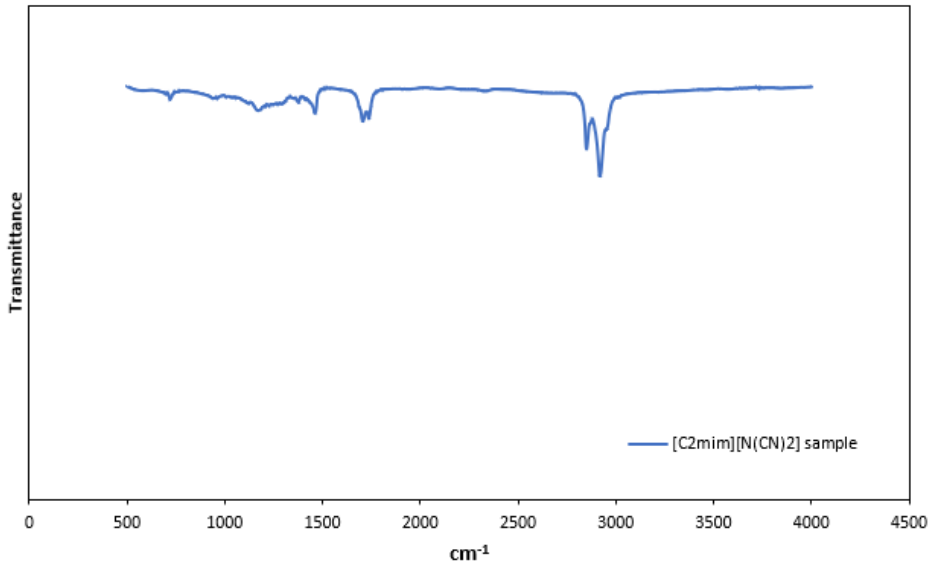
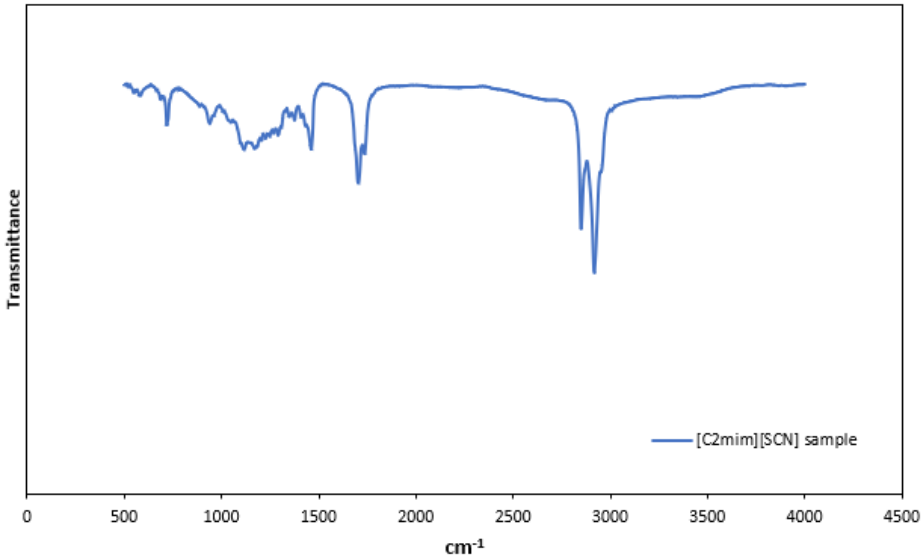
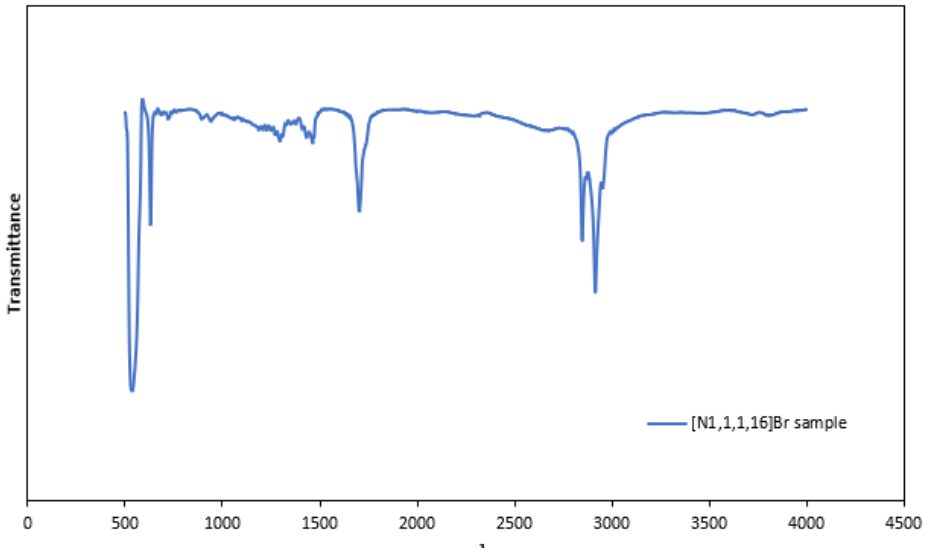
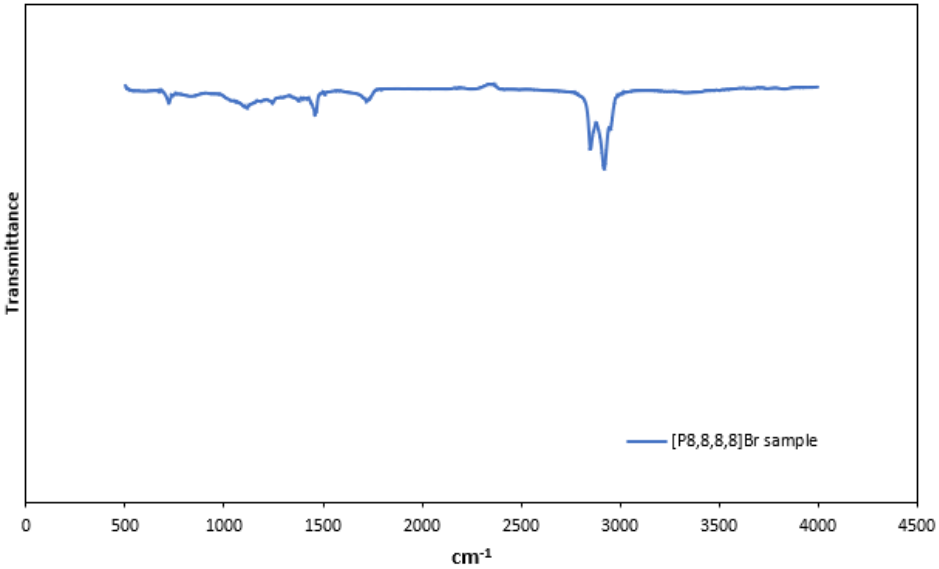
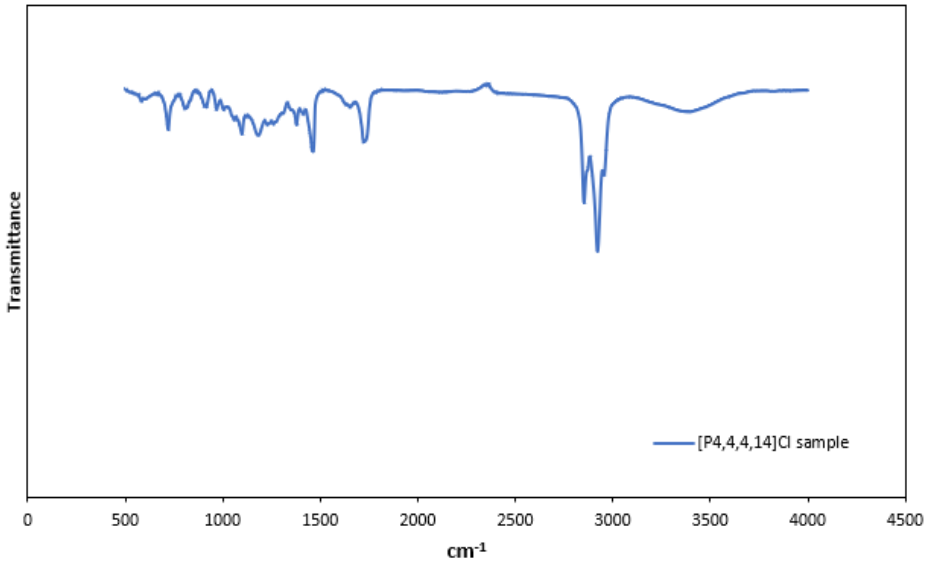
1000
1500
2000
2500
3000
3500
4000
4500


Transmittance
[C2mim][N(CN)2] sample
0
500
1000
1500
2000
2500
3000
3500
4000
4500
cm-1


Transmittance
[C2mim][SCN] sample
0
500
1000
1500
2000
2500
3000
3500
4000
4500
cm-1


Transmittance
[N1,1,1,16]Br sample
0
500
1000
1500
2000
2500
3000
3500
4000
4500


Transmittance
[P8,8,8,8]Br sample
0
500
1000
1500
2000
2500
3000
3500
4000
4500
cm-1


Transmittance
[P4,4,4,14]Cl sample
0
500
1000
1500
2000
2500
3000
3500
4000
4500
cm-1