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A STUDY TO IMPROVE THE EXTRACTION YIELDS OF POLY-3-HYDROXYBUTYRATE FROM BURKHOLDERIA SACCHARI CELLS AVOIDING CHLORINATED SOLVENTS.

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Extended abstract

Quest'opera ha approfondito lo studio delle tecnologie disponibili per l'estrazione dei polimeri biodegradabili detti poli-idrossialcanoati (PHA) dai micro organismi produttori. I PHA stanno recentemente attirando l'interesse della comunità scientifica e delle industrie poiché uniscono proprietà meccaniche simili ai polimeri termoplastici sintetici (poli-propilene) ai vantaggi della completa biodegradabilità.

Gli aspetti peculiari dei PHA, che li distinguono dalle altre bio-plastiche, sono la totale biocompatibilità (in campo medico, le suture riassorbibili sono fatte con questo materiale), ma soprattutto la possibilità di produrre il polimero partendo da substrati di basso pregio, come cellulosa, lignina e carboidrati complessi, consentendo finalmente di ridurre i costi di produzione, il che li rende un'alternativa reale alle plastiche derivate dal petrolio. Le tecniche di produzione dei PHA si basano infatti su fermentazioni condotte da batteri che accumulano il polimero all'interno della membrana cellulare sotto forma di granuli che fungono da riserva di energia: questi sono poi recuperati e purificati dal materiale cellulare che li circonda. In particolare, il polimero studiato in questa tesi proviene da uno stesso batch di batteri liofilizzati del ceppo *Burkholderia sacchari,* contenente il 65.68% in peso secco di poli idrossi-butirrato (P3HB), il più semplice e più studiato dei PHA.

Scopo principale del lavoro è stato lo sviluppo un nuovo metodo di estrazione e purificazione dei granuli polimerici che fosse economico, efficace, rispettoso dell'ambiente e facilmente industrializzabile. L'obiettivo finale infatti è l'abbandono delle vecchie tecniche di estrazione basate su solventi clorurati, che costituiscono l'ostacolo per la diffusione dei PHA per motivi sia economici sia ambientali. Si stima appunto che il 60-80% del costo totale di produzione sia legato proprio alle complesse operazioni d'estrazione, ma soprattutto, la pericolosità nei confronti dell'ambiente dei solventi clorurati (in particolare il cloroformio) è un evidente paradosso per un materiale che voglia affermarsi in virtù delle sue proprietà "green".

Il lavoro sperimentale sviluppato in questa tesi, svolto presso i laboratori IBB/CEBQ dell' Istituto Superior Tecnico di Lisbona (all'interno del programma di scambio Erasmus), ha seguito principalmente due approcci distinti. Inizialmente si è studiata la possibilità di purificare il polimero con la tecnica della digestione dei batteri in soluzione acquosa alcalina. Le membrane cellulari sono state sciolte in una soluzione allo 0,2N di NaOH, così da liberare i granuli di polimero, lavati poi dallo scarto cellulare con una soluzione acquosa di tensioattivi (Sodium Dodecyl Sulfate). La tecnica della digestione e lavaggio delle membrane si è dimostrata efficace, portando il polimero finale ad una purezza del 98% con rese di estrazione paragonabili a quelle del cloroformio, tuttavia il polimero, al momento della fusione, si è degradato rapidamente, carbonizzandosi prima di raggiungere la temperatura di fusione. Sebbene questo fenomeno inatteso lasci spazio a nuove

ipotesi sulla natura microbiologica della degradazione dei PHA, di cui ancora poco si conosce, il metodo è stato abbandonato.

La seconda modalità di estrazione ha riguardato invece la dissoluzione selettiva del polimero in solventi cosiddetti "green", rispettosi dell'ambiente. Per la selezione di questi è stato seguito un nuovo metodo sistematico, basato sulla lista di 110 solventi industriali redatta dalla multinazionale GSK. La lista associa ad ogni sostanza un punteggio in base alle sue prestazioni ambientali, valutate con i metodi di Life Cycle Assessment e di Footprint ambientale, metodi moderni ed affidabili. Un'equazione predittiva basata sul modello di Hansen è stata poi utilizzata per stimare la solubilità del P3HB nei solventi con punteggio ambientale più alto: ne sono stati selezionati quattro (cicloesanone, fenetolo, dimetil-etere, anisolo), sottoposti successivamente a valutazioni sperimentali di solubilità. Di questi, il cicloesanone e l'anisolo hanno dimostrato le migliori prestazioni, consentendo di ridurre a un quarto i volumi di solvente richiesto per l'estrazione del PHA, con rese maggiori rispetto alla tecnica basata sul cloroformio, riducendo inoltre il tempo di estrazione a soli 15 minuti (contro le 36 ore richieste dal cloroformio).

L'ultima parte della tesi, svolta presso l'università di Padova, è stata finalizzata a valutare il vantaggio economico di un processo di estrazione con cicloesanone su grande scala. Un impianto per la produzione di 50 000 ton/anno di P3HB è stato simulato con l'ausilio del programma Pro/II, dimensionando e stimando il costo delle apparecchiature attraverso correlazioni economiche di letteratura e contattando direttamente le aziende produttrici ove possibile. Dal confronto dei risultati del design concettuale con i dati di un impianto esistente (Metabolix Telles, USA), è emerso che questa nuova tecnica non solo è una valida alternativa all'estrazione con cloroformio, con indiscutibili vantaggi ambientali, ma potrebbe ridurre sensibilmente il costo del P3HB. La progettazione di processo condotta con un approccio approssimato ma conservativo, ha infatti portato ad una stima del costo d'estrazione che lascia spazio a considerazioni ottimistiche, permettendo inoltre di identificare gli aspetti che, con ulteriori studi, possono perfezionare il metodo ed ottimizzare al meglio il processo industriale.

Abstract

This thesis addresses the possible improvements of the available technologies for the extraction of the biodegradable polymers named poly-hydroxyalkanoates (PHA) from the micro-organisms producers.
The main purpose of the work is to develop a new extraction method for poly hydroxy-butyrate (P3HB), which is simultaneously economical, effective, environmental friendly and easy to industrialize. The final goal is to abandon the old chloroform-based extraction technique, which is hindering the diffusion of PHA plastics both for economical and environmental reasons.
The experimental work includes two different ways. The first one is the cell membrane disruption in aqueous alkali solution (NaOH 0.2N) to free the polymer granules contained, with subsequent cell debris washing with surfactant (Sodium dodecil sulfate). The second one regards the polymer selective dissolution in green solvents. All the experiments were performed on a single batch of lyophilized bacteria of the strain *Burkholderia sacchari*, containing 65,68% of dry weight of P3HB.
The membrane-digestion and washing technique proved to be effective as it raised the polymer purity up to 98% with extraction yields comparable with chloroform ones. However the final product showed an insufficient melting behavior, due to fast degradation reactions that burned the polymer. The study of this unpredicted phenomenon opened new questions over the microbiological nature of PHA degradation, still little understood. Anyway aqueous extraction had to be abandoned.
To select a suitable green solvent instead of chloroform, a new systematic approach was followed. A recent predictive method based on Hansen solubility model was used to screen among a list of 110 industrial solvents, whose environmental performances have been evaluated by the pharmaceutical company GSK with the modern and reliable methods of Life Cycle Assessment and Environmental Footprint. Four solvents were selected and undergone to the experimental solubility tests. In particular, cyclohexanone and anisole showed the best performances, reducing the required solvent volumes to one fourth of the chloroform ones, with higher yields, requiring only 15 minutes for the extraction.
The economical viability of cyclohexanone extraction was then proved with a process simulation of a big scale plant for 50 000 ton/year of P3HB, comparing the results of conceptual design with the real data of an existing plant (Metabolix Telles, USA). In spite of a number of approximation, the final values are useful to have an order of magnitude of the production costs and permit to identify the points to be further studied to improve the method, in order to achieve an optimized process.

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Introduction

Biodegradable plastics are one of the most remarkable solutions to the problem of pollution related to goods disposal, but their diffusion is still very limited. The reasons are both economical, as oil derived plastics maintain lower costs and do not require food crops to be produced (which bonds biodegradable plastic price to the price of grain), and of technical quality of the product. In fact the mechanical properties of bio-polymers are often lower than synthetic plastics ones. However, a family of biodegradable polymers is recently gaining the attention of chemical industries and public institutions: poly-hydrxyalkanoates (PHA). They have mechanical properties comparable to the most used thermoplastics (PE and PP), have the same possibilities of compounding, very good environmental resistance though being completely biodegradable, but most of all, PHA can be produced via fermentation starting from low quality carbon substrates, so solving the problem of food-chain overlap. The European Union, with the 7^{th} framework programme, funded the so called "Bugworkers project" with the purpose of improving the technology behind PHA. Bugworkers international consortium numbers many European small and medium enterprises, larger industries, research institutes: each of these is focusing on different aspects of the assignment of making PHA a wide spread consumer material. Among these institutions, the Institute of biotechnology and Bioengineering (IBB) of Istituto Superior Tecnico in Lisbon is studying the production and recovery of poly 3hydroxy-butyrate using cultures of *Burkholderia Sacchari*, fermenting a straw hydrolisate.

This thesis was developed in this context, focusing on the downstream process, i.e. the operations that allow recovering the P3HB grains inside the microorganism, by removing impurities and cellular debris. The main goal was to develop a new polymer extraction technique abandoning the old ones based on chloroform, which obviously cannot be accepted any longer due to its harmfulness, for health and environment.

The first chapter provides a complete prospect of the available extraction techniques, better describing the context of PHA with the reasons that still hinder its diffusion.

The second chapter is dedicated to the experimental analysis of one of the two polymer purification approaches studied in this thesis, both promising for effectiveness, low cost, good environmental performances. The first approach is based on cell membrane digestion by NaOH solution and surfactant washing of the debris. The development of the method, improving other existing techniques, and the evaluation of the performances are reported in detail.

Chapter three presents the second approach, that is solvent extraction with green chlorine-free solvents. These solvents were first analyzed with thermodynamic predictive methods so to choose the most P3HB selective, then solubility was evaluated experimentally.

In the last chapter, a process simulation for the P3HB production is carried out, so to evaluate the economic large-scale applicability of the best extraction technique previously studied. The downstream plant for solvent extraction was designed providing commercial quotations of the equipment and estimating the cost of the required utilities. These data allow quantifying the cost of P3HB extraction, which affects the overall polymer cost by about 70% (this is the real bottleneck for PHA diffusion). The results obtained are compared to the real prices of PHB produced by the American company Telles, leader of PHA production until the year 2012.
The results provided in this work are promising and hopefully will help PHA to become a real solution to protect the environment from non-biodegradable plastic disposal.

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Chapter 1

Biodegradable plastics from Poly-hydroxyalkanoates, state of the art.

1.1 The role of PHA

Plastic, due to its versatility, easy workability and durability, has become one of the most diffuse materials, giving a great improvement to the quality of life of the people. At the same time, the stability of most of the oil-derived plastics has raised much concern for the environment. At the present only a small amount is recycled or incinerated, the rest becomes a troublesome inheritance accumulating in the soil, with long lasting deleterious impact.

One of the environments most threaten by plastic debris is the Ocean, where pollution consequences has different kinds of effects (Derraik 2002). First, the intrinsic toxicity of chlorinated plastics on aqueous organisms; but also the accumulation of plastic films in the guts of fish and (as a consequence) seabirds (Blight and Burger 1997), compromising their digestive apparatus; then the deleterious effect of floating garbage, that transports for thousands of kilometers "invader" species contaminating different eco-systems. Accumulation of plastic on the seafloor alters the gas-exchange mechanisms; plastic ropes, lines and packaging bands are a danger for big sea animals, that remain entangled. Plastic demand is increasing due to the development of the Asian regions (Plastics-Europe 2008), so the contrast of the dramatic effects of plastic garbage has became a compelling matter.

In the last years research has focused on this issue, studying a way to substitute oil derived polymers with bio-degradable materials. One possible solution can derive from the family of bio-polyesters called Polyhydroxyalkanoates (PHA) which are synthesized by some bacterial species.

The first PHA, poly-hydroxybutyrate, was discovered in 1926 by the French microbiologist Lemoigne from a culture of *Bacillus megaterium* (Lemoigne 1926).

Today the family of PHA has been enriched with more than 80 monomer units (Kunasundari and Sudesh 2011) synthesized by more than 300 genres of *archaebacteria* and *eubacteria* (Lee 1996), both under anaerobic and aerobic conditions (Zinn *et al.* 2001). These long chains of polyesters are a reserve of carbon and energy for the cells. They accumulate under the form of spherical water-insoluble granules of 0.2-0.5 µm inside the cytoplasmatic membrane (Khanna and Srivastava 2005).

Not all the different PHAs have proven useful for technical application and also, among the large number of producing microorganisms, only a few offer good biopolymer productivities and yields when cultivated in bioreactors. These microorganism are roughly divided into two main categories (Nawrath *et al.* 1995) according to the characteristics of the specific PHA they produce. The former category collects microorganisms that produce short chain acids monomers (3, 4, 5 carbons), for example 3 hydroxybutyric acid, which polymerizes giving the so called P3HB or poly 3-hydroxy butyrate, the simplest and most studied poly-hydroxyalkanoate (figure 1.1).

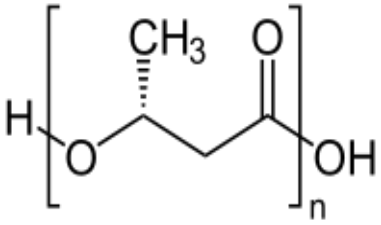


Figure 1.1. *Chemical structure of poly-3-hydroxybutyrate (P3HB)*

The latter category collects bacteria producing medium-long monomers (6 up to 14 carbons). Typical examples of the first class are *Alcaligenes eutrophus* and *Rhodospirillum rubrum*, while examples of the second class are bacteria like *Pseudomonas oleovorans* (Nawrath *et al.* 1995). For example *A. eutrophus* can accumulate the homo-polymer PHB up to 85% of its dry weight, making it a very good source of polymer. By changing the carbon source and/or by the addition of precursors it is possible to obtain different co-polymers: the addition of valeric acid to the fermentation broth induces the production of P(3HB-co-3HV), the first commercialized PHA, named BioPol by Imperial Chemical Industries (ICI) (Byrom 1990).

In the nineties, research improvements followed two parallel ways to select the best bacteria: the first one focused on the productivity of the microorganism, the second one tryed to reduce the cost of fermentation, exploiting less expensive substrates. Usually, bacterial cells start to synthesize PHA under limitation of a nutritional element as N, P, Mg, K, O or S, and excess of carbon: feeding strategies must be tailored for each type of microorganism to obtain the highest production yield (Yup Lee and Nam Chang 1995). However, one example of a very good producer is *Alcaligenes latus*, of the same genus of *A. eutrophus*, which showed continuous PHB production associated with growth, needing less specific feed strategies. This enables single stage fermentation and shorter overall culture periods (Tamer *et al.* 1998).

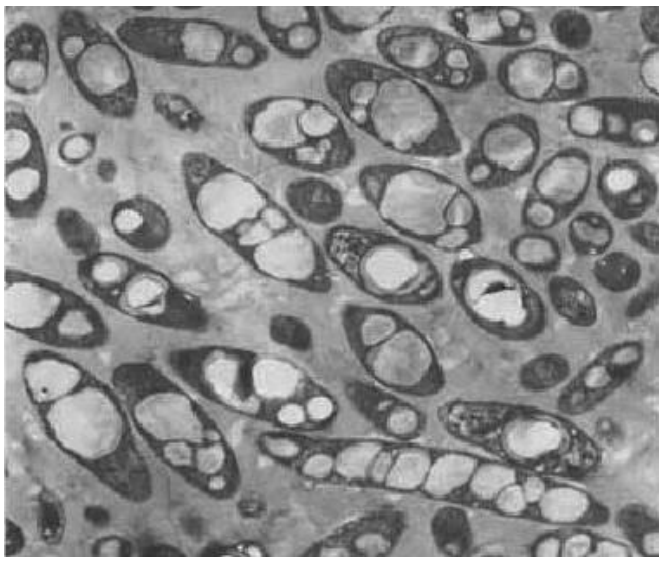


Figure1.2 *TEM micrograph of recombinant E. coli accumulating P3HB in spherical shaped granules inside the membranes.(Yup Lee and Nam Chang 1995)*

Furthermore, the isolation of DNA genes encoding the production of PHA gave the possibility to create transgenic bacteria with optimal qualities. It is the case of recombinant *Escherichia coli* (figure 1.2), which accumulates more than 90% of its weight of polymer and facilitates the recovery due to the fragility of its membranes (Choi *et al.* 1998). However the cited bacteria need valuable substrates to grow, typically glucose or sucrose: this makes a high scale industrial application less attractive, as reported for *A. eutrophus* (Nawrath *et al.* 1995). The authors showed that this production strategy, with a price of glucose of 0.55 USD/kg required in the proportion of 3.3 grams per gram of polymer, would deal to a minimum cost for P3HB of 1.8 USD/kg, considering only the feed and not the rest of the process. This price is unsustainable also if compared to other bio-plastics. Hence, the efforts have been concentrated on bacteria able to degrade less expensive molecules such as cellulose and xylose. This is the case of the European research program 'Bugworkers' (Appendix 2) which provided funds for an intensive analysis on microorganisms able to grow on ligno-cellulosic hydrolysates. In the context of this project *Burkholderia sacchari*, first isolated in sugar cane Brazilian fields, proved to be a good PHA producer starting from straw hydrolysates (Cesário *et al.* 2012). In appendix1 more specific information about *B. Sacchari* are provided, as this bacterium is the source of the polymer here addressed.

1.2 Application and economics

Even if the polymeric nature of PHA was clear since the discovery, only in the 80s research has started to consider these polymers as a possible source of commercial plastic (Steinbüchel *et al.* 1998). The properties of some of the most known and best engineered PHA are summarized in table 1.1 (Jacquel *et al.* 2007; Khanna and Srivastava 2005) together with those of the most common commercial plastics.
These properties are good enough to consider PHA for possible applications in the field of bulk packaging (films, bags, coated boards, disposable food service-ware, molded products), with the advantage of using a material with high environmental performances.
The two most relevant characteristics making PHA so suitable for an ecologic future are these:

- biodegradability, as PHA are completely degraded to water and carbon dioxide (Holmes 1988), which eliminates the problems of disposal
- renewability, as these polymers derive from the fermentation of natural biomass, without necessity of fossil sources (Chanprateep 2010).

Table 1.1. *Comparison of PHA different properties with some of the most used commercial plastics.*

Polymer	Melting point [ºC]	Young's modulus [GPa]	Tensile strength [GPa]	Elongation to break [%]
P(3HB)	179	3.5	40	5
P(3HB co 3HV) 3%mol	170	2.9	38	-
P(3HB co 3HV) 9%mol	162	1.9	37	-
P(3HB co 3HV) 14%mol	150	1.5	35	-
P(3HB co 3HV) 20%mol	145	1.2	32	50
P(3HB co 3HV) 25%mol	137	0.7	30	-
P(3HB co 4HB) 64%mol	50	30	17	591
P(3HB co 4HB) 90%mol	50	100	65	1080
Polypropylene	170	1.7	34.5	400
Polyethyleneterephtalate	262	2.2	56	7300
Polystyrene	110	3.1	50	-
LDPE	130	0.2	10	620

Moreover, some PHA have been shown to have a good biocompatibility, demonstrating then applicability in medical field, for example to produce sutures, artificial skin, scaffolds for cartilage, vascular fabric, etc (Zinn *et al.* 2001).

In reality, the market of PHA is still limited, due to its high price. PHAs are indeed far to be wide spread because of the concurrent petroleum-based plastics. The first company that produced PHA at higher scale (approximately 1000 t/year) was Zeneca Bio Products, a subsidiary company of ICI, which was able to sell the already cited copolymer "BioPol", at the commercial price of 16 USD$/kg (Choi and Lee 1997). The price lowered and nowadays, after Monsanto bought the rights for Biopol and then sold it to the American company Metabolix, PHB plastics were sold at a price around 5 USD/kg ([1,2]), but this was still not enough to take a firm position in the market. Metabolix and its partner ADM ceased their collaboration in 2012 ([5]), closing the biggest plant producing PHA in the world. Other companies managed to produce bio-polymers of the family of PHA (Jacquel *et al.* 2008; Chanprateep 2010) and they are reported in table 1.2.

Table1.2. *Overview of the main producers of PHB, with volumes of production and commercial price of the bio-plastics.*

Polymer	Manufacturer	Nation	Volume [ton/year]	Price [€/kg]
PHB	Mitsubishi Gas Chemical Company Inc	Japan	60 000	2.5-3.0
PHB	Metabolix Telles	USA	50 000	4[(1,2)]
PHB	PHB Industrial Company	Brazil	60 (Burkholderia sacchari)	[2.20[(3)]]
PHB	Biotechnology Co	Germany	50 (2003)	3.0-5.0
PHB	Tianan biologic, Nigbo	China	10,000	3.26
PHBHV	P&G	USA	20 000 /50 000	[1.85([4])]
PHBHV	Lianyi Biotech	China	2 000	3.70
PHB	Meredian (P&G technology)	USA	3 plants for 9 100[(7)]	[1.60[(6)]]

Values in square brackets are target prices for plants in construction.

To be really competitive, the price should lower to less than 1USD/kg, but this is hard for a number of reasons. The first element of the price is the volumetric productivity attained. From this point of view much has been done to reach optimal process performances. Then, prices also depend on the type and quality of the carbon source supplied to the culture. Like for other renewable bio-technologies (such as bio-fuels), sources of high quality nutrients could raise conflicts due to the overlap with human and animal feed. There is hence the need of synthesizing PHA from low quality agro-food byproducts. From this point of view, there are already existing plants working in Brazil with sub products of the sugar cane production chain. However, the operations of separation and purification of the polymer after the fermentation step represent from 60% to 80% of the total cost of the final product (Jacquel *et al.* 2007).

At the state of the art, an optimal way of extraction of PHAs still has to be defined.

1.3 Downstream recovery methods for PHA

In the literature a number of recovery techniques are proposed, which can be divided into two big families: mechanical and chemical processes. The latter can be further divided into two main approaches: a first one that dissolves the polymer and separates the non-soluble cell debris, and the other one that breaks and solubilizes the cellular matter and recovers the polymer as non-soluble residual. Below, a short overview reports the most used recovery techniques with their main advantages and their drawbacks. The quality of the method is quantified through the following parameters (Choi and Lee 1997):

- *Recovery yield*, defined as the ratio between the polymer obtained and total amount of polymer contained in the cell.
- *Purity,* defined as the ratio of the amount of the PHA to the total amount of dry matter after recovery.

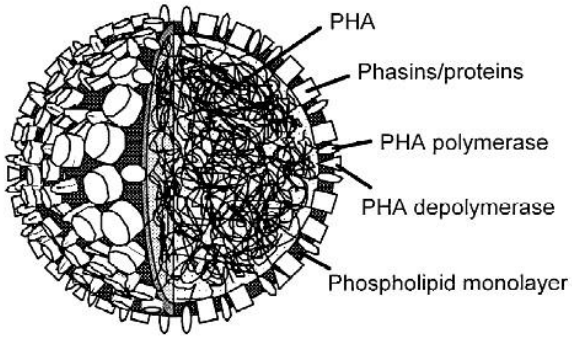


Figure 1.3. *Representation of the microscopic structure of a PHA granule as they appear inside the micro-organisms. The aim of the purification is the removal of cellular membranes but also the proteic material that surrounds the granule itself (Zinn et al. 2001).*

1.3.1 Solvent extraction

The aim of the method is to dissolve the granules of PHA in a very selective solvent and to remove the cellular debris (figure 1.3) through filtration. Then the precipitation of the polymer is induced through the addition of a non-solvent. According to the process for the P3HB, best results have been reached with chlorinated solvents, mainly chloroform and dichloroethane. These solvents are strong enough to break the cell membranes, and the

recovery yield can be enhanced through pretreatments that weaken the membranes, for example with a methanol pretreatment, or sonication, or microwave thermal shock (Jiang *et al.* 2006; Lee *et al.* 2010). Unfortunately this technique is not suitable for a large scale recovery process for many reasons:

- chloroform is a polluting chemical, highly volatile, bio-accumulating, toxic and suspect carcinogenic: it should be avoided in industrial applications;
- the concentration of the polymer in solution should be maintained lower than 5% w/v, otherwise the viscosity becomes too high: this causes large consumption of solvent (Jacquel *et al.* 2008);
- the consumption of the non- solvent, methanol or ethanol, consequently is very high too (five times the volumes of chloroform), which further rises again the costs.

However, chloroform allows reaching the highest purity, so this method is often used as a standard to compare the performance of other solvents or other extraction techniques. Obtaining a high purity polymer is also a must when the properties are to be assessed. Chloroform and the other chlorinated solvents have another important advantage: they are effective both for homo-polymers and co-polymers, and the process does not reduce PHA chain length. This is because the chloride atom of the solvent causes a specific polar interaction with the carbon of the polymer that holds the carbonyl function, enhancing solubility (Jacquel *et al.* 2007). Several studies have been carried out to develop a predictive model of the solubility of P3HB,varying temperature, molecular weight of the polymer and type of solvent (Terada and Marchessault 1999). These studies were later improved by Jacquel *et al* (2007), who obtained an empirical equation for estimating P3HB solubility (and another for co-polymers as well), evidencing the limits of a predictive modeling approach. In fact, even if the equation for the P3HB homo-polymer solubility can be reliable within certain limits, developing a good model for co-polymer becomes a tricky issue. The paper reported how much the solubility is affected by the nature of the monomers and by their relative composition: this adds two more complex variables to the ones considered for the monomers. As a consequence, very bad solvents for P3HB proved to be better ones for co-polymers, and the opposite as well. The aim of this kind of research was to give a tool helping to find solvents less harmful for the nature, avoiding chlorinated compounds. Unfortunately other solvents do not have the same good performances as chloroform and need higher processing temperature, with higher energy consumption and the risk of damaging the polymer. This is the case of 1,2 propylene carbonate (Fiorese *et al.* 2009) which allows to reach 95% of yield and a maximum purity of 84% with the extraction performed at 130^{o}C for 30 min. Still, by rising temperature to 140^{o}C, a decrease of the chain length to one third is caused. So, at the state of the art, when

excluding the chlorinated solutions, solvent methods are neither cheap nor really effective. Research is still investigating to find a solvent fulfilling these three qualities: highly selective, eco-friendly, inexpensive.

1.3.2 Chemical digestion of cell membrane

This other practice is based on the fact that PHA are not soluble in water and this can be the starting point for a good separation. The digestion method consists in treating the cells with a solution that causes the breakage of the phospholipidic membranes, typically modifying the pH. In this way the granules of PHA get free and, because they are heavier, tend to sttle down. To enhance the separation velocity, this step is performed in a centrifuge, discharging the supernatant which carries all the organic debris. The earlier techniques used bleach solutions of sodium hypochlorite to induce the disruption of membranes (Berger *et al.* 1989), but it was demonstrated that in this way polymer suffered severe degradation, with reduction of the average molecular weight to one half (Ramsay *et al.* 1990; Hahn *et al.* 1994). The technique was made less aggressive combining bleach with chloroform (Hahn *et al.* 1994), obtaining purities higher than 94%, or pre-treating the cells with a surfactant solution, attaining lower purities (Ramsay *et al.* 1990) . The method was not developed any more until the solution of hypochlorite was substituted with aqueous alkali solution (NaOH or KOH) which gave good results (Choi and Lee 1997; Choi and Lee 1999). It was noticed that the chain length is not reduced significantly if the digestion is performed for a short time (1h) at lower temperature (30^{o}C); secondly, the recovery yield and the purity are higher than 90%; thirdly, alkali solution digestion, which involves very common chemicals and simple operations, is the cheapest recovery way for PHA. In the same study, Choi and Lee (1999) developed a process simulation to estimate the cost of P3HB of 95%purity in a plant for 100 000ton per year. Their optimistic result gave a P3HB production cost of 3.66$/kg, which was far lower than the best optimized process working at the time. Anyway this method can be further improved, mainly to reach better purities and higher yields, as 10% of loss is not a good performance. In this thesis the first experimental campaigns will verify the results of Choi and Lee and try to refine their method.

1.3.3 Surfactant cell dissolution

The mildest way to disrupt cell membranes, which allows even to preserve the amorphous state of the polymer granule, is the surfactant treatment. When cells are suspended in a tensioactive aqueous solution the surfactant molecules start to accumulate between the lipidic bilayer of membranes. As soon as the saturation is reached, the membranes inflate and disrupt, forming the typical micelles that carry in solution lipids, proteins and cell organelles. PHA does not dissolve and can be separated through centrifugation (Ramsay *et al.* 1990). The major drawbacks of this technique (Yang *et al.* 2011) are that it is difficult to obtain good yields (about 86%) and purities (92% maximum), that surfactants are expensive chemicals and that an industrial application would eventually result in high volumes of wastewater, difficult to treat.
A hypothetical process was designed to reduce the impact of surfactant technology (Chen *et al.* 2001). It involves recycling five times the solution of surfactant and the addition of a chelating agent (EDTA disodium salt, to improve the effect of surfactant), before regenerating the dirty solution. This allows reaching purity higher than 96%, but the yield remains lower than 92%. Also, the suggested regeneration step can be easily performed at a lab scale, but it is less suitable for high industrial scale. In fact they lowered the wastewater pH to induce debris precipitation using hydrochloric acid (expensive and uneasy chemical) and then filtered the solution through activated carbon absorption. If considering a higher volume of wastewater so contaminated, this is not the cheapest solution. In conclusion, even if this method has been improved reducing the volumes of process water, it is still too expensive and less effective to be applied in industry.

1.3.4 Mechanical cell disruption

This method, like those using surfactants, does not change the structure of the granules, preserving the amorphous state of the polymer. There are mainly two types of equipment (Tamer *et al.* 1998) used to break cells in the pharmaceutical industry: high pressure homogenizers and bead mills. A homogenizer consists of a high pressure pump (60-95MPa) which forces the cell slurry through two parallel slots of 100µm. The loss of head and the kinetic shock cause the breakage of cells. Bead mills instead consist of a cylindrical grinding chamber which hosts inside a concentric variable speed rotor (2800rpm) and glass spheres (425-600 µm). The equipment has to be kept cooled down; the cell slurry is slowly pumped through the grinding chamber and filtrated at the exit to retain the beads. Both disruptions are carried out in continuous steady state conditions.

More, mechanical methods need shorter processing time if compared to their chemical counterparts. Bead mills gave satisfactory yield values only after more than five passages (it depends on the nature of microorganism), making this process very energy demanding. In addition a bead mill is a very sensitive piece of machinery, difficult to scale up and susceptible to beads fluidization, that cancels the grinding effect.
Homogenizers too are difficult to handle, as they are effective only for a narrow window of pressure and temperature, and they are sensitive to viscosity. The pumps are energy consuming and, as compared to bead mills, have lower performances with dilute solutions. Yield does improve with higher biomass concentrations, but as viscosity is higher too, the homogenizer becomes susceptible to clogging and frequent blockage.
As a consequence, these methods are widely used in pharmaceutical industry to recover active compounds, high valuable products which justify expensive recovery equipment, but they are not so suitable for PHA extraction purposes.

1.3.5 Other techniques

The technique that ICI applied in its large-scale process involved a first thermal treatment to weaken the cells and then an enzymatic dissolution of the membranes (Holmes and Lim 1990), which gives good performances in terms of yields and purities, but results very expensive as proved by the first commercial prices (16 $/kg). The cost of BioPol has been sensibly lowered in the years (Choi, Lee 1997), but mainly thanks to scale up and appliance of GMO technology. Other approaches for recovery of polymer are based on the density difference between PHA and the other components of bacterial cells.
The last method here reported is called aqueous-two-phases-system separation. The purpose is to induce in the cellular lysate a split of phases to concentrate in one the PHA, phenomenon achieved adding a particular solution of PEG800 and potassium phosphate (Divyashree *et al.* 2009). However, this technique is still little understood, and it is far to be industry applied.

1.*3.6 Aim of the thesis*

This thesis has been developed in two different moments, with different work approaches and purposes. The first part of the work was carried out in the IBB/CEBQ laboratories of Istituto Superior Tecnico of Lisboa, under supervision of prof. Manuela Regalo da Fonseca: it is an experimental study which takes its origins in the research projects promoted by the Bugworkers project (appendix 2).
The purpose was to develop, among the existing PHA recovery methods, new techniques which could substitute chloroform solvent extraction both for laboratory-scale application and also industrial-scale application. In particular, the analysis was focused on P3HB homo-polymer extraction from *Burkholderia sacchari* bacteria, a recently-discovered strain, not yet investigated from the point of view of extraction technique optimization. The recovery methods were tested and improved trying to maximize the "*3 E*" qualities, that is to design a method both "*Economic*", "*Effective*" and "*Eco-friendly*". As a consequence the experimental part of the thesis focused on aqueous extraction techniques and "green solvent" techniques, optimizing their efficacy and environmental sustainability.

The second part of the thesis was developed in the University of Padova, under the supervision of prof. Alberto Bertucco, and had the aim of evaluating the economical validity of the best working technique studied in Lisbon. Therefore a large scale plant for P3HB recovery was designed and simulated, so to have an overall cost estimation of the final product. In this way the commercial applicability of the new technique could be estimated, pointing out those aspects that need more study to have the best improvements.

Chapter 2

Cellular membrane digestion and surfactant washing

The technique here reported had the aim of putting together the advantages of the extraction methods based on NaOH digestion and surfactant membrane disruption. The original protocol of recovery, described on the beginning of paragraph 2.2 has been changed and optimized to enhance the purity of the final polymer. The explanation of the final optimized technique follows, while the specific elucidations of all modifications are reported in detail in the paragraph 2.3. Paragraph 2.1 describes all the experimental procedure of characterization and evaluation of the substrates recovered. The final considerations over the method and the consequences deriving from the experimental evidence are reported in paragraph 2.4.

2.1 Characterization techniques

2.1.1 Measure equipment

Mass

Balance 1: CPA64 Sartorius (standard deviation= ± 0,1 mg)
Balance 2 AA250 Denver instruments company (standard deviation= ± 0,1 mg)

Volume

Pipette 1: Eppendorf research 5000µL
Pipette 2: Eppendorf research 300 µL
Pipette 3: Thermo scientific 1000 µL

Temperature

Thermometer: FLUKE 51 K/J (thermocouple iron-costantan: range -200ºC/760ºC)

Composition

Gas chromatograph: 5890 Series II Packard Bell.

Column: HT5 of SGE filled with 5% Phenyl (equiv.) Polycarborane-siloxane

Post-processing program: Lab Solutions-GC Solution Analysis (copyright 2000/2004 Shimadzu), version 2.30.00.

2.1.2 Methylation procedure.

The samples to be injected in the GC were prepared with the simplest and most used method for P3HB content evaluation (Braunegg *et al.* 1978). To process the sample inside the GC column, the polymer has to be broken first, because the long chains would have very long retention time and possibly could clog the column. At the same time, the forming olygomers are methylated, thus enhancing the separation capacity of the column. The addition of an Internal Standard (hexanoic acid) has the purpose of reducing the effect of the error of injection in the GC, as the known concentration of internal standard allows to rescale the results, before calculating the purity through a calibration curve (see paragraph 2.1.3). The sample preparation method is now summarized.

1. The powder of the extracted and dried polymer is put inside 10 mL Pyrex® centrifuge tubes, in a quantity lower than 0.020g, to respect the range of validity of the calibration curve. Preferably, the measured quantity should be around 0.01g of powder;
2. the volume of 2ml of chloroform ($CHCl_3$ analytical reagent grade. Fisher Scientific) is added, to dissolve the polymer. This leads to a solution of polymer and chloroform of about 5g/L;
3. in the same tube 2 ml of the methylation solution is added, whose composition is
    - 97mL CH_3OH
    - 3 mL H_2SO_4 purity of 96%
    - 330µL $C_5H_{11}COOH$ (exanhoic acid)
4. the tubes are firmly closed and put for 5 hours at 100ºC in an oven. The tubes are shaken every hour to enhance the dissolution-methylation of the polymer.
5. the samples are removed from the oven and let to cool down. Then, 2 mL of an aqueous solution of $CaCO_3$ (60g/L) is added to the samples. This is to neutralize the acidic solution, which could damage the GC.

6. The tubes are shaken and then centrifuged for 5 minutes at 4000 rpm, helping the split of the phases in the tube. The upper phase is an aqueous solution of the remaining reactants, the heavier one is chloroform carrying the methylated polymer and the standard. A volume of 200 µL is taken and transferred in a GC vial, ready for the injection.

This is the protocol adopted to produce all the results which will be discussed later.
In some points it differs from the original protocol previously used in the lab, with the attempt to reduce an experimental error which had not been relevant until the beginning of this work. The former protocol was used to measure values of purity always lower than 85%, so the measurements were mainly qualitative and high precision was not required: such a test mainly dealt with the determination of the polymer content in different batches of fermentation and it was enough to make a comparison. Instead, in this work the values of purity are very close to the 100%, so the difference in few percentile points can be crucial.
The former protocol was analyzed to focus the points which could cause larger errors:

- Originally 5mg of polymer were dissolved in 1 mL of chloroform, and it was used 1 mL of both the methylation and neutralization solutions. In this way the quantity of powder was very close to the sensitivity zone of the balance. To overcome this problem, a single flask of a larger quantity with polymer-chloroform solution (5g/L) was prepared, and then distributed in the test tubes. The scatter of the result was reduced, but this procedure should have been repeated for all the samples, involving a huge amount of work for each measurement. So it was preferred to simply duplicate the quantities of both polymer and solvents.
- Even if the pipette volume measurement is very precise, the amount of chloroform could vary, changing the real concentration of the solutions. In fact chloroform has a surface tension lower than water, and it is very volatile. The transfers of chloroform with pipette have to be made with care, to reduce evaporation and dripping.
- The former protocol distinguished two separated steps, first for the complete dissolution of the powder in chloroform, then for the addition of the methylation solution. It was noticed that stirring the tube to enhance dissolution caused the dispersion of the powder among the walls of the tube, leading to a partial loss of the sample and fake results (concentration lower than real). Due to this, both chloroform and methylation solution are now added at the same time, and the tubes are put in the oven without agitation. The shaking comes later, when the tubes are already hot.

- The sample volume of the lower phase after centrifugation was required to be 200 µL. The reason was to have possibility of diluting the sample in case of too high concentration, which could happen when measuring an unknown quantity of polymer during fermentations. In this work this occurrence is not possible, as values can be equal/lower than the quantity of powder measured. Instead, the very low volume of the sample, 200 µL, makes stronger the effect on the final result of evaporation (concentration higher than real). As a consequence, 600 µL of chloroform were sampled.

The estimated error of the method, expressed with the standard deviation of 18 samples, is around 5%, as reported in paragraph 2.3. Hence, this measurement method is no more reliable when trying to distinguish purities higher than 95%. So GC has been used as a first technique to give an indication of purity, comparing different protocols of extraction, but then, if the results were sufficiently high, to the produced polymer the melting test was applied (paragraph 2.1.4).

2.1.3 Gas chromatograph calibration curve

To convert the results on the final chromatogram to the required purity data, a calibration curve is needed. Polymer purity is not provided automatically as the detector of the GC is a simple FID (flame ionization detector). The detector sends to the recording computer the signal of the amount of species passing through in the unit of time, producing the chromatogram. The calibration allows correlating a certain value of purity with the ratio between the area lying under the P3HB peak and the area under the peak of the internal standard. The purpose of using the ratio with the Internal Standard is to reduce the effect of injection error on the final result. In fact the ratio between the areas remains the same even if the injected volume fluctuates: differently the value of P3HB area (and so the final purity) changes whenever the micro-syringe does not measure the proper volume of 1µL. The remaining peaks on the chromatogram are neglected, as they correspond to solvents and other impurities brought to the sample through the methylation procedure.
The calibration curve has been produced correlating the known concentrations of methylated solutions of pure polymer to the results of their GC analysis. Table 2.1 reports the experimental points which will give the curve, shown in figure 2.1.

Table 2.1. *Experimental points used to obtain the calibration curve. The columns "area" refer to the the area below the line of the cromatograms, related to the amount of the considered specie.*

Sample ID	P(3HB) (mg)	V CH_3Cl (mL)	Area IS	Area 3HB	3HBME/IS	C 3HB (g/L)
blanc			42130,4	0	0	0
1	6,2	2	42130,4	14039,4	0,333	3,1
1	6,2	2	42204,4	13896,6	0,329	3,1
2	7,0	2	45895,6	18584,8	0,405	3,5
2	7,0	2	43205,2	16845,2	0,390	3,5
4	11,5	2	41168,2	23761,5	0,577	5,8
4	11,5	2	41626,1	25124	0,604	5,8
Sample 2	12,3	2	43666,8	30624,8	0,701	6,2
Sample 2	12,3	2	40330,9	27208	0,675	6,2
6	14,1	2	42485,1	33645,1	0,792	7,1
6	14,1	2	43070,3	33778,4	0,784	7,1
Sample 1	15,6	2	37126,1	32250,2	0,869	7,8
Sample 1	15,6	2	40869	35435,6	0,867	7,8
7	16,8	2	42047,6	41237,2	0,981	8,4
7	16,8	2	40184,6	39477,6	0,982	8,4
8	17,4	2	39582,2	39738,8	1,004	8,7

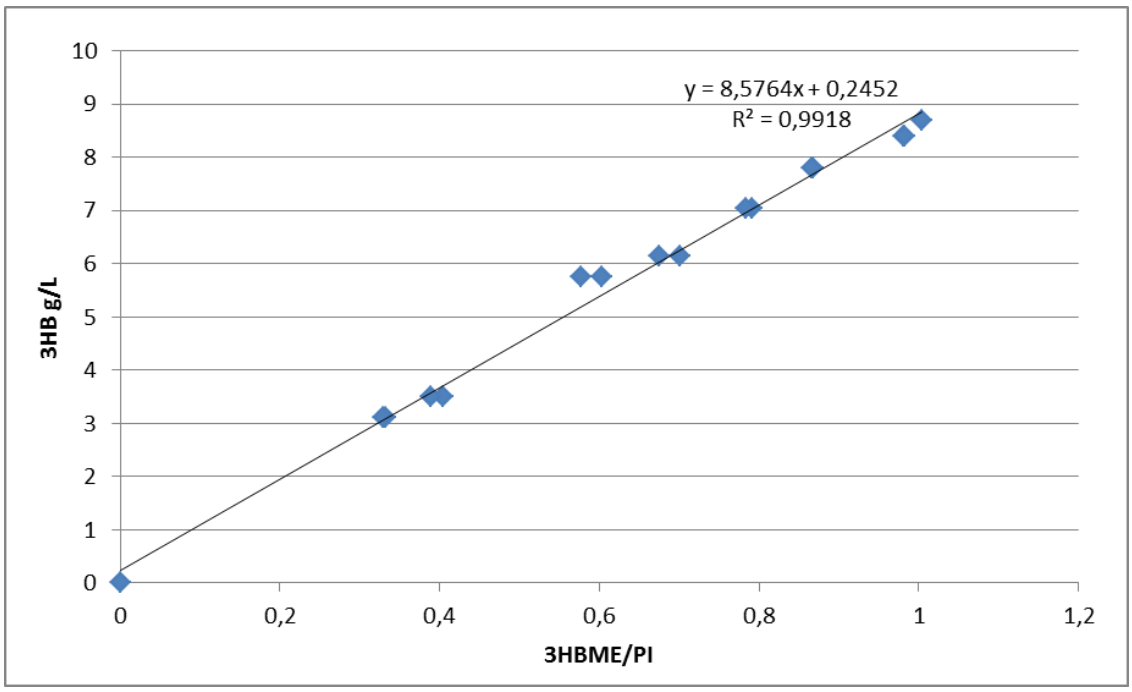


Figure 2.1. *Graphical representation of the calibration curve. The blue dots are the experimental points regressed to obtain the analytic expression.*

Interpolating the experimental points, the equation of the calibration curve is obtained:

$$C_{P3HB}\left[\frac{g}{L}\right] = 8.576 \cdot \frac{AreaP3HB}{AreaIS} + 0.245 \qquad (2.1)$$

C_{p3HB} is the detected concentration of polymer. The final purity of the extracted polymer samples is given by the ratio between C_{P3HB} and C_{mat}, where the last one is the concentration of the extracted material put in the chloroform solution for methylation (weighed mass of the sample / volume of chloroform).

These data have been obtained with the GC working conditions summarized in table 2.2, which were used for all the analyses.

Table 2.2. *Working conditions of the Gascromatograph 5890 series II Packard Bell for all the measurements performed.*

Temperature of the oven	60ºC
Temperature of injector	120ºC
Temperature of detector	150ºC
Retention of carrier	5.22 s^{-1}
Type of carrier	Nitrogen

2.1.4 Melting temperature test

Due to the fact that the polymer presents high index of crystallinity (Mitomo and Doi 1999), it is possible to have an indirect evidence of the chain length variations after the extraction process. This is achieved through the comparison between the melting points of the digestion-recovered polymer and a reference sample (in this case the polymer obtained from chloroform extraction): a lower melting temperature means lower chain length and damaged polymer. These measurements result to be fast and easy to make, following the method described in chapter 2.3.5.5 of the manual "Polymer Synthesis: Theory and Practice" (Braun *et al.* 2013) . Actually the technique here applied is much simplified, but it is enough to give a quick indication of the melting temperature and the flow behavior of the polymer. All the steps for the measurement of crystallization temperature are omitted (a polarized light microscope is needed) and instead of a Kofler hot-block, a simple hot plate (with control of temperature) was used to give heat to the sample on the glass slide. The picture in figure 2.2 shows the equipment for the measurements.

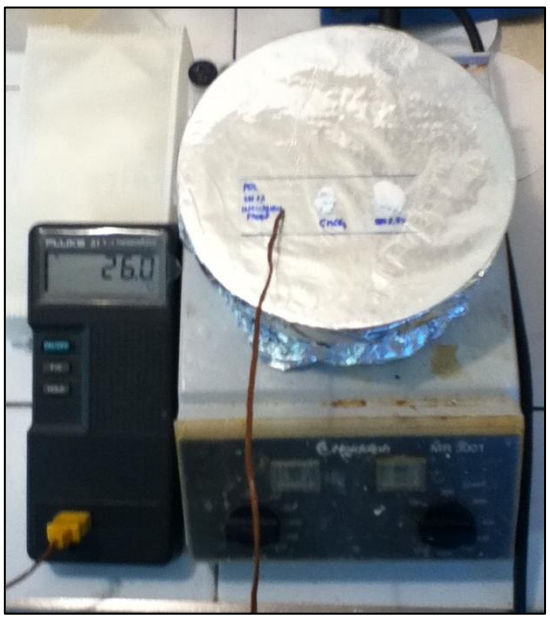


Figure 2.2. *Melt test equipment, composed by a hot plate supporting the glass slide with the samples and a digital termocoulple iron-costantan.*

The steps are so summarized.

1. A small amount of powder of the polymer to test is put on a microscope glass slide, together with the one extracted with chloroform for comparison.
2. The slide is put on a heated plate at room temperature. Then the plate is switched on.
3. The temperature on the surface of the slide is monitored with a digital thermocouple in contact with the glass.
4. The observer should take note of the temperature at which the following phenomena appear: smoke, powder darkening, presence of smell, burning of the sample.

The temperature of melting was taken down, together with the onset other phenomena (smoke, change of colors, presence of smell, …).

According to a qualitative criterion of evaluation, the more the melted polymer resembled the chloroform extracted one, the better it was ranked for its quality.

In fact, there is not a universal qualitative standard for the commercial P3HB yet, as different applications require both very pure and lower quality polymers. For sure, as a thermoplastic material, an higher melting temperature is preferable, while a slight change of color of the polymer is commonly tolerated, as for the one commercialized by the Brazilian PHB industries. Obviously, a brownish, bad smelling polymer should be refused. This qualitative criterion helps to evaluate the results of the test.

2.2 Digestion of membrane: materials and methods

2.2.1 Source of polymer and micro-organism

All the experiments have been performed using the same batch of lyophilized cells of *Burkholderia sacchari*, identified by the code F2-21/11/12. These cells contain only the homopolymer (P3HB). Further information about bacteria and the fermentation conditions are reported in Appendix 1.

2.2.2 Recovery by membrane digestion: base procedure

The protocol was first defined by Choi and Lee (1999), who investigated cheap techniques for disrupting cell membrane avoiding solvents. They tested a number of chemicals (HCl, H_2SO_4, NaOH, KOH, NH_4OH) and surfactants (commercial names: AOT, CTAB, SDS, Triton-X 100 and Tween 20) to digest the non-polymeric cell material. Their best results were reached with NaOH: 98.5% of polymer purity, 91.3 % of recovery yield, low chain length loss. They worked with a culture of recombinant *E. coli* with a high content of P3HB, 77% of the dry weight.

From their results, the basic method of recovery was designed in the IST lab for *B. sacchari*. These are the steps of the first procedure developed.

1. Rough pellets of lyophilized *B. sacchari* cells are reduced in powder using a hand mortar.
2. The cells are suspended in a NaOH solution of 0.2N, with a proportion of 5% weight on volume (5g of cells in 100ml of solution), inside a large bottom flask.
3. The flask is put for 10 minutes in a HT Infors incubation shaker at the speed of 170 rpm and temperature of 30ºC. This is the proper digestion step.
4. The aqueous slurry of disrupted membranes and polymer is transferred in pre-weighed plastic centrifuge tubes (producer: VWR), 15 ml for each, and then put for 10 minutes at 14000rpm in a lab centrifuge (Eppendorf 5810R). Then the supernatant can be separated and discarded, interrupting the digestion (that could damage the polymer).
5. The solid pellet at the bottom of the tube is suspended in a volume of 15 ml of distilled water, centrifuged again for 10 minutes at 14000 rpm. The dirty water is discarded, and this washing operation is repeated.

6. After the discard of the last washing water, the tube is put into a 60ºC oven for 48h to dry the sample.
7. Eventually, the weighed mass of recovered matter is milled and prepared for the characterization step.

2.2.3 Recovery by membrane digestion: surfactant improved technique

Surfactants have the ability of disrupt cellular membranes and isolate the hydrophobic molecules of lipids inside micelles, making them soluble in water. It is possible (Yang *et al.* 2011) to use surfactants for the digestion, so to save the chain length of polymer, differently from the alkali solution technique. However this leads to lower performances in purity and recovery yield.
The idea at the base of NaOH method improvement is that a surfactant can be used in the washing steps, making the cleaning of the cell debris easier. In facts *B.Sacchari* presents membranes much more complex than the recombinant *E. Coli*, so the simple water washing cannot give high enough purities.
The main differences introduced in the original procedure concern the washing steps, as the digestion phase is unmodified. The steps of the optimized extraction method are listed here below.

1. The 5% weight on volume suspension of lyophilized cell in the 0.2N aqueous solution of NaOH is put for 10 minutes at 30ºC in the shaker at 170rpm. The slurry is transferred into the pre-weighed tubes and then centrifuged for 10 minutes at 14000rpm; the supernatant is discarded.
2. A solution of SDS, sodium dodecyl sulfates (Merck Schuchardt OHG), and distilled water is prepared. The critical micellar concentration (Khan and Shah 2008) of SDS is 0,0082M in H_2O (25ºC), which corresponds to 0.236% w/v: the solution for the washing is meant to be about ten times the critical micelle concentration (i.e. 2.5% w/v), to be aligned with the proportions used in the literature (10-20 times CMC).
3. The pellet of polymer is resuspended in 15ml of the SDS solution; it is well stirred and then centrifuged again.
4. The supernatant is discharged. The solid residual is resuspended in 15ml of distilled water and centrifuged. The supernatant is discarded. This washing is performed another time.
5. The sample in the tube is put to dry in the oven at 60ºC for 48 hours.

6. The weighed solid material after recovering is milled and prepared for the next analysis.

The surfactant helps in separating the debris, which precipitates together with the polymer after the first centrifugation. However, sodium dodecyl sulfate is very foamy, so the surfactant washing is performed inside the centrifuge tubes, adding a step after the digestion. In principle the surfactant could be added directly to the digestion broth, inside the flask. This possibility, which could reduce of one step the extraction procedure, has not been investigated as the foam could render difficult the transfer and the measurement of the 15ml sample. On the opposite, the foam that forms inside the tubes during the resuspension of the sample helps the dispersion of debris and collapses completely with centrifugation. The so designed method allowed reaching the best purities, comparable with the ones of the chloroform technique.

2.3 Results

2.3.1 Characterization of SDS washed samples.

After performing the separation according to the base procedure derived from literature, the work was focused on the development of an improved technique based on the use of surfactant. As this approach was new, different procedures were explored before finding the best method. The best ones have been repeated and studied in detail. The former experimental campaign is sumarized in table 2.3, which reports the differences in the washing procedure after extraction. If not otherwise indicated, the other steps of the procedure are the same as the method in paragraph 2.2.3.

Table 2.3. *Summary of the washing protocols investigated for the first screening of purification techniques.*

Sample ID	***1st washing***	***2nd washing***	***3rd washing***	***Note***
9/4 S1	15mL H_2O	15mL H_2O	Not done	
9/4 S2	15mL SDS 2.5%	15mL H_2O	Not done	
9/4 S3	15 mL SDS 5%	15mL H_2O	Not done	
9/4 S4	15mL H_2O	15mL SDS 2.5%	15mL H_2O	
9/4 S5	15ml SDS 2.5%	15mL H_2O	15mL H_2O	
9/4 S6	15mL SDS 5%	15mL H_2O	15mL H_2O	
25/3 2*	15mL SDS 2.5%	15mL H_2O	Not done	Between the two washings, no centrifuge. Gravity sedimentation for 30 min.
25/3 2 #	15mL SDS 2.5%	15mL H_2O	Not done	Between the two washings, two centrifugations (20 min)
25/3 1	15mL H_2O	15mL H_2O	15mL H_2O	This is the reference, performed as the old procedure prescribes.
12/4 I	7.5 mL digested solution + 7.5 SDS 5%	15mL H_2O	15mL H_2O	SDS solution was added directly to the digested broth. To remove the first centrifugation step.
12/4II	7.5 mL digested solution + 7.5 SDS 2.5%	15mL H_2O	15mL H_2O	As sample 12/4 I
12/4 III	15mL H_2O	15mL H_2O	15mL H_2O	Reference for the three water washing experiences.
12/4 IV	15mL H_2O	15mL H_2O	7.5 mL H_2O + 7.5mL Acetone	Check another step to dissolute lipids

The variables investigated in this campaign were the amount of SDS to add to the washing solution, the order of the steps, the effect of centrifugation on the final value (i.e. if the enhanced gravity cause the debris to precipitate with polymer) and the utility of another step with a selective solvent for lipids.

The results of the characterization of the obtained dry matter are reported in table 2.4.

Table2.4. *Results of dried polymer GC characterization after the extraction with protocols of table 2.3.*

Sample ID	Methylated mass [g]	Total solid conc. [g/L]	Area P3HB	Area IS	Ratio	P3HB conc. [g/L]	Purity %	Mean Purity %
9/4 S1	0,0078	7,8	23203,8	32294	0,72	6,4	82,13	81,32
9/4 S1	0,0075	7,5	24111,9	34245,7	0,70	6,04	80,51	
9/4 S2	0,0053	5,3	30011,4	52532,2	0,57	4,90	92,44	92,98
9/4 S2	0,0057	5,7	23549,4	37884,7	0,62	5,33	93,52	
9/4 S3	0,0072	7,2	30239,9	36239,9	0,83	7,16	99,39	95,23
9/4 S3	0,0067	6,7	28228,5	39673,7	0,71	6,10	91,07	
9/4 S4	0,0071	7,1	28351,7	39882,2	0,71	6,10	85,87	96,70
9/4 S4	0,0082	8,2	36529,4	35531	1,03	8,82	107,52	
25/3 2*	0,0064	6,4	26126,3	45008,5	0,58	4,98	77,78	81,23
25/3 2*	0,0062	6,2	26122,3	42671,9	0,61	5,25	84,68	
25/3 2#	0,0058	5,8	26075,3	44704,4	0,58	5,00	86,25	88,96
25/3 2#	0,0079	7,9	30178,3	35736,8	0,84	7,24	91,67	
12/4 I	0,0055	5,5	22914,4	38915,6	0,59	5,05	91,81	87.864
12/4 I	0,0055	5,5	18511,1	36323,4	0,51	4,61	83,91	
12/4 II	0,008	8	41202,2	47250,7	0,87	7,48	93,48	93,61
12/4 II	0,0058	5,8	20763	34296,2	0,60	5,43	93,74	
12/4 III	0,0076	7,6	28621,7	43492,8	0,66	5,64	74,26	84,06
12/4 III	0,0083	8,3	33016,3	36346,6	0,91	7,79	93,86	
12/4 IV	0,0073	7,3	35776,1	53128,4	0,67	5,77	79,11	81,28
12/4 IV	0,0065	6,5	22474,3	35529,2	0,63	5,42	83,46	
25/3 1	0,0046	4,6	18036,9	41158,1	0,44	3,76	81,70	82,75
25/3 1	0,0069	6,9	27536,5	40838,9	0,67	5,78	83,81	

Sample 9/4 S5 and Sample 9/4 S6, not reported in the table, gave average purities of 95% and 100% respectively: as these were the best achieved results considering also the yields of extraction, these washing techniques were further studied.

The recovery yields were then calculated according to the results of purity. Defining Y the recovery yield, X the purity of the recovered polymer, x the polymer content inside the cells, equation 2.2 is the relation used for calculations.

$$Y = \frac{X \cdot m_{recovered}}{x \cdot m_{cell}} \tag{2.2}$$

where m_{cell} is the amount of cells loaded in the centrifuge tubes (volume * cell concentration) and $m_{recovered}$ is the amount of solid matter weighed after the sample exsiccation. The yield so calculated is the real one, considering for x the amount of polymer in the cells obtained from with GC analysis (65.68%). The relative recovery yield

considers instead the performance of the extraction technique compared to the results of chloroform extraction (*x* is considered 0,53: as explicated in chapter 3), that is the reference technique. Table 2.5 summarizes the results of GC analysis of the extracted polymer.

Table 2.5. *Recovery yields for the extraction protocols of table2.3. Absolute recovery yield is calculated considering the total amount of the polymer inside the cells, relative recovery yield is calculated considering the maximum amount of P3HB which can be extracted with chloroform a 4°C.*

	Weight Total	Weight tube	Weight recovered material	Amount of cells	Amount of polymer in cell 65.68%	Final purity	Absolute recovery yield %	Relative recovery yield
	[g]	[g]	[g]	[g]	[g]		%	%
9/4 S1	12,0065	11,468	0,5385	0,75	0,4926	81	89	110
9/4 S2	11,906	11,464	0,442	0,75	0,4926	93	83	103
9/4 S3	11,904	11,473	0,431	0,75	0,4926	95	83	103
9/4 S4	12,047	11,635	0,412	0,75	0,4926	97	81	100
9/4 S5	**11,9125**	**11,498**	**0,4145**	**0,75**	**0,4926**	**95**	**80**	**99**
9/4 S6	**11,993**	**11,548**	**0,445**	**0,75**	**0,4926**	**100**	**90**	**112**
25/3 2*	11,749	11,449	0,3	0,75	0,4926	81	49	061
25/3 2#	12,0318	11,5136	0,5182	0,75	0,4926	89	94	116
25/3 1	5,6122	5,3427	0,2695	0,35	0,22988	83	97	121

The techniques that gave the best purities proved to be good also in terms of yields, equal or slightly better than the ones with chloroform solvent extraction. Only the procedure without centrifugation caused a big loss of the polymer.

The following conclusions were derived from the analysis of these samples:

- adding other steps of simple water washing doesn't make difference. The purity obtained with two water washings is not substantially worse than with three;
- using the surfactant, results get far improved. The difference on result between SDS 5% or SDS 2.5% solution is negligible. So it was decided to use the lower concentration one, for reasons of both cost and waste-water issues;
- variation in the centrifugation patterns does not change the results (sample 25/3 2*; 25/3 2# and 9/4 S2), on the contrary, the gravity sedimentation of sample 2* caused a loss of polymer and a lower Yield;
- the addition of a step with acetone washing does not change the purity substantially. As it could instead increase the overall cost of the product, it was abandoned;

- the attempt to add the SDS solution directly to the broth of digestion did not give very good results, even if it could have reduced the centrifugation steps (and consequently the cost of the final polymer)

The best results were obtained if the samples were first added with supernatant and then water-washed twice. As already said, this technique was widely repeated in the following experiments to obtain more significant results: the data, not reported before, are summarized in table 2.6.
About the experimental technique, the same batch of digested cells was divided in 6 centrifuge tubes, subjected to the same cleaning procedure. Three samples of the dried polymer for each tube were then taken and methylated separately.

Table 2.6. *Results of GC caracterization and purity of the recovered solids after SDS 2.5% solution treatment and two subsequent water washings. Six repetitions of the protocol.*

Date	Sample ID	Methylated mass [g]	Total solid conc. [g/L]	Area P3HB	Area IS	Ratio	P3HB conc. [g/L]	Purity [%]	Mean Purity [%]
08-05-2013	41a	0,009	4,5	17685	39054,8	0,45	4,13	91,74	
08-05-2013	41b	0,0151	7,55	33820,9	38253,7	0,88	7,83	103,67	97,71
08-05-2013	41c	0,0121	6,05	Sample lost during methylation					
08-05-2013	42a	0,0132	6,6	27361	39072,4	0,70	6,25	94,70	
08-05-2013	42b	0,0103	5,15	22007,5	36888,4	0,60	5,36	104,10	99,30
08-05-2013	42c	0,0108	5,4	23432	39356,3	0,59	5,35	99,09	
08-05-2013	43a	0,0115	5,75	24232,9	40901,6	0,59	5,33	92,63	
08-05-2013	43b	0,0108	5,4	20676,8	37510,8	0,55	4,97	92,08	95,77
08-05-2013	43c	0,0122	6,1	26729,2	38117,7	0,70	6,26	102,60	
08-05-2013	44a	0,0086	4,3	19035,2	37729,3	0,50	4,57	106,32	
08-05-2013	44b	0,0131	6,55	27149,8	38115,2	0,71	6,35	97,00	100,19
08-05-2013	44c	0,0142	7,1	30362	39097,3	0,78	6,90	97,25	
08-05-2013	45a	0,0104	5,2	18078,7	35246,1	0,51	4,64	89,30	
08-05-2013	45b	0,0125	6,25	23806,4	33431,7	0,71	6,35	101,63	97,677
08-05-2013	45c	0,0087	4,35	18092,1	36983,9	0,49	4,44	102,07	
08-05-2013	46a	0,0133	6,65	27635,7	38127,3	0,72	6,46	97,16	
08-05-2013	46b	0,0127	6,35	26289	36741,8	0,71	6,38	100,49	98,82
08-05-2013	46c	0,015	7,5	Sample lost during methylation					

The following table 2.7 reports the purity of the samples obtained with SDS 5% solution.

Table 2.7. *Results of GC caracterization and purity of the recovered solids after SDS 5% solution treatment and two subsequent water washinsg. Six repetitions of the protocol.*

Date	sample ID	Methylated mass [g]	Total solid conc. [g/L]	Area P3HB	area IS	Ratio	P3HB conc. [g/L]	Purity % [%]	Mean purity [%]
07-05-2013	31a	0,0142	7,1	32408,4	45886,5	0,706273	6,30	88,76	
07-05-2013	31b	0,0114	5,7	26166	42551,4	0,614927	5,51	96,82	94,84
07-05-2013	31c	0,0097	4,85	22574,9	42519,7	0,530928	4,80	98,93	
07-05-2013	32a	0,0135	6,75	26035	38775,8	0,671424	6,00	88,93	
07-05-2013	32b	0,0109	5,45	26729	43940,4	0,608301	5,46	100,22	98,85
07-05-2013	32c	0,0166	8,3	45327,4	44841,3	1,01084	8,91	107,40	
07-05-2013	33a	0,0124	6,2	27077,4	40085,5	0,675491	6,04	97,39	
07-05-2013	33b	0,0158	7,9	39560,9	44999,8	0,879135	7,78	98,54	99,19
07-05-2013	33c	0,0132	6,6	34641,8	45958,1	0,753769	6,71	101,66	
07-05-2013	34a	0,0108	5,4	22638,1	36977	0,612221	5,49	101,77	
07-05-2013	34b	0,0109	5,45	27477,7	45223,6	0,607596	5,46	100,11	100,18
07-05-2013	34c	0,0124	6,2	29762	43469,4	0,684666	6,12	98,66	
07-05-2013	35a	0,0127	6,35	27852,6	37514,7	0,742445	6,61	104,13	
07-05-2013	35b	0,0132	6,6	33264,4	43884,5	0,757999	6,74	102,21	101,21
07-05-2013	35c	0,0172	8,6	40768,3	43051,1	0,946975	8,36	97,28	
07-05-2013	36a	0,011	5,5	24910,8	40315,2	0,617901	5,54	100,80	
07-05-2013	36b	0,0149	7,45	Sample lost during methylation					101,86
07-05-2013	36c	0,0112	5,6	28395,9	44124,8	0,643536	5,76	102,93	

The overall means for the two washing techniques are summarized in table 2.8. It is possible to quantify properly the accuracy of the data thanks to the number of repetitions.

Table 2.8. *Average purity and standard deviation for the washing protocols with SDS 2.5% and 5%.*

	Average purity	Standard deviation
SDS 2.5	98.24%	4.92%
SDS 5	98.26%	4.59%

The results indicate that the solution with 2.5% of SDS is enough, saving chemicals and reducing the difficulty of the wastewater treatment. As it can be easily seen, the numbers of both table 2.6 and 2.7 present a high variability, giving an overall standard deviation of around 5%. As the economic value of the polymer derives from its purity, these few percentage points are very relevant. The procedure of methylation was so changed to try to

reduce the scattering of the results. From the analysis of the protocol, the most error-affected step appeared to be the first weighing of the sample, as the measured values of 0.0100g are close to the scales measurement error. Hence a higher amount of polymer (0.1027g, ten times the standard measurement) has been dissolved in 20mL of chloroform. From this solution 12 mL in 6 tubes (2mL for each) were sampled and then the normal methylation was performed; results are shown in table 2.9. This lead to a reduction of the standard deviation, confirming the hypothesis of the balance error. The average results are close to the previous one, as shown in table 2.10.

Table 2.9. *Results of GC characterization and purity of therecovered solids after SDS 2.5 % washing. To reduce the scattering of the results, methylation was performed on six samples from the same batch of extracted polymer, dissolved in 10 times the standard proportions to reduce the scale error.*

Date	sampl e ID	Methylated mass [g]	Total solid conc. [g/L]	Area P3HB	Area IS	Ratio	P3HB conc. [g/L]	Purity [%]
14-05-2013	71	0,10579	5,29	21241,5	35496,4	0,598413	5,38	101,65
14-05-2013	72	0,10579	5,29	18969,5	34027,1	0,557482	5,03	95,02
14-05-2013	73	0,10579	5,29	18921,3	34420,4	0,549712	4,96	93,76
14-05-2013	74	0,10579	5,29	18550,7	32496	0,570861	5,14	97,19
14-05-2013	75	0,10579	5,29	19293,8	33608,9	0,574068	5,17	97,71
14-05-2013	76	0,10579	5,29	19586,3	33626,4	0,582468	5,24	99,07

Table 2.10. *Average purity and standard deviation for the washing protocol with SDS 2.5% reducing the scale error.*

	Average purity	Standard deviation
SDS 2.5	97.40%	2.58%

There is still an error, which is hard to be reduce, as it derives from the sum of pipette variability, GC error, evaporation of the solvent and all the unpredictable errors linked to the long procedure of sample preparation. This is the limit of the GC analysis method, which uggested to develop the "melting test".

In summary, table 2.11 reports the calculated recovery yields for these two extraction techniques with SDS at 2.5% and 5%.

Table 2.11. *Recovery yields for the extraction protocols with SDS solutions at 2.5% and 5%. Absolute recovery yield is calculated considering the total amount of the polymer inside the cells, relative recovery yield is calculated considering the maximum amount of P3HB which can be extracted with chloroform a 4°C.*

	Weight Total [g]	Weight tube [g]	Recovered material [g]	Cell mass [g]	Polymer purity [%]	Absolute recovery yield [%]	Relative recovery yield [%]
SDS2.5%	11,8829	11,4607	0,4222	0,75	98	84	104
	11,8851	11,4622	0,4229	0,75	99	85	106
	11,9315	11,5224	0,4091	0,75	96	80	99
	11,8981	11,5053	0,3928	0,75	100	80	99
	11,9574	11,555	0,4024	0,75	98	80	99
	11,9069	11,5056	0,4013	0,75	99	80	100
SDS5%	11,9641	11,5079	0,4562	0,75	95	88	109
	11,9233	11,4967	0,4266	0,75	99	86	106
	11,9708	11,5517	0,4191	0,75	99	84	105
	11,9618	11,4765	0,4853	0,75	100	99	122
	11,9207	11,4888	0,4319	0,75	100	88	109
	11,9213	11,4569	0,4644	0,75	100	94	117

It is possible to see how the extraction yields are comparable (or even better) with the ones of chloroform techniques.

2.3.2 Other surfactant washing.

SDS gave a good result in terms of final purity, so a campaign of experiments was carried out to check the performance of another surfactant, a non-ionic one. According to the same criteria adopted to choose SDS (i.e. low cost and market availability), the non-ionic surfactant named Triton X 100 (AppliChem ® Union Carbide Company) was used. Two aqueous solutions were prepared, the first with 0.15% w/v the second with 0.3% w/v, respectively corresponding to ten times and twenty times the critical micellar concentration (CMC=0.00023M/L which corresponds to 0.0144% w/v). These solutions were used in the washing patterns described in table 2.12.

Table 2.12. *Washing protocols investigated for the screening of purification techniques with surfactant Tryton X 100.*

Sample ID	1st washing	2nd washing	3rd washing	Info
30/4 T1	15mL TritonX 0.15%	15mL H_2O	15mL H_2O	Same procedure of SDS, changes only the surfactant.
30/4 T3	15mL TritonX 0.3%	15mL H_2O	15mL H_2O	Same procedure of SDS, changes only the surfactant.

Unfortunately the results were unsatisfactory if compared to SDS performance (table 2.13), so Triton X 100 was abandoned.

Table2.13. *Results of GC characterization and purity of the recovered solids afterTryton X 100 washing.*

Date	sample ID	Methylated mass [g]	Total solid conc. [g/L]	Area P3HB	Area IS	Ratio	P3HB conc. [g/L]	Purity [%]
30/4	T1	0,0111	5,55	16804,1	30275,8	0,555034	4,76	85,76
30/4	T1	0,0112	5,6	19482,9	34545,1	0,563984	4,84	86,37
30/4	T3	0,0095	4,75	19572,3	41696,8	0,469396	4,02	84,75
30/4	T3	0,01	5	18980	39676,5	0,478369	4,10	82,05

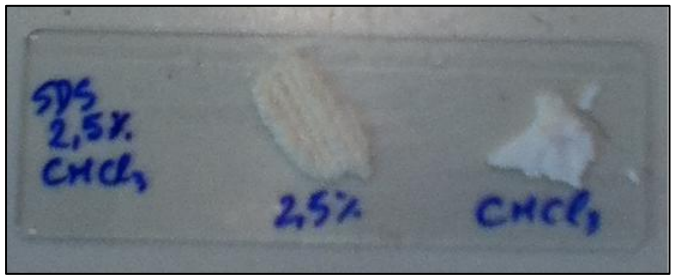
2.3.3 Melting temperature test.

The samples which showed the highest purity according to the GC were tested on the hot plate. Unfortunately the results were far to be considered acceptable, as pictures 2.3 and 2.4 clearly show.
The first sample analyzed was the polymer obtained with three washings, beginning with SDS solution at 2.5%. The control extracted with chloroform (named $CHCl_3$) is put on its side, to give a comparison. In table 2.14 the record of the phenomena observed during the test is reported.

Table 2.14. *Record of the phenomena occurred during SDS 2.5 % sample melt test.*

Temperature	**Observation**
60ºC	A bad sugary smell can be sensed.
80-100ºC	The sample starts to become yellowish. But the powder keeps its granular shape.
120ºC	The sample becomes dark yellow and starts to smoke. The granules soften but do not melt properly. Granules can still be distinguished.
150ºC	The sample is burning. The granules release a small amount of a bubbling liquid that soon disappears. A thick smoke rises from the sample, which loses mass. In the end it remains a small amount of dry char. The control sample softens and starts to melt.
160 ºC	The control sample melts completely. It starts to darken a little bit, due to traces of impurities. It has the behavior of a viscous fluid (no smoke).

It is clear that the sample washed with SDS, which had an estimated purity of 98%, is far to be comparable to the chloroform extracted one. The melting did not even occur (figure 2.3).



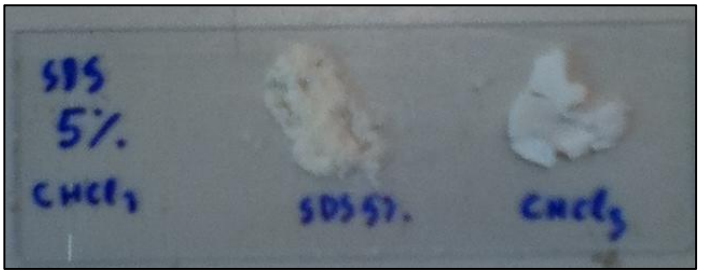
(a)

(b)

Figure 2.3. comparison between the sample of P3HB purified with SDS 2.5% (left) and the reference extracted with chloroform (right), before the heating(a) and after(b)

The experiment was repeated for a sample of polymer extracted with SDS solution at 5%. The steps of the melting and the phenomena observed are exactly the same of the previous sample: sugary smell, yellowing, smoking, darkening and the final burnt residue. Hence, only the comparative pictures have been reported (figure 2.4).

With the aim to understand better this unexpected phenomenon, the test was repeated again, putting the polymer in a sealed tube with an inert (nitrogen), so to avoid any oxidation reaction. In this case there was no smokes or loss of the mass of the sample, but the granules darkened in the same way, releasing a sticky fluid, molten polymer, but completely dark and useless. This is the proof that the polymer still contains impurities subjected to thermal degradation: in the presence of oxygen, these impurities are involved in reactions that alter the nature of the polymer, causing its fast degradation.



(a)

(b)

Figure 2.4. comparison between the sample of P3HB purified with SDS 5% (left) and the reference extracted with chloroform (right), before the heating (a) and after (b)

The fast degradation, described in the literature as "carbonization", is said to be still little understood (Yu *et al.* 2013), as deriving from complex interaction between the polymer and the cellular material. This specific reaction mechanism could be difficult to discover in detail, but the overall behavior can be explained with the available information from polymer science.

It might be considered strange that only 2% of impurities are enough to cause this dramatic change in the whole polymer; but in reality this is not unusual at all. Polymer technology show how a very low amount of radicals in a plastic material can determine a very fast ageing in normal use conditions; moreover, during recycled PET reprocessing,very low amounts of reactive species (radicals, monomers, chlorinated compounds, PVC) even cause the total degradation of the chains (Levchik and Weil 2004; Modesti 2012). P3HB, in particular, is a polyester, one of the most reactive polymeric chain, and the high temperature enhances the effects of the degradation reactions. The smoke occurring during the melting in air demonstrates that the reaction involves oxidations, which are typically radical reactions. Radicals can cause a chain mechanism involving the degradation of the entire polymer and the kinetic of these reactions is sustained by temperature. As a consequence, this mechanism, started by the low-temperature degradation of impurities, leads to the complete denaturation of P3HB at temperatures even lower than its melting

point. This is why the residual appears like a granular, burnt char (carbonaceous residue of thermal degraded polymers), as the granules of milled polymer are denatured before melting point.

A more detailed analysis, performed with DSC (nitrogen atmosphere, thermal ramp of 10ºC/min up to 220ºC), allows knowing the precise temperature of this first endothermic degradation of the impurities: this happens between 46 and 60 degrees, and continues slightly until the curve reaches the melting zone (figure 2.5). These low temperatures show that impurities have biological origin.

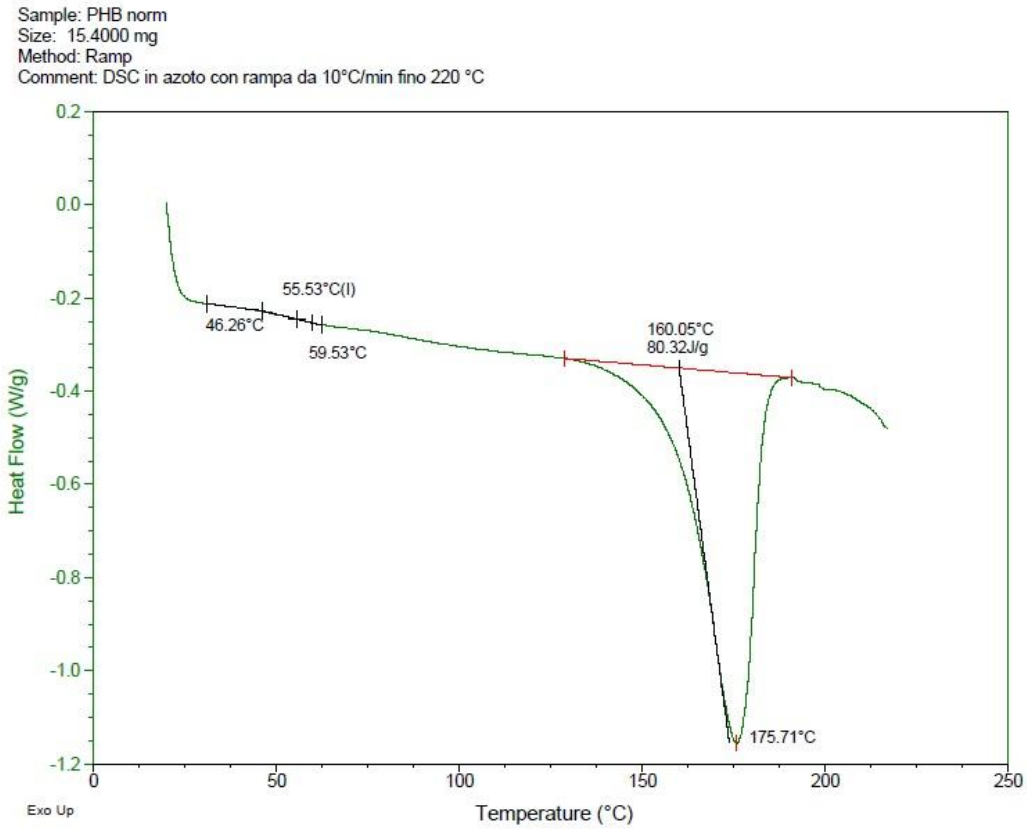


Figure 2.5. *Differential scanning calorimetry in Nitrogen (thermal ramp 10°C/min up to 220°C) of a P3HB sample extracted with SDS 2.5% washing. Analysis performed in the Department of principles and plants of Chemical Engineering "Sorgato", of University of Padova under authorization of professor Modesti Michele.*

The possibilities for the nature of these organic substances are these (Zinn *et al.* 2001):

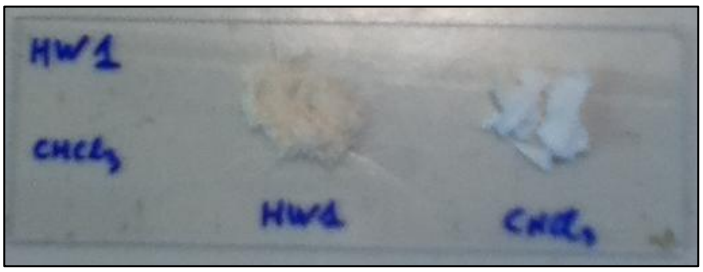
- lipids, which have a similar structure to the PHB, even if they should be dissolved by surfactants;
- proteins, that suffer thermal degradation (and are typically soluble in water);
- some residual of SDS, which is unstable to high temperature.

To exclude some of the alternatives, the procedure of extraction was repeated in more severe conditions. First, the polymer was washed with hot boiling water (100ºC), which, together with the surfactant, should remove both lipids and proteins. To be even safer, another washing step was added, so to remove any possible residual of surfactant. Table 2.15 reports the washing steps in the more severe conditions.

Table 2.15. *Modification of the washing protocol to enhance cleaning performance*

Sample ID	1st washing	2nd washing	3rd washing	4th washing
HW1	Hot SDS 2.5% solution (15mL)	Hot water (20mL)	Hot water (20mL)	Hot water (20mL)
HW3	SDS 5% solution (15mL)	Hot water (20mL)	Hot water (20mL)	Hot water (20mL)

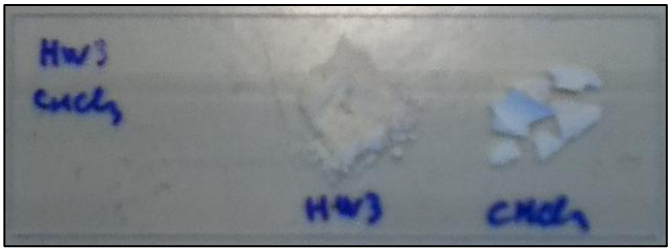
In spite of these attempts, the results did not change at all (figure 2.6; 2.7).



(a)

(b)

Figure 2.6. *Comparison between the sample of P3HB purified with SDS 2.5% in hot boiling water (left) and the reference extracted with chloroform (right), before the heating (a) and after(b)*



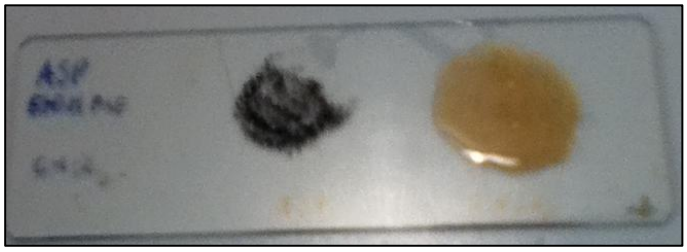
(a)

(b)

Figure 2.7. *Comparison between the sample of P3HB purified with SDS 5% in hot boiling water (left) and the reference extracted with chloroform (right), before the heating (a) and after(b)*

Another attempt to understand the reasons of polymer burning, was pretreating the cells with methanol, before the digestion procedure. The literature (Jiang *et al.* 2006) reports that treating the lyophilized cells with alcohol (methanol or ethanol) improves the results of extraction as has a very selective action in dissolution of lipids. So, before the usual extraction and purification with SDS at 2.5%, the batch of cells of 5g (extraction ID: 7/6 ASP) was first suspended in 100mL of methanol for 24 hours (shaken at 170rpm, 30ºC), then filtered. The final powder of polymer (white and apparently pure like others) was subjected to the melt test (figure 2.8).

(a)



(b)

Figure 2.8. *Comparison between the sample of P3HB purified with SDS 2.5% after 24h of ethanol pretreatment (left) and the reference extracted with chloroform (right), before the heating (a) and after(b)*

Again, nothing changed in the melting behavior.

A last attempt was performed washing with methanol the powder after the normal extraction. A sample of 100mg of polymer extracted with SDS2.5% and 2 water washing was resuspended in 0.5 mL of methanol, centrifuged, and then the methanol was discharged. This operation was repeated twice on each sample.
Eventually, the polymer burned like the others.

2.3.4 Explication of the phenomena.

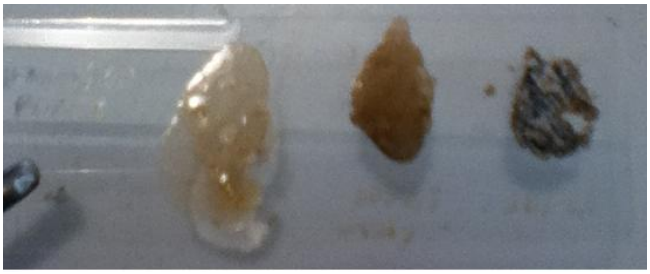
The results are contradicting all the hypotheses: washing with hot water and surfactant should have removed the proteins, methanol should have removed lipids, but the final effect is still the same. On the other hand, there are no other possibilities, the impurities are biological matter and the washing techniques applied at least should have improved the final result.

So, the right question was not, “what are the impurities still present?”, but “why the polymer still burns though the improved washing methods?”.
Apparently all the tested methods, even the most aggressive, do not remove the amount of debris (about 2%) responsible of the bad melting.
The answer should not be found in the methods, but in the polymer.
In fact the common point to all extraction was the use of NaOH, a technique that is known (Yu *et al.* 2013) to change the crystalline structure of the polymer grains. As soon as the grains enter in touch with water, when the membranes are broken by NaOH, the polymer rearranges its structure from amorphous to highly crystalline, with a grade of crystallinity up to 70% (Mitomo and Doi 1999). This fact can help to understand why the washing proved to be ineffective. The following theory was developed.

The enzymes, proteins, lipids and all the cellular matter present around and inside the grains get stuck into the crystals of P3HB. The polymer itself, with its water insolubility, obstruct the effect of the washing agents. In this way, polymer grains are washed outside, removing the most of the impurities, but the debris inside the grains remains untouched until the end.

To prove this hypotheses an experiment was designed and run. The crystalline structure has to be changed to allow debris being released; this can be obtained by plastifying or dissolving the polymer crystals in a solvent, in this case, chloroform. A sample of the powder obtained with the technique of SDS at 2.5% was put in a flask with chloroform (0.4g of polymer in 40mL, 1% solution). After 48h at room temperature with agitation the polymer lost its crystalline structure and was completely dissolved. The solution was then filtered, according to the extraction method for solvents described in chapter 3. The polymer, precipitated, filtrated again and dried, was subjected to the melting test. The results are shown in figure 2.9.
The remarkable sample is the one in the middle: it became brown, but it melted without smoke or any of the other phenomena. The reasons of the darkening are two: a small amount of debris passed through the filter, or a slight degradation of the polymer occurred (as subjected a very high number of operation, between NaOH extraction, washings and then all chloroform extraction procedure).

(a)



(b)

Figure 2.9. *Comparison between the sample of P3HB purified with SDS 2.5% (right), purified with SDS 2.5% then dissolved and filtered in chloroform (centre) and the reference extracted with chloroform (left), before the heating (a) and after(b). The central samples liquefies, after becoming slightly brownish.*

The second possibility is sustained by the fact that the melting temperature of the control sample was about of 160ºC, while the central sample melted already at 145ºC. In any case, **there was no dramatic degradation and the polymer melted and flowed.** The powder which was not dissolved and filtered, the sample on the right, degraded as always.
Moreover, during the chloroform purification a thin film of yellowish residuals remained in the filter, a proof that some debris was present even if the powder appeared white and clear: the dissolution opened the crystals and let the impurities come out.

2.4 Conclusions

The techniques investigated have proved highly effective in incrementing the purity of the final polymer, raising it from the 81% of the old method based on two water washings, up to the 98% of the method based on SDS solution at 2.5%.
Unfortunately, the recovered polymer, white and apparently without any impurity, during the test to measure the melting temperature started to burn and smoke, becoming a dark char at 150ºC.
The phenomenon was inexplicable until the characteristic crystalline structure of the P3HB was reconsidered. In fact the dynamics of crystallization are very fast when the polymer enters in touch with water, so the following hypothesis was developed: **the membranes and proteins (enzymes) remain trapped inside crystallized granules of PHB after cell digestion.**
This simple idea explains a number of phenomena which were observed.

- The polymer is washed and rinsed very strongly, removing debris thanks to mechanical stirring, chemical aid of surfactant and physical effect of hot boiling water. The excess of the washing media, compared to the mass of real polymer, should remove everything. Nonetheless, the cleaning is only outside the very small crystals of polymer: what remains stuck in the regular patterns of the crystals, is not reached by the washing agents, as polymer is insoluble in water.
- The thermocalorimetric analysis shows negative curves at about 40-90 ºC, denoting the presence of biological species, which typically denature in this range of values. These substances, lipids and proteins, are the only plausible cause of the poor melting performance of the polymer. They should be cleared by the combined effect of supernatant and hot water, unless they could not be reached.
- Chloroform extraction works better than this method because, besides the solvent's high selectivity, the crystalline structure does not occur during extraction because the polymer is plastified by the solvent.
- Further washing of the already white polymer are pointless. This was demonstrated using hot water, adding a washing step with water or with methanol. All these techniques washed the outside of the granules only.
- The theory of the trapped-inside debris helps to explain why the burnt powder, after heating, remains with the shape of a dark and bruised dry powder. The decomposition of debris molecules, very near and between the crystal reticules, weakens the structure and causes the overall polymer degradation directly before melting. Decomposition of impurities is an oxidation, which produces smoke and starts radicalic reactions of polymer degradation accelerated by temperature: one

proof of this statement derives from the samples heated in nitrogen. Under inert gas the sample could melt a little before degradation, as the chain mechanism of degradation was not started. Impurities decompose and caused the denaturation of the very reactive ester bonds of the polymer, which becomes a carbonaceous char. Many commercial plastic show this behavior, becoming char after high temperature degradation (it is actually a phenomenon obtained artificially with additives, to make fireproof polymer).

This theory finds support not only from the listed indirect evidence, but from an experiment on purpose. Chloroform was used to dissolve the washed polymer, opening the crystal structure: in this way the impurities stuck inside the granules were released and filtered away. Most of the remained debris was removed and the polymer showed again a normal melting behavior, comparable with the polymer directly extracted from cells with chloroform.

It has been demonstrated, and explained, that the water-based extraction techniques are not suitable to recover P3HB homo-polymer from cells of *B.sacchari*.
It can be replied that none of the literature articles published on these topics encountered this issue, and, on the contrary, Choi and Lee considered enough a purity of 95% for the simulation of their process. These facts can be easily explained. First, Choi and Lee work was based on another type of bacterium, recombinant *E. coli*, while this work was developed with *B. sacchari*, which presents more complex systems of membranes: this could be a reason of the different behavior of the polymer after extraction. However it can also be that they did not melt the polymer in the way here experimented, as this practice is never mentioned in the reference literature for this extraction methods. The few melt tests encountered in literature (Yu *et al.* 2013) were all performed in DSC under nitrogen atmosphere, which does not allow observing the proper carbonization, as oxygen is missing.
This is the difference between this work, with a very applicative approach, and the more theoretical surveys on PHA recovery available in the literature. In fact the results of Choi and Lee, Yang, Chen (and others who investigated aqueous digestion extraction) are all based on the GC analysis of the purity, without mentioning any further downstream operation (from melting to extrusion). Also, the process simulation proposed by Choi and Lee, based on experimental data, was not put in practice with a pilot plant.
The aim of this work was not to question the previous results, but unfortunately it has been shown that high purity is not enough, if the polymer denatures when melted.

For sure other steps could be added to the procedure of extraction and purification, to avoid or slow down the crystallization.
However, other steps mean further expenses for polymer purification, making it pointless the base idea of developing a cheap and safe recovery method.
Extraction based on aqueous solution has not found industrial application yet, at least with the simple structure proposed by Choi and Lee, the only one that grants very low costs and eco-friendly performances.

However the method currently proposed should not be abandoned. Its main values, cheapness and low impact, with a proved efficacy in purification, were hindered only by the peculiar crystalline structure of P3HB. It means that the method could be applied for the recovery of other bio-polymers, that have the same cellular origin, but do not crystallize. *B.Sacchari* and other bacteria (Nawrath *et al.* 1995) have been so far studied because they are able to produce directly co-polymers, for example P3HB-HV, characterized by lower grades of crystallization (Mitomo and Doi 1999). Moreover, solvent extraction techniques are easily applicable to the homo-polymer.Iinstead, the variance in the structure of co-polymer makes it impossible to select a universally valid solvent (Jacquel *et al.* 2007). So, aqueous cell digestion could be the simplest and most versatile extraction technique for low-crystallized co- polymers.

Chapter 3

Solvent extraction of homo-polymer P3HB

The use of selective solvents to recover a specific compound is very common in the pharmaceutical industry. However it is often an expensive solution, motivated by the high value of the recovered ingredient. Also in the case of PHA, the most effective technology for the recovery of P3HB is based on solvents, in particular on chloroform, with great concerning about environment. This solvent is already highly regulated, subjected to the BAT (best available techniques) indications and included in a list of solvents to be replaced in the future, as reported in the European Union risk assessment report for chloroform (2007). Consequently, in the last years the research has been oriented in finding a substitute for chloroform. This new solvent should have three base characteristics:

- ✓ Low price: in this way P3HB can become a wide spread common use polymer
- ✓ Efficiency: to optimize the process minimizing losses
- ✓ Eco-sustainability: this is the most important, according also to the ecological idea at the base of a bio-polymer.

Paragraph 3.1 describes the predictive method developed by Jacquel et al (2007) to estimate the solubility of P3HB in different solvents: this is an essential tool to screen among many possibilities, as it allows to simulate the behavior of the solvents, saving much time in experimental sessions.
In paragraph 3.2 the criteria to select the best available solvents (according to ecological performances) are discussed: simulation results are reported in paragraph 3.3. Paragraph 3.4 describes the experimental methods for the solvent extractions, performed in the lab, to obtain the results of paragraph 3.5, then commented in paragraph 3.6.

3.1 Thermodynamic model of solubility.

The equation used to predict the solubility of P3HB is a thermodynamic correlation which is a function of temperature, polymer molecular weight and cristallinity (Jacquel *et al.* 2007). This model is theoretically based on the original work of Hildebrand (1936), who introduced the solubility parameter δ_t after the thermodynamic definition of the cohesive

energy density: two materials present affinity (solubility) if their δ_t parameters are similar. The model was further improved defining a second parameter to represent also the polar interaction (Blanks and Prausnitz 1964), as Hildebrand model was suitable only for non-polar systems. In 1967 Hansen proposed a more refined method with three parameters: the dispersion component δ_d, then δ_p for polar interaction and δ_h specific for hydrogen bonding. A 4-parameters model was later published (Beerbower *et al.* 1984), more rigorous However Jacquel *et al* (2007) demonstrated that the last model works better than the Hansen one only for certain solvents, while for others (esters in particular) the 3-parameter method is more reliable. Therefore the Hansen predictive method has become a widely used instrument in polymers, paint and coating technology and it is easy to find large data banks of the parameter values (Hansen 2007). Since this, the Hansen model here was here applied as well.

The behavior of two substances is described with the *solubility radius*: the smaller the radius, the higher the miscibility. The expression for the three dimensional model of Hansen solubility radius is:

$$ {}^{kj}r = \sqrt{\left[4\left({}^{k}\delta_d - {}^{j}\delta_d\right)^2 + \left({}^{k}\delta_p - {}^{j}\delta_p\right)^2 + \left({}^{k}\delta_h - {}^{j}\delta_h\right)^2\right]} \qquad (3.1) $$

Where *i* and *j* identify the two species.

The Hansen parameters, if not available, can be predicted, for example by the Krevelen methods or the theory of Hoy, based on group contribution theory as mentioned by Jacquel *et al* (2007); however the accuracy is not very high. On the contrary, the group contribution correlations can lead to real evaluation errors, as already pointed out by the authors (pag 2709) referring to the previous article of Terada and Marchessault (1999). Moreover, during this thesis work, it was noticed an error in table 22.6 (page 464) of a published manual (Belgacem and Gandini 2011), that reported a group contribution prediction for cyclohexanone in stark contrast to the experimental evidence reported in paragraph 3.5. So, to calculate the Hansen solubility parameters of the polymer, Jacquel preferred to use an experimental method, based on a baricentric data regression technique. The measured solubilities of a population of 20 different solvents and their Hansen parameters (from literature) are the input data of this method. Accordingly, the values obtained for P3HB are referred to a polymer of Mw of 690 000 at 50ºC and are shown in table 3.1.

Table3.1. *Experimental values of Hansen Solubility parameters, calculated by Jacquel et al.2007 for P3HB homopolymer with MW of 690 000 at the temperature of 50°C.*

δ_d [MPa^{0,5}]	δ_p [MPa^{0,5}]	δ_h [MPa^{0,5}]
19.3	5.3	6.3

The experimental solubility as a function of the calculated radius showed an exponential decay relation, which can be represented by the equation

$$s = s_0 + A \cdot \exp(-Br) \tag{3.2}$$

Where s_0, A and B are regression parameters.

Measuring the solubility of samples of different chain length (Mw=4,500; 80,000; 250,000; 690,000; 1,300,000) at different temperatures (between 30ºC and 70ºC) allowed to obtain the predictive equation for PHB solubility, which has been used in this work for solvent selection. The final form of the equation proposed by Jacquel, with all the calculated parameters is:

(3.3)

$$s\ [g/L] = \left[141.7 + 0.454 \cdot \exp(0.063 \cdot T) + 292.8 \cdot \exp\left(-0.210 \cdot 10^{-4} \cdot Mw\right)\right] \cdot \exp(-0692 \cdot r)$$

The equation 3.3 was tested on 175 different solvent-polymer systems, leading the authors to the following main conclusions:

- halogenated solvents show higher solvation efficacy, due the strong polar interaction between the chloride atom of the solvent and the carbon that holds the carbonyl function in the polymer;
- to develop a general predictive equation valid also for co-polymers of P3HB, a new variable depending on composition is introduced. The equation becomes complex and gives unreliable results. In fact, the variability is too high, as the type of co-polymer and the final structure of the chain strongly change the behavior of the polymer-solvent system.

The crystallinity of the polymer was not investigated in deep, as the authors used P3HB of the same producer (Jangsu Nantian Group), which had the same type of crystallinity. Unfortunately crystal properties varies according to the species of bacteria used for polymer production.

Figure 3.1 shows the scattering of predicted and experimental values of the equation.

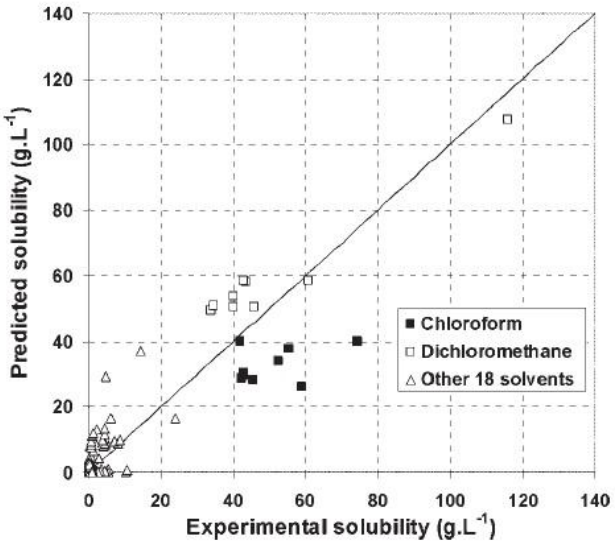


Figure 3.1. *Scattering diagram of the error between the predicted and the experimental solubility. The diagonal corresponds to perfect matching between prediction and measure.175 points, temperature between 30°C and 70°C, Mw between 4500 and 1300000 , (Jacquel et al. 2007).*

It is clear that the accuracy of the method is not high, considering also the fact that only two of the investigated solvents show high solubility and the rest of the calibration is based on very low values. Anyway, this method is enough to make a preliminary screening among the possible solvents, so to give indications of a substitute for chloroform and the other harmful halogenated solvents.

3.2 Selection criteria for the solvents

Although there are no doubts over the necessity of the development of a green solvent technology, the definition of "green" is not single nor absolute. Indeed, there's plenty of misunderstanding when dealing with environment protection. As a consequence, a strong evaluation criterion of "green performance" is needed to select a list of solvents, suitable for P3HB extraction. This criterion should be well-motivated and reliable, based on scientific information, avoiding arbitrary judgments, a procedure not trivial at all. Luckily it has already been done (Curzons *et al.* 1999). The "Solvent selection guide" provided in the cited article derives from the long-standing experience of GlaxoSmithKline Beecham (GSK). This company decided to share its knowledge on solvents to create a tool-kit for engineers and chemists to select the best solvents, reducing human health impacts, process

safety risk and multiple environment impacts. Beyond the credits of R&D department of GSK for the huge work of data-collection, the value of this guide, and in particular the second version released (Henderson *et al.* 2011), is remarkable. In facts it is based on risk evaluation reports, on the modern approaches of Life Cycle Assessment (LCA) and Environmental Foot-print Analysis. These evaluation method grants the highest level of objectivity and completeness for the analysis, giving a reliable statement over the solvents analyzed.

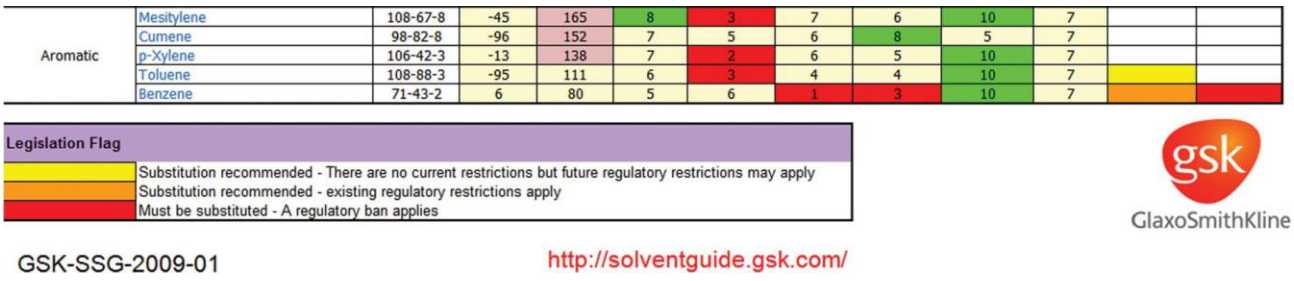
The guide has been thought to be easy to consult, so the solvents were evaluated according to nine main categories, associated to a color (green, yellow, red) and to a score (from 1, very poor, to 10, highest mark). The score is given according to the assessment procedure for process data, safety indications and legislation. The use of colors is a help for the user: green for scores higher than 8, red for lower than three and yellow the others.

The categories of interest resuming the evaluated properties of the solvents are:

1. **Waste**: the evaluation includes the sub-topics of recycling, incineration (complexity of process, possible subproducts), VOC (potential of emission of volatile organic compounds), biotreatment issues
2. **Environmental impact**: it refers to fate and effects on the environment considering water and air
3. **Health**: it accounts for the possible acute and chronic toxic effects on humans of the solvents, and all the parameters of exposure allowed by law (Example: VHR vapor hazard rating, OEL occupation exposure limit ...)
4. **Flammability & explosion**: it deals with issues affecting storage and handling of the solvents (evaluation based on flash point, conductivity, autoignition temperature, peroxydes formation …)
5. **Reactivity and stability**: it accounts for factors affecting the stability of the solvent
6. **Life cycle score**: it reports the overall (environmental) impact of the solvent from the production to the disposal, with a sight also to associated energy and raw material consumption.
7. **Legislation flag**: it is not related to a score, but simply to a color, points up the necessity of substitution of the solvent (as a regulatory ban applies) or its recommended substitution (regulatory restriction applied or future regulation)
8. **EHS flag**: red flag highlights the worst solvents for Environment Health and Safety (benzene and chloroform for example)
9. **Boiling and Melting point**: the highest (>120ºC) and the lowest (<40ºC) boiling points are marked with red color, as well melting points higher than 17ºC (issue for rheology). Even if this is not a category so directly involved with EHS, it can be

useful to pointing out possible issues (for example high volatility or at the opposite high power consumption for distillation).

Figure 3.2 shows the final layout of the GSK solvent selection guide for aromatic compounds.



Aromatic	Mesitylene	108-67-8	-45	165	8	3	7	6	10	7		
	Cumene	98-82-8	-96	152	7	5	6	8	5	7		
	p-Xylene	106-42-3	-13	138	7	2	6	5	10	7		
	Toluene	108-88-3	-95	111	6	3	4	4	10	7		
	Benzene	71-43-2	6	80	5	6	1	3	10	7		

Figure 3.2. *Solvent selection guide layout, aromatic section. On the right it is possible to see the logo of Glaxo Smith Kline, which promoted the guide realization.*

At this point a criterion to choose among the list of 110 solvents evaluated in the Guide must be given, so to perform the simulation for P3HB.
The choice was in favor of the solvents that did not present any red flag, to be sure of avoiding environmental harmful solutions, but also to avoid problems on the health and safety areas.

3.3 Results of the simulation

With the aid of an Excel datasheet (Microsoft office professional plus 2010® v. 14.0.6129.5000 32bit) the equation of Jacquel was implemented for the no-flag solvents. The values of the Hansen solubility parameters, if not differently indicated, were obtained from the handbook (Hansen 2007). Table 3.2 shows the final results of the simulation:

Table 3.2. *Results of the predicted solubility for some of the selected solvents of GSK Solvent Selection Guide. In grey the results for chloroform, reference for comparison, in green the best solvents are indicated.*

Solvent	δd Mpa$^{0.5}$	δp Mpa$^{0.5}$	δh Mpa$^{0.5}$	Hansen radius adim	solubility g/L
omo-polymer P3HB	19,3	5,3	6,3		
chloroform	17,8	3,1	5,7	3,768	11,225
water	15,5	16	42,3	38,318	0,000
2-ethyl hexanol	15,9	3,3	11,8	8,972	0,306
glycerol	17,4	12,1	29,3	24,283	0,000
cyclohexanol	17,4	4,1	13,5	8,229	0,512
ethylene glycol	17	11	26	21,018	0,000
1,4 butanediol	16,6	15,3	21,7	19,139	0,000
isoamyl alcohol	15,8	5,2	13,3	9,900	0,161
benzyl alcohol	18,4	6,3	13,7	7,681	0,749
2-pentanol	15,6	6,4	13,3	10,245	0,127
1-butanol	16	5,7	15,8	11,575	0,051
methanol	15,1	12,3	22,3	19,379	0,000
1-propanol	16	6,8	17,4	13,001	0,019
t-butyl acetate [9]	15,75	3,68	12,89	9,822	0,170
butyl acetate	15,8	3,7	6,3	7,181	1,059
ethyl lactate	16	7,6	12,5	9,343	0,237
propyl acetate	15,3	4,3	7,6	8,166	0,535
dimethyl carbonate	15,5	3,9	9,7	8,443	0,442
methyl lactate [10]	16	8,3	16,8	12,760	0,022
ethyl acetate	15,8	5,3	7,2	7,058	1,153
ethyl propionate[10]	15,5	6,1	4,9	7,769	0,704
ethyl formate	15,5	8,4	8,4	8,472	0,433
cyclohexanone	17,8	6,3	5,1	3,382	**14,662**
cyclopentanone	17,9	11,9	5,2	7,253	1,007
2-pentanone MIBK	15,3	6,1	4,1	8,335	0,476
3-pentanone [11]	15,75	7,57	4,7	7,624	0,779
propionic acid	14,7	5,3	12,4	11,039	0,073
acetic anhydride [10]	16	11,7	10,2	9,986	0,152
acetic acid	14,5	8	13,5	12,300	0,031
Cumene [10]	18,1	1,2	1,2	6,970	1,225
di(ethylene glycol)	16,6	12	20,7	16,775	0,001
Phenetole (#)	18,4	4,5	4	3,028	**18,733**
tri(ethylene glycol)	16	12,5	18,6	15,706	0,003
sulfolane	18,4	16,6	7,4	11,495	0,053
DEG monobutyl ether	16	7	10,6	8,059	0,577
anisole	17,8	4,1	6,7	3,256	**16,004**
diphenyl ether	19,6	3,2	5,8	2,241	**32,310**

dibutyl ether	15,3	3,4	3,3	8,753	0,357
1,2-dichlorobenzene	19,2	6,3	3,3	3,169	**16,999**
1,2,4-trichlorobenzene	20,2	6	3,2	3,652	**12,163**
chlorobenzene	19	4,3	2	4,455	**6,978**
trichloroacetonitrile	16,4	7,4	6,1	6,172	2,128
chloroacetic acid	17,7	10,4	12,3	8,500	0,425
gamma butyrolactone [10]	18	16,6	7,4	11,647	0,048
propylene carbonate	20	18	4,1	12,965	0,019
diethyl succinate [12]	16,1634	4,092	8,184	6,660	1,517

(#)(Williams and Kuklenz 2009)

The solvents highlighted are the ones that showed the best solubility properties according to the model. To reduce again the number of solvents on which perform the experimental analysis, it was decided to exclude all the chlorinated ones, even if considered "green" by the GSK analysis. This choice was done due to the hostility that chlorinated substances encounter among public opinion, even if not so harmful. The solvents eventually used for P3HB extraction are listed in table 3.3:

Table3.3. *Summary of the main properties of the green non-chlorinated solvents investigated, compared to chloroform.*

Solvent	Melting point	Boling point	Density
[Chloroform]	-63ºC	61ºC	1.492 g/cm3
Anisole	-37ºC	154ºC	0.995 g/cm3
Phenetole	-30ºC	170ºC	0.966 g/cm3
Cyclohexanone	-47ºC	155ºC	0.947 g/cm3
Diphenylether	25ºC	259ºC	1.073 g/cm3

3.4 Solvent extraction, materials and methods

The results deriving from a laboratory scale experience are often different from industrial process ones, and the same could be said about the methods: it is difficult to simulate, with volumes of the order of 10^{-1}g of polymer recovered, the real unit operations with industrial scale equipment. Anyway the methods here presented are useful to evaluate the relative

effectiveness among the solvents. If the investigated one is similar or even better than chloroform, it is worth to be further studied and maybe considered for a scale-up design.

3.4.1 P3HB recovery: chloroform extraction technique

The protocol here described follows the indications presented in the work by Choi and Lee (1997), with some improvements introduced by professor Fonseca's researchers, who apply this protocol daily. In facts this is the fastest way to recover relevant amounts of pure PHA without reducing sensibly polymer weight. The polymer extracted in this way is used for further analysis, but also as a comparison to other extraction techniques (for example, the calibration curve is made using chloroform-extracted P3HB). The steps of the protocol are:

1. An amount of 0,5g of dry lyophilized cells is put in a glass flask with 30 mL of chloroform ($CHCl_3$ analytical reagent grade. Fisher Scientific) and a magnetic stirrer (covered by teflon). The flask is sealed and put over a stirrer plate for 36h at 4ºC. The low temperature grants that the chain length is preserved.
2. The suspension of chloroform and cell debris is vacuum filtered with Buchner equipment (Kitasako flask, hydraulic guard, Buchner filter, vacuum pump as showed in picture 3.3), using cellulose filters (Rotilabo Rundfilter typ114A, cellulose membrane, diameter 90mm. 3-5 µm retention)
3. The recovered solution of polymer is added with 120mL of an anti-solvent (4 parts the amount of the solvent), in this case ethanol (purity 96%, Fabrica de alcool Vieira&irmão.lda).
4. The polymer precipitates, so the suspension of chloroform-ethanol and polymer is filtered again, this time on a pre-weighed filter of the same type.
5. The filter is recovered and put on a glass plate under a fume hood so to remove residuals of solvents via evaporation. The filter is weighed again to have an indication of the amount of polymer extracted. To have a measurement even more precise the filter could be exsiccated in oven at 60ºC for 12hours.

Figure 3.3. *Buchner filtration equipment. On the left the vacuum pump.*

The volume of 30mL for 0,5g of polymer is high: it corresponds to a concentration of about 8.3 g/L (considering a polymer content of 50%) and gives the saturation concentration. Higher concentrations lead to a very viscous slurry difficult to handle, as after saturation the addition of more polymer eventually gives the formation of a gel. This point is very important when scaling the process up. Another issue worth to be discussed regards the amount of anti-solvent required. In literature a volume of 4 times the solvent one is suggested, which means very high amounts of liquids for kg of polymer recovered, which substantially increases production costs.

3.4.2 P3HB recovery: other solvents extraction

Besides the used indexes of extraction-performance (recovery yield and purity), for any new solvent it is important to investigate the time and the temperature to reach the best results. Another issue that can strongly influence the quality of the polymer is the pretreatment performed on the dry lyophilized cells. Chloroform does not need any pretreatment to grant good results, but the efficacy of a solvent can be far improved with a simple and costless operation, making it suitable even if not so good at a first glance. The following technique developed for solvent experimental screening allows optimizing these variables, through repetition in different conditions.

1. An amount of 0.6g of cells is put with 40mL of the investigated solvent, inside a balloon flask (100mL volume) with a magnetic stirrer (Teflon covered).
2. The sealed balloon is immersed in an oil bath with temperature control (figure 3.4). The stirring grants the homogenization of the extracting solution.
3. The suspension is filtered under vacuum (same procedure of chloroform) to remove the debris.
4. The polymer-solvent system is precipitated with the addition of the anti-solvent (4 times the solvent volume) and the suspension is then filtered again on the pre-weighed cellulose filters.

Figure 3.4. *Solubility measurement equipment. The temperature on the hot stirred plate is decided and controlled by the digital immersion thermometer*

This amount of solvent would lead to a concentration of about 9.85g/L of polymer in the solution (the P3HB content in the cells is of 65,68 %). According to the prediction, this value should be under the maximum solubility: at the moment this is not relevant, as the purpose is simply to have a comparison between the investigated solvents and chloroform: if the solvent does not present any problem, the technique can be optimized.
This layout of the experiment permitted to easily change the temperature of the extraction and the duration of the process. Another variable investigated was the type of pretreatments performed on the cells, to enhance the recovery. The results are discussed in the next section.

3.5 Results

3.5.1 Solvent extraction efficacy

The screening of the extraction efficacy gave the results summarized in table 3.4. The data reported do not consider diphenyl-ether as, after some preliminary tests, this possibility was abandoned, even if the solvent was able to dissolve large amounts of polymer. In facts diphenyl- ether has a very high boiling point (259ºC), so the further step to remove it from P3HB is very complex, due to the impracticability of a simple evaporation. As the drying in this case is a very long and tricky operation, especially from an industrial point of view, the attention was focused on the other solvents.

Table 3.4. *Results for the first experimental analysis on the purchased solvents. Extraction performed on 0.6g of cells.*

Solvent	Temperature [ºC]	Time [h]	Cell amount [g]	Recovery yield %	Relative yeld (cloroform 4ºC) %	Relative yeld (cloroform 60ºC) %	Chain Mw [aum]
cyclohexanone	130-120	2	0.6	84,3	104,5	95.6	$5.9\ 10^5$
	130-120	1	0.6	85,7	106,2	97.2	$6.4\ 10^5$
	70-60	2	0.6	0,3	0,3	0.3	na
anisole	130-120	2	0.6	72,1	89,4	81.8	$6.5\ 10^5$
	130-120	1	0.6	85.3	105.7	96.7	$5.2\ 10^5$
	70-60	2	0.6	2,4	3,0	100.3	na
phenetole	130-120	2	0.6	76,8	95,2	87.1	$6.3\ 10^5$
	130-120	1	0.6	83,7	103,7	94.9	$4.8\ 10^5$
	70-60	2	0.6	0,8	1,0	0.9	na

Molecular weight measured by Technalia ([29]) with Resipore columns (Cloroform 25°C- patron poly styrene). Chloroform extracted polymer at 4°C for 36h gives $5.6\ 10^5$ of molecular weight.

The Recovery yield has been calculated considering the P3HB cell content of 65.68%, while the relative yields compare the investigated technique respectively, to the recovery of the standard extraction at 4°C with $CHCl_3$ and to an extraction performed at 60°C with $CHCl_3$ according to the procedure of paragraph 3.4.2.

The extractions performed in a range between 120°C and 130°C gave good results for all the solvents: the lower values of 89,4% and 95,2% for anisole and phenetole (at 120-130°C, 2 hours) are not precise (and in contrast with theoretical expectation: short time methods seem to extract more than the longer treatments). In facts the calculated yields are based on mass measurements of samples that present a significant variability due to the

experimental technique: it is easy to lose part of the sample due to fouling of the funnel (or the flasks). This leads to conservative results, as the real recoverable amount is always a little higher. Anyway, the losses are not predictable and the results can vary a little bit, as the numbers show.
Nonetheless, these results are adequate considering the aim of this first level of analysis. The most important evidence obtained is that 60ºC is not enough to have a proper extraction. The reason is probably an inadequate breakage of the membranes: in fact thermal disruption of the cell occurs at 120ºC, letting the polymer free to be fast dissolved; instead, at 60ºC the membranes are not broken and the solvent is not effective. Chloroform, with its strong polar interaction due to chlorine, manages to break the cells without any external aid, while the other solvents are not strong enough. Therefore a way to weaken the cell membranes was ideated to facilitate the solvent work, avoiding the extraction at high temperature. Among the literature techniques of cell pretreatment, the cheapest and easiest to scale-up were tried, that are alcohol pretreatment and pre-digestion with NaOH solution.
A last consideration on the results of table 3.4 regards the molecular weight analysis. It is shown how these new solvents do not damage the chain length and the quality of the polymer, giving a product comparable with the chloroform extracted one.

3.5.2 Pretreated cells extraction

The first investigated pretreatment was the one with ethanol.
An amount of 3g of lyophilized cells was added with 3ml of ethanol: the system remained 18 hours at room temperature. Then ethanol was evaporated to obtain a dry pretreated powder. The extraction was performed as before, with 0,6g of cells per 40mL of solvent.
The extraction yields so obtained were very low, comparable with the one without treatment, for each of the tested solvent.

A second attempt was performed using pre-digested cells, which means cells that were subjected to a protocol of digestion with NaOH, similar to the one described in chapter 2. The dry lyophilized cells were suspended in the amount of 0.6g in 12mL of NaOH solution 0,2N, directly in the centrifugation tubes. After 5 minutes of stirring the tubes were centrifuged at 12000 rpm for 10 minutes; the supernatant was discarded and the solid residue was used for the solvent extraction. In this case the residue was not dried, as

drying gives a solid pellet very tough and hard to mill. The solvent extraction of the pre-digested embranes gave the results showed in table 3.5.

Table 3.5. *Results for pre-digested cells. Extraction performed on 0.6g samples of cells.*

Solvent	Temperature [ºC]	time [h]	Cell amount [g]	Recovery yield %	Relative yield (cloroform 4ºC) %
Cyclohexanone	60	1	0.6	14,3	17,7
Anisole	60	1	0.6	15,9	19,7
Phenetole	60	1	0.6	0,3	0,3

Apparently the yields did not improve. One reason could be the slow dissolution kinetics: the aqueous digestion solution could induce a fast crystallization which makes the dynamics of dissolution slower. Another reason, more relevant, is the immiscibility of water in the solvents: after centrifugation the cells residue is humid and this amount of water hinders the proper dispersion of the sample, preventing the contact with the solvent. It was observed that the water-wetted cellular material, due to surface tension and glass wettability, scattered over the walls of the balloon, while only a little bit of it was properly dispersed in the solvent. As a consequence, the surface of contact between solvent and cells was strongly limited, reducing the efficacy of the method.

As the idea of finding an easy and affordable pre-treatment did not give the expected results, this way was abandoned, focusing on the optimization of the extraction at higher temperature.

3.5.3 Solvent extraction optimization

The first improvement in the extraction process had the aim of reducing the time of the operation: the benefits involve both productivity, and quality of the polymer, as the faster the process the longer the chain length. The experiment was repeated for cyclohexanone with a contact time of half an hour, and the results were similar to the previous of the same solvent for 1-2 hours (confirming the values, despite the method's error). The time was then further reduced to 15 minutes, without lowering the performances. Values around 85% of absolute recovery are to be considered as the maximum achievable with this lab technique for cyclohexanone.

It was noticed that the solution P3HB-cyclohexanone at 120ºC was still fluid, differently than with chloroform that gets viscous already when using 0,6g of cells. The extraction was repeated, with the same volume of 40mL of solvent, but four times the amount of cells (2,4g). The extraction time was reduced to 30 minutes and then 15 minutes. The final values of the extraction are reported in table 3.6, where it is possible to see that the indexes of extraction performance continue to be very good, better than chloroform extraction. With the higher amount of cells, all the solvents reached saturation, becoming viscous, but the workability for cyclohexanone and anisole did not change so much, and the filtration was normally performed. This was not true for phenetole, which produced a gel that clogged the filter, with consequent reduction of the extraction yields.

Table 3.6. *Results for optimized extraction protocols: reduction of the dissolution time and increment of the amount of cells dissolved. In bold, the results used for process simulation of chapter 4.*

Solvent	Temperature [ºC]	time [h]	Cell amount [g]	Recovery yield %	Relative yield (cloroform 4ºC) %	Relative yield (cloroform 60ºC) %	Chain Mw [aum]
cyclohexanone	130-120	0.5	0.6	84.2	104.4	95.5	$6.1\ 10^5$
	130-120	0.25	0.6	82.9	102.7	94.0	na
	130-120	0.5	2.4	81.8	101.4	92.8	na
	130-120	**0.25**	**2.4**	**86.4**	**107.1**	**98.0**	$6\ 10^5$
anisole	130-120	0.5	0.6	83.8	103.8	95.0	$6.8\ 10^5$
	130-120	0.5	2.4	79.0	97.9	89.6	$6\ 10^5$
	130-120	0.25	2.4	84.3	104.5	95.6	$4.5\ 10^5$
phenetole	130-120	0.5	2.4	27.8	34.4	31.47	$5.6\ 10^5$
	130-120	0.25	2.4	33.7	41.8	38.25	$5.2\ 10^5$

Molecular weight measured by Technalia ([29]) with Resipore columns (Cloroform 25°C- patron poly styrene). Chloroform extracted polymer at 4°C for 36h gives $5.6\ 10^5$ of molecular weight.

Molecular weight analysis shows that these techniques respect the chain length of the polymer, giving results even better than the standard extraction technique with chloroform at 4°C. As a consequence, the extraction performed with anisole or cyclohexanone should be preferred to the chloroform: for the shorter time of the operation, for the higher yields, for the longer chain length and finally because consumption of solvent and antisolvent can be reduced to one quarter (at least).

The results of cyclohexanone, due to its lower price, will be considered as a base indication for the development of a process simulation, with the aim of obtaining an estimation of the overall cost of new chlorine-free extraction technique.

3.6 Conclusions

The solvent selection guide of GSK proved to be absolutely useful, in line to its purpose of giving a fast instrument to select better solutions for environment. Without this precious help, the work would have been much slower and more incomplete.

The selection, based on the predictive method of Jacquel, was obviously less rigorous, due to the errors deriving from statistical approximation an from the application on "not tested" solvents. But also in this case, the experimental evaluation applying the protocols for 49 green solvents of GSK list would have been an impossible task. The predictive equation excluded the worst solvents, with some approximation for sure, but saving time and money.

Eventually, among the number of solvents reported on GSK selection guide, only four have been selected for the experimental analysis of P3HB extraction. The best results were obtained with cyclohexanone and anisole, while phenetole did not show an extraction performance as good as the other two. Diphenyl ether was excluded since presented a low processability, due to its high boiling temperature (of 259ºC).

Cyclohexanone, cheaper than anisole, has been investigated in deep, obtaining extraction yields higher than the ones of the reference method, based on chloroform extraction at 4ºC. The only drawback is that chloroform extraction can be held at room temperature, while the non-halogenated solvents need 120ºC. However this fact does not mean automatically higher process costs, because the lab-scale optimization demonstrated that the time of the extraction can be reduce to 15 minutes and the initial concentration of cells can be quadrupled without any loss of performance. The higher energy costs could be overcome by the lower consumption of solvent and anti-solvent, with great savings in the solvent recovery by distillation. For this reason a process simulation will be designed n chapter 4, so to quantify properly the economic applicability of a chlorine-free process of recovery, based on cyclohexanone.

Chapter 4

Process design and economical evaluation

4.1 Preliminary considerations

In the previous chapters efficiency in extraction and the good environmental performances have been proved for the studied solvents, however, the economical applicability still has to be addressed. The aim of this chapter is to provide a first-design possibility of a large scale polymer-recovery plant, based on cyclohexanone extraction. The conceptual design of the process is followed by a cost evaluation analysis, so to quantify the overall contribution on the final price of P3HB, and also to identify the most expensive steps of the process.

The volume of production has been decided to be 50 000 ton/year of P3HB, with the purpose of comparing the final results with the price of Mirel®, PHB based product of the former Telles, joint venture of Metabolix and ADM. In fact Mirel price was the true commercial price of a large scale produced PHA, differently from other companies that publish only previsions or unconfirmed data.

It has to be said that the final results should be considered as a mere indication of the order of magnitude of the polymer production price. This is due to the still lacking knowledge about the properties of the system P3HB-cells, in particular as far as the unit operations to remove the water from the cells after fermentation are concerned. Therefore, until the following aspects are not investigated, the shape of the process could change sensibly:

- **Moisture acceptable content of dry cells before polymer extraction.** In this work it was experimentally proved that the suspended cells cannot be treated directly with the solvents, as water immiscibility hinders the mass transfer. However a small amount of residual moisture could be accepted, determining the required grade of drying and the overall cost. Furthermore, the consequence of interaction of water with the two solvents has to be checked, as they gives azeotropes with both of them and could possibly change polymer solubility.
- **Thermosensitivity of the polymer contained in the wet cells.** The experiments performed in this work showed a high thermosensitivity of the dry polymer, which

decomposed at low temperatures in the presence of debris. However this behavior could be different with wet cells and should be quantified to choose among different types of dryer.

- **Physical properties of the aqueous slurry at different concentration [rheology, density, abrasiveness, specific heat capacity, …].** These properties affect the sizing, the materials and the cost of all the equipment and should be known to have the most specific and realistic cost estimation.
- **Possibility to flocculate the cell aqueous suspension.** This practice, used in similar processes applied in the fields of microalgae and waste-water remediation, allow to use cheaper unit operation and enhance the process of solids separation.
- **Physical properties of the polymer-solvent solutions at different concentration [rheology, density, abrasiveness, specific heat capacity, …].** These properties are determinant to design properly the clean polymer recovery units.
- **Optimization of P3HB precipitation.** To induce the precipitation of the dissolved P3HB, laboratory protocol recommend an ethanol volume proportion of four times the volume of the solution. This grants the necessary excess of the anti-solvent and a good precipitation, but a too large excess can increase costs of the scaled up industrial application.

The first part of the process (paragraph 4.2), concerning the cells drying, was designed according to the "worst conditions" of the above, meaning that the most expensive equipments for a thermosensitive and delicate material were selected. The second part of the process (paragraph 4.3), concerning solvent extraction, did not suffer any lack of information, so it was simulated properly, with a result applicable in all conditions and determining the largest part of the downstream cost.
As a consequence the final values obtained gave a realistic and conservative estimation of the price of the polymer (paragraph 4.4).

The block diagram of the designed process is reported in Appendix 4.

The economic relations used to estimate the cost of utilities (cooling water, steam, electricity) are summarized in Appendix 3.

4.2 First section: inlet and dewatering

The first section of the P3HB recovery plant (appendix 4, for the whole sight) is shown in figure 4.1.

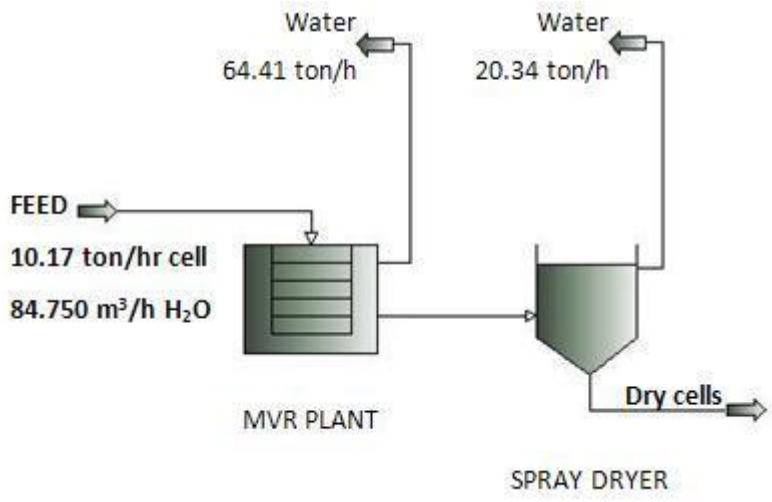


Figure 4.1. *Dewatering and drying section of the P3HB recovery plant.*

The presented unit operations can be considered as separated sub-processes : the *mechanical vapor recompression evaporation plant (MVR)* performs the first dewatering, and the *spray drying unit* removes the residual water and produces a granulate of dry cells. Both these technologies are specific and tailor-made, based on proprietary know-how.
For this reason, many thanks are due to dr. Feliciano Masola, proposal engineer of the company GEA Process Engineering S.p.a, Italian branch of GEA Group AG (figure 4.2).

GEA Process Engineering

Figure4.2. *Logo of Gea Process Engineering, of the German company GEA Group AG.*

GEA Group AG acquired in 1993 the Danish company A/S Niro Atomizer ([28]), one of the first companies who produced spray dryers since 1933. A/S Niro Atomizer changed its name in Gea Niro in 2008, and soon became a leader company in solutions for granulation and exsiccation, making part of the strong group Gea Process Engineering.

Dr. Masola provided equipment technical details of crucial importance, which became the base of the design and quotation of P3HB plant facilities. Information from similar case-studies (when available), from commercial catalogues and from literature were included in the analysis.

4.2.1 MVR/TVR plant.

The feed to this section is the fermentation broth coming from the upstream process, consisting of a slurry with concentration of 120 g/L of cells at 40 °C. These values are in line with the experimental results obtained by the research group of prof. Fonseca, presented in Istanbul conference acta (Cesário *et al.* 2012). In terms of total solid percentage, the concentration is 12% TS (which corresponds to 10.71% w/w tot), too low to proceed water evaporation. The thermosensitivity of the polymer excludes the simplest solutions, such as tunnel and drum dryers. Another aspect that excludes other low temperature dryers is the big inlet flowrate.
In these conditions, dewatering should be achieved through an evaporator with mechanical vapour recompression technology, which grants high volumes and safeguards the polymer. This solution, widely applied in food industry (dairy, beverages,…), is far more effective and economical than multi-effect evaporators or thermal recompression plants ([13]), so it was chosen for this simulation. The main element of a MVR plant is one or more falling film evaporation towers, where the water contained by the inlet slurry is evaporated at low temperature. Figure 4.3 shows an example of the layout of an evaporation plant.

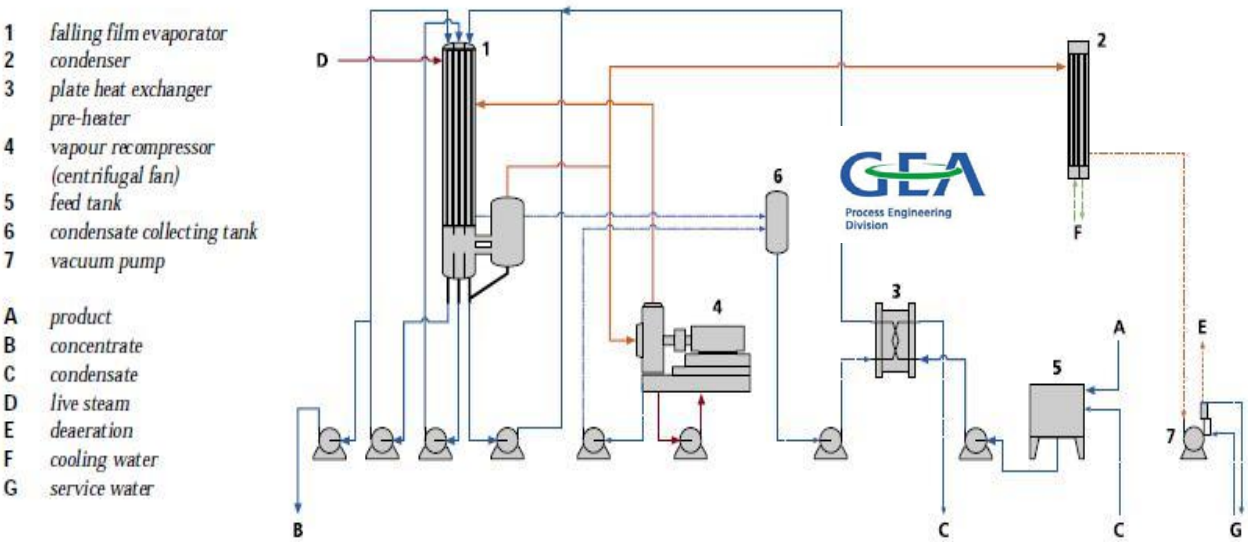


Figure 4.3. *Process flow diagram of a Gea evaporation plant with Mechanical Vapour Recompression. General layout of a single stage evaporator.*

The main characteristic of MVR technology is that the steam used to evaporate water is not condensed (with high energy losses), on the contrary, its energy content is recovered recompressing it with a mechanically driven compressor. The advantages of this solution are:

- Low specific energy consumption
- Gentle evaporation of the product (small temperature gradient)
- Short residence time in single effect evaporators
- Low specific operating cost

Typical ranges of concentrations for diary plants are from 7-12%TS up to 48%TS ([14]), similar to the performances required for *B. sacchari* drying; however a higher concentration in a single step is also possible.

For example, GEA Niro evaporators allow reaching an outlet concentration higher than 60%TS, according to the properties of the concentrate that should not become too viscous. In the specific, GEA Niro evaporator *1 MVR 1 WPC 80*, whose performance specifications are summarized in table 4.1, was used as a base of calculation.

Table 4.1. *Technical data of 1MVR WPC80 with TVR finisher and flash cooler*

Feed	46.860 kg/hr
Heating temperature	72°C
Concentration rate	18%TS to 62%TS
Evaporation rate	33 260 kg/hr
Electricity for compressor	344 kWh/h
Electricity for pumps	61 kWh
Steam consumption	2350 kg/hr
Relative steam consumption TVR (kg steam for kg of evaporate)	0.080
Total purchase cost	3 085 000 €

An outlet concentration of 50% TS was considered enough for this step, so the material balance could be written for water.

Water content in the outlet is given by

$$m_w^{out} = \frac{m_{solid}}{0.50} = \frac{10.17}{0.50} = 20.34 \qquad [ton/hr] \tag{4.1}$$

The evaporation rate required is given by

$$m_{evap} = m_w^{in} - m_w^{out} = 84.75 - 20.34 = 64.41 \qquad [ton/hr] \tag{4.2}$$

For the cost estimation of the equipment the rule of the 6/10 was used (Peters *et al.* 2004), as the evaporation capacity of 1 MVR 1 WPC 80 is lower than the required one. This procedure of rescaling introduces an approximation, however the high quality of the initial data gives better reliability (i.e. real updated price of an industrial equipment, directly from the producer).
The correlation among the purchase cost (PC) and the different capacities is expressed by equation 4.3.

$$\frac{PC_X}{PC_{WPC80}} = \left(\frac{m_{evapX}}{m_{evapWPC80}} \right)^{0.6} \tag{4.3}$$

Therefore, the calculated purchase-cost for *B. sacchari* evaporator was 4 586 419 €, which corresponds to 6 126 539 $ (18th September 2013 conversion rate). The calculation of the grass root module cost, needed to estimate the capital investment, was performed according to the indexes of Peters *et al* (2004): the detailed procedure for the calculation of other direct and indirect expenses is reported in appendix 3. The estimated capital investment is 4.4275 times the purchase-cost, that is 27 125 251$.

The annual operating cost of *B. sacchari* evaporation section was obtained simply rescaling GEA Niro equipment duties to the different evaporation rate: this is acceptable because the steam and the electricity required by the process are directly proportional to the energy required for evaporation.

Yearly electric power consumption is given by:

$$E_X = E_{WPC80} \frac{m_{evapX}}{m_{evapWPC80}} = 784.3 \ [kWh/h] = 6870468 \quad [kWh/year] \tag{4.4}$$

Where E is the power consumption in kWh/h and the number of working hour per year are 8760. With a cost of electricity estimated of 0.05$/kWh (appendix 3), the yearly cost of pumps and fan was 343 523 $/year.
Considering the WPC80 ratio of steam usage (0.08kg/kg) still valid in the larger evaporator, the steam consumption could be so calculated.

$$E_X = m_{evapX} \cdot 0.08 = 5152.8 \ [kg/h] = 45138528 \quad [kg/year] \tag{4.5}$$

Where *S* is the steam consumption in kg/h. With a estimated cost of steam of 0.01232$/kg (appendix 3), the yearly cost of steam for evaporation was 556 106$/year.
Table 4.2 summarizes all the costs for the evaporation plant.

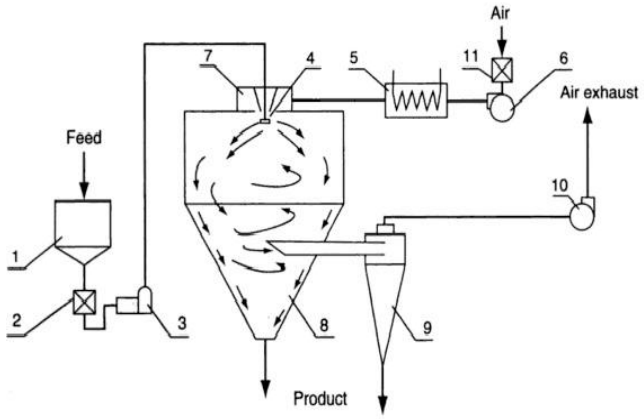
Table 4.2. *Cost summary for the evaporation section.*

Fixed capital investment	**27 125 251 $**
Yearly electricity cost	343 523 $/year
Yearly steam cost	556 106 $/year
Total utility cost	**899 629 $/year**

As possible alternatives to this step, the first grade of concentration could be reached through centrifugation, for example through MBUX equipment of Alfa Laval ([15]), followed then by a semi-batch step of filter press. However, these alternatives will be explorable only once the missing data for the process are known.

4.2.2 Drying

Spray drying has some advantages over other simpler drying processes, such as tunnel drying or drum filtering. First, it allows to obtain a dry granulate easy to convey (small spherical grains), moreover the grains offer a large interface, which enhances the dissolution. In this way a grinding step is avoided, which is energy demanding (Barbosa-Cánovas *et al.* 2006) and could damage the polymer; furthermore the spray drying is suitable for thermosensitive materials.
Dimensioning and then quoting this device is particularly complex at the stage of conceptual design: the construction variables are so many that a pilot-scale plant is often required to determine the proper working conditions. Another issue in spray dryers design is that many different layouts are available, adding so many degrees of freedom that only specialists can propose the most effective solutions.
The easiest configuration is the one displayed in figure 4.4 (Mujumdar 2006).



1. Feed tank
2. Filter
3. Pump
4.Wheel atomizer
5. Heater
6. Fan
7. Air distribution
8. Drying chamber
9.Cyclone
10. Fan

Figure 4.4. *Process flow diagram of a standard spray dryer for viscous slurries (Mujumdar 2006)*

The feed is pumped through the atomizer (in this case a wheel atomizer, for viscous slurries) and the so formed small droplets fast dry in contact with co-current hot air inside the drying chamber. Most of the solid particles accumulate at the bottom of the funnel-shaped chamber, the rest is recovered through a series of cyclones that remove solids from the exhaust air, leaving the process. Countercurrent air chambers, mixed current, spray nozzles atomizer, fluid beds, multi-step processes, are only some of the possible alternatives available in the market (GEA Niro catalogue for spray dryers 2013, [16]). The precious suggestion of dr Masola of GEA Niro not only gave the right input to select the type of dryer, but also provided the basic technical data to proceed with estimation, like for the evaporator. Dr. Masola proposed a solution specifically addressed to cell drying, a spray drier plant (CDI 800), followed by a post treatment in fluidized bed (VIBRO FLUIDIZER ®). The technical data of interest for this simulation are listed in table 4.3.

Table 4.3. *Technical data of CDI 800 spray dryer plant with Vibro fluidizer® bed.*

Feed	7 566 kg/hr
Concentration rate	50%TS to 97%TS
Evaporation rate	3 701 kg/hr
Heating power requirement	6.953MW
Steam consumption	500 kg/hr
Total purchase cost	4 500 000 €

For P3HB plant the lowest possible moisture content is required. For simplicity of calculation the outlet moisture concentration was assumed equal to 0%.
The evaporation rate is

$$m_{evap} = m_w^{in} - m_w^{out} = 20.34 - 0.0 = 20.34 \qquad [ton/hr] \tag{4.6}$$

Also in this case the GEA Niro equipment capacity is lower than the required, so the purchase cost for *B. sacchari* drying plant was approximated again through 6/10 rule.

$$\frac{PC_X}{PC_{CDI800}} = \left(\frac{m_{evapX}}{m_{evapCDI800}} \right)^{0.6} \tag{4.7}$$

The final purchase cost so calculated was of 12 509 260 €, corresponding to 16 709 759 $ (18/09/13 change rate). The estimated grass root capital investment was 4.4275 times the purchase cost, that is 73 982 457$. The complexity of the equipment is the reason of this huge capital cost.
The duty requirements were calculated as proportional to the evaporation rate: as expressed below.
For the yearly consumption of steam:

$$S_X = S_{CDI800} \frac{m_{evapX}}{m_{evapCDI800}} = 2747.90[kg/h] = 24071656 \qquad [kg/year] \tag{4.8}$$

For the yearly consumption of fuel (air heating):

$$G_X = G_{CDI800} \frac{m_{evapX}}{m_{evapCDI800}} = 38.21 \qquad [MW] \tag{4.9}$$

Where *S* is the steam consumption in kg/h and *G* is fuel power required in MW.
With a steam cost of 0.01232$/kg, the yearly steam cost becomes 296 562 $/year. Considering a fuel cost of 3.788 $/GJ, the yearly heating cost becomes 4 564 504 $/year.
Table 4.4 summarizes the costs of the spray drier unit.

Table 4.4. *Cost summary for the spray drying section*

Fixed capital investment	**73 982 457 $**
Yearly heating fuel cost	4 564 504 $/year
Yearly steam cost	296 562 $/year
Total utility cost	**4 861 066 $/year**

4.2.3 Possible alternatives

The performances of the evaporator, in terms of capacity and gentleness of the operation, are difficult to be reached by other technologies, but the investment for the spray dryer should be reconsidered. The very high equipment cost makes interesting also other solutions that were rejected because of their lower capacity. In particular, the semi batch filter presses allow reaching output concentrations higher than 70%TS, in one case up to 90%TS (Siemens J-Vap ®- [17]) in a single step. Then a short grinding step of the dry residue (porous and frail) and a shorter air drying could lead to a dry granulate similar to the one obtained with spray dryers, but with consistent savings in the equipment investment and the heating expense. Figure 4.5 shows the layout of a standard filter press.

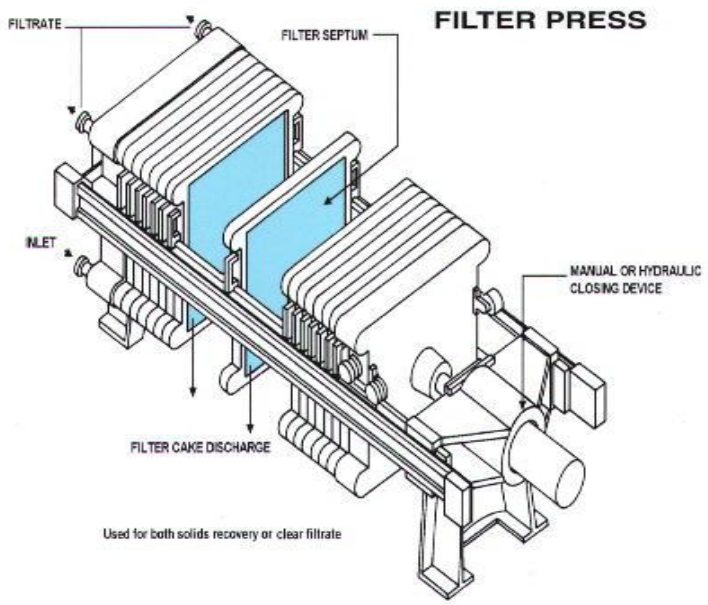


Figure 4.5. *Filter press layout. The slurry is pumped in the filter septum between the frames of the plates. The hydraulic closing device provides the pressure that squeezes the liquid, obtaining a dry cake with concentration of the order of 70% TS. ([18])*

The order of magnitude of the cost for a filter press plant to process 30 ton/hr of slurry with 50%TS is about 2 411 000$, by personal communication (Eastwood 2013) of CDE filter press producing company ([19]). This cost is so reduced with respect to the spray dryer, that even if the expenses for electricity are higher, this alternative may become attractive.
However for pursuing filter presses alternative, as for the other possible solution, it is required to investigate the points presented in paragraph 4.1.

4.3 Second section: cells dissolution and polymer recovery

The second part of the process performs the extraction of the P3HB contained in *B. sacchari* cells and it is illustrated in picture 4.6.

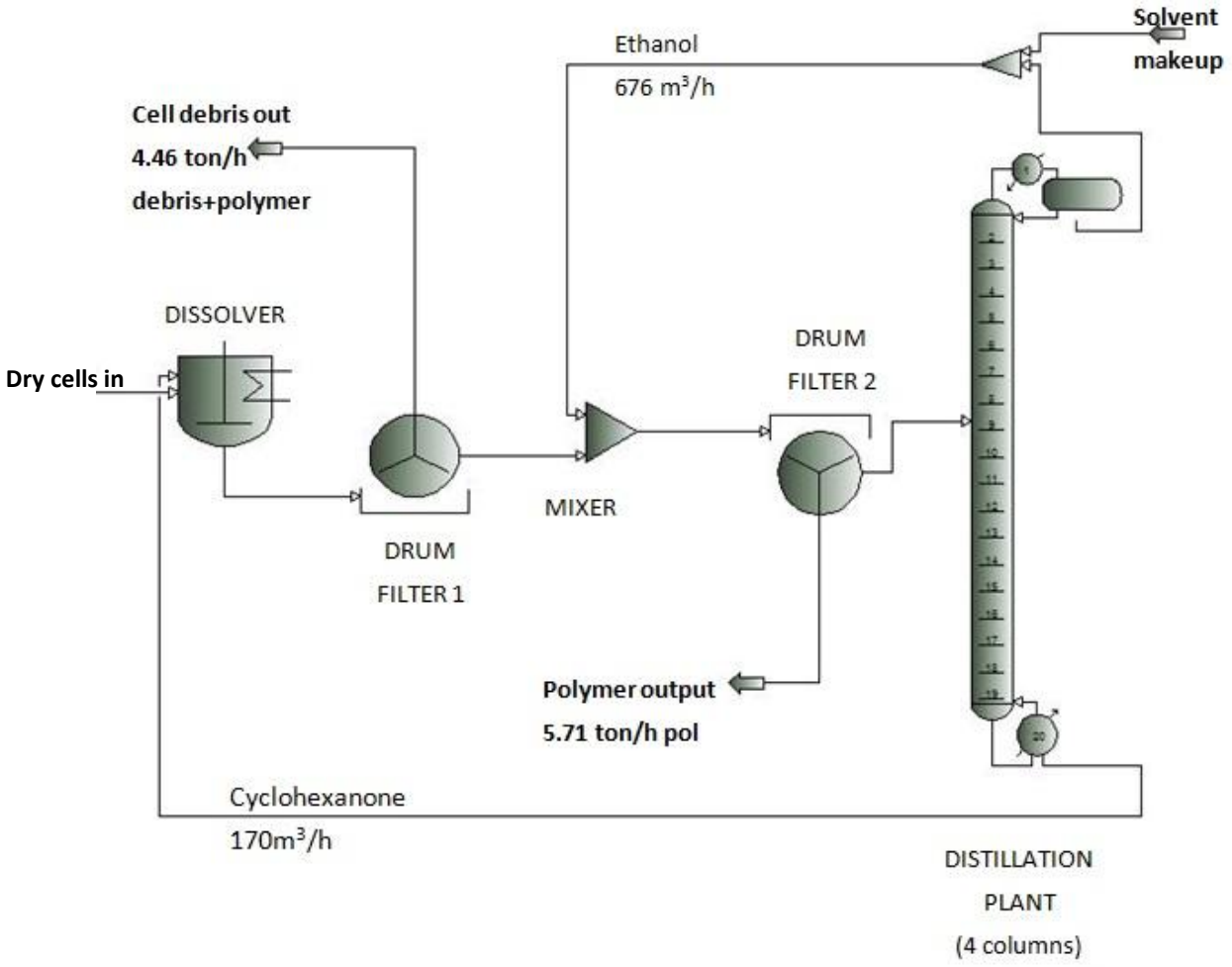


Figure 4.6. *Second section of the plant, where P3HB is extracted from the dry cells with cyclohexanone and ethanol. The solvents are then recovered through distillation.*

For this section of the plant it was possible to perform the material balances separately for the solid and the liquid phase. Solids occur only in the dissolver, where cells are mixed with hot cyclohexanone; in the first filter, where the cell debris is removed; in the precipitator; in the second filter that removes the precipitated polymer. These devices where quoted as explained before, with data both from the literature and from commercial information (paragraph 4.3.2). However, as the "solid phase" is completely removed after the second filtration and its amount is very low if compared to the flowrates of the solvents, it was possible to design the whole closed circuit of use and recovery of the solvents ignoring the presence of the solid phase. The simulation of the solvent recovery system was performed with the process simulator PRO/II (© Invensys Systems inc1994-2011 version 9.1.1). Material and energy balances were applied to have the most realistic estimation of energy and solvent consumptions (paragraph 4.3.1).

4.3.1 Solvent recovery and recycle

The solution Thermodynamics

Due to obvious environmental and economic reasons, solvents have to be recycled in a closed circuit, reducing to the minimum their consumption: the key operation of this cycle is the distillation, which allows to separate the mixed solvents. The binary mixture of cyclohexanone and ethanol, whose properties are reported in table 4.5, does not present separation issues.

Table 4.5. *Summary of cyclohexanone and ethanol properties.*

		Cyclohexanone	Ethanol
Normal Boiling Point	°C	155.6	78.32
Density	Kg/m3	951.83	793.83
Mol Weight		98.14	46.07

In fact figure 4.7 shows a binary diagram without azeotropes and large volatility of ethanol with respect to cyclohexanone: very good conditions to perform classical distillation.

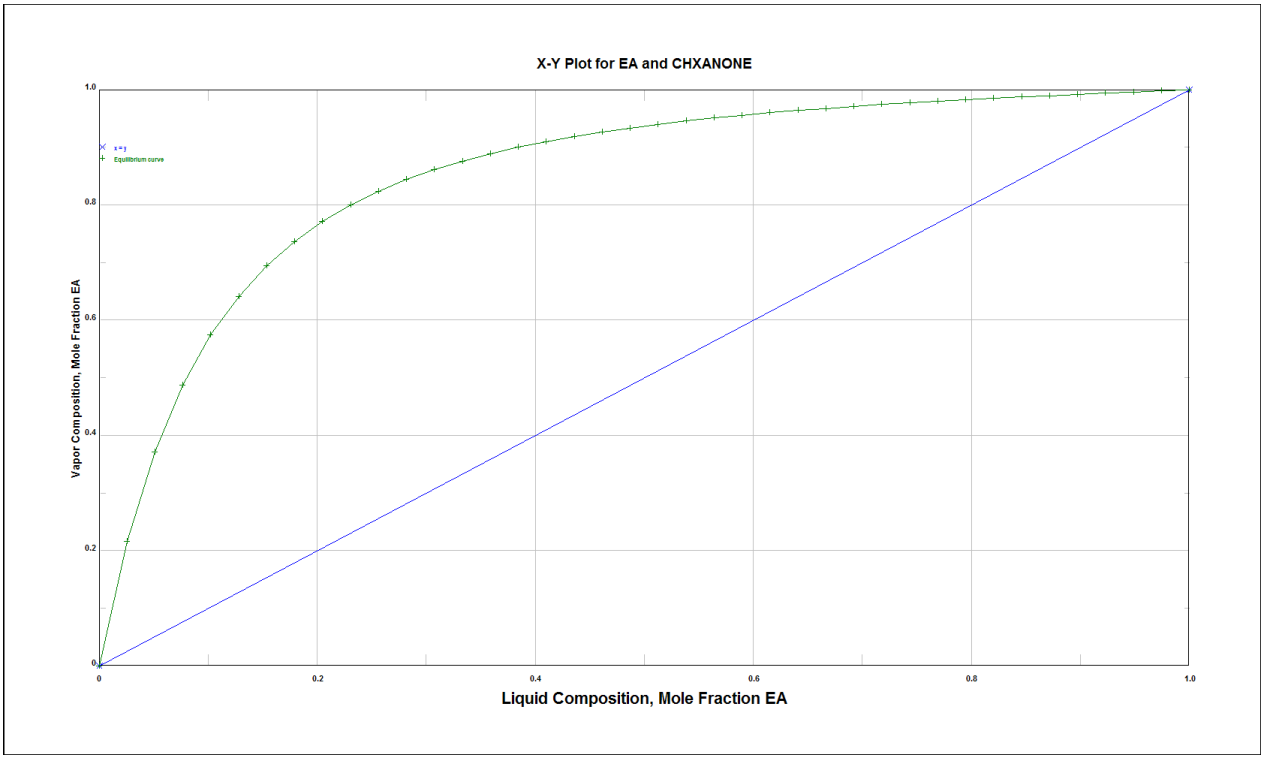


Figure 4.7. *Liquid-vapour composition diagram for the binary solution cyclohexanone- ethanol.*

These data were generated by thermodynamic model SRK 01, on which the simulation of the whole process is based.

Process flow diagram and stream table

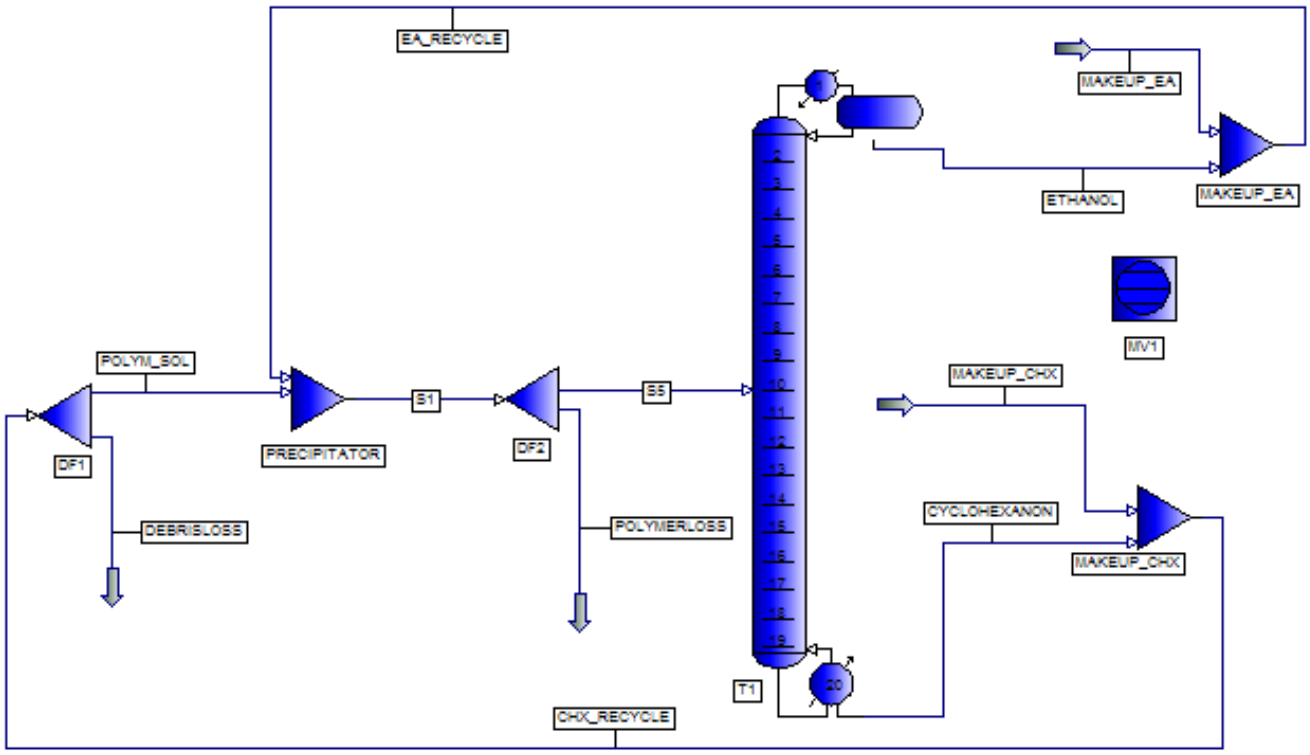


Figure 4.8. *Process flow diagram for the solvent recovery facility.*

Table 4.6. *Stream table for the Process flow diagram of the solvent recovery section, presented in figure 4.8.*

Stream Name		POLYM_ SOL	CHX_ RECYCLE	EA_ RECYCLE	DEBRIS LOSS	POLYMER LOSS	MAKEUP_ CHX	MAKEUP_ EA	S1
Temperature	°C	152,98	152,98	78,45	152,98	81,66	21	21	81,66
Total Std. Liq. Rate	*m3/h*	170,18	170,70	685,83	0,52	0,77	0,71	2,12	856,00
Total Mass Rate	*Kg/h*	161827,76	162323,15	545848,26	495,39	634,56	675,07	1685,62	707676,02
Total Molar Rate	*Kgmol/h*	1657,61	1662,68	11750,07	5,07	12,02	6,88	36,59	13407,68
Total Molar Comp. Rates									
EA	*Kgmol/h*	16,51	16,56	11663,23	0,05	10,47	0,00	36,59	11679,74
CHXANONE	*Kgmol/h*	1641,10	1646,12	86,84	5,02	1,55	6,88	0,00	1727,94
Total Molar Comp. Fractions									
EA		0,01	0,01	0,99	0,01	0,87	0,00	1,00	0,87
CHXANONE		0,99	0,99	0,01	0,99	0,13	1,00	0,00	0,13

The process flow diagram of figure 4.8 shows only one distillation column for intelligibility sake: the real simulation was performed with 4 distillation column in parallel, because a single column was not structurally possible (i.e. too large plate diameter required).

Performance specifications and degrees of freedom for convergence

Differently from the diagram of the entire process (Appendix 4), the outlets of the solvent cycle simulation are the losses of the solvents which remain inside the polymer cake after separation. The inlets consist in the solvent makeup streams. PRO/II simulation represents these losses with the split unit operation: the outlet amount is a fixed quantity, related to the separation performances of the drum filter and to the polymer throughput (5.71 ton/h). The moisture content assumed inside the solid after separation was 10% (weight fraction). The analytical relations that link the solvent loss to the stream of clear cyclohexanone are:

$$Debris^{out}\ [kg/h] = P_{cell}(1-0.65) + P_{cell}\,0.65(1-0.864) \tag{4.10}$$

$$lossDF1 = \frac{0.4384}{9} P_{cell} = 495.389\ [kg/h] \tag{4.11}$$

$$Polymer^{out}\ [kg/h] = P_{cell}\,0.65 \cdot 0.864 \tag{4.12}$$

$$lossDF2 = \frac{0.5616}{9} P_{cell} = 634.55\ [kg/h] \tag{4.13}$$

Where P_{cell} is the cell mass flowrate in kg/hr (P_{cell}=101700kg/hr), 0.864 is the polymer extraction yield, 0.65 is the polymer content in the cells.

The first performance specification for the convergence of the algorithm is that the stream CHX_RECYCLE has to be of 1643.77 kmol/hr, as required to have the right cell concentration. The second process specification is that the amount of ethanol of the stream S4 (volumetric flowrate) has to be four times the volumetric flowrate of the solution coming from the first drum filter (S2). With these two specification, two degrees of freedom to reach the convergence are needed: the flowrates of the two makeup streams, composed by pure solvent were selected as such.

It has to be noted that 10% of solvents inside the recovered solid cakes after filtration is too low according to standard filters performances, and it is too high considering the

resulting daily solvent losses. However it was impossible to quantify properly the residue of the cake at this stage of design, so these data are to be considered as a first approximation. With 10% or even higher percentages of solvent residues, P3HB could not be commercialized, so further evaporation /stripping operations should be considered, with the recycle of the recovered solvents. The sake of this simulation, however, was to estimate the largest cost contributions, so these points remained in the background. The most expensive equipment, in fact, was the one involved with the distillation of ethanol-cyclohexanone mixture.

Column design results

The distillation column is provided with total condenser and reboiler.
As the simulator gives only the overall energy consumption of the whole facility, the necessary properties for cost analysis of the single devices had to be calculated separately.
The condenser exchange surface is given by

$$A_{cond}\ [m^2] = \frac{Q_c}{U_{cond} \cdot \Delta T_A} \tag{4.14}$$

where Q_c is the cooling energy , ΔT_A is the average temperature difference between the two sides of the exchanger, U_{cond} is the overall energy transfer coefficient, assumed of 600 W/m^2/°C (Sinnot 1993) .
The water consumption of the condenser is

$$W_{cond}\ [kg/s] = \frac{Q_c}{c_{pw} \cdot \Delta T_w} \tag{4.15}$$

where c_{pw} is the specific heat of water (4186 kJ/kg/°C) and ΔT_w is the temperature difference between the outlet and the inlet water (in this case 45-35=10 °C).

The reboiler exchange surface is given by

$$A_{reb}\ [m^2] = \frac{Q_h}{U_{reb} \cdot \Delta T_A} \tag{4.16}$$

where Q_h is the heating energy , ΔT_A is the average temperature difference between the two sides of the exchanger (assumed to be 10°C), U_{cond} is the overall energy transfer coefficient, assumed of 4 kW/m^2/°C (Sinnot 1993)

The steam consumption of the reboiler is

$$S_{reb}\ [kg/s] = \frac{Q_h}{\lambda_s} \tag{4.17}$$

Where λs is the latent heat of vaporization of the steam used for the process. As the temperature to reach in the reboiler is at least 163°C, to provide a minimum thermal gradient of 10°C, it was decided to use superheated steam at 7 bar, whose properties are reported in table 4.7.

Table 4.7. *Thermodynamic properties of superheated steam at 7bar.*

Boiling point	Density	Liquid water enthalpy	Steam enthalpy	Latent heat of vaporization
°C	kg/m^3	kJ/kg	kJ/kg	kJ/kg
165	3.667	697.07	2761.98	2064.92

It is noteworthy that the values obtained for both the exchangers are conservative, as based on a conservative estimation of the exchange area (Sinnot 1993).

The results of the calculations and other useful data are summarized in table 4.8, together with all the properties valid for each of the four column of the process.

Table 4.8. *Properties of the distillation column. The plant is designed with 4 identical column in parallel.*

General Equipment data		
Nr theoretical trays		20
Feed tray		10
Maximum tray diameter	mm	4700
Reflux ratio	Refl/dist	0.03
Flooding percentage		80%
Reboiler		
Reboyler duty	kW	33817
Temperature	°C	153.46
Outlet Flowrate	Kmol/hr	413.43
Molar composition		99% cyclohexanone
Steam consumption	Kg/s	16.37
Area exchanger [1]	Ft^2	9095
Condenser		
Condenser duty	kW	-33803
Temperature	°C	78.65
Outlet flowrate	Kmol/hr	2933.62
Molar composition		99.99%ethanol
Cooling water consumption	kg/s	807.53
Area exchanger[1]	Ft^2	15693

1. Area in ft^2 required for cost evaluation relations

Cost analysis

The solvent recovery is without any doubt the most energy demanding unit operation, hence the most expensive for the entire process. The equipment costs were calculated according to Guthrie module costing correlations (Douglas 1988).
The grass root cost correlations of Guthrie for the column shell, the reboiler and the condenser are

$$C_{shell}\ [\$] = \left(\frac{M\ \&\ S}{280}\right) \cdot 101.9 \cdot D^{1.066}\left[(n_t - 1)h_t + 15\right]^{0.802}(3.4275 + 1) \tag{4.18}$$

$$C_{reb}\ [\$] = \left(\frac{M\ \&\ S}{280}\right) \cdot 101.3 \cdot A^{0.65}(3.4275 + 1) \tag{4.19}$$

$$C_{cond}\ [\$] = \left(\frac{M\ \&\ S}{280}\right) \cdot 101.3 \cdot A^{0.65}(3.4275 + 1) \tag{4.20}$$

where both the exchangers are of floating head type; *M&S* is Marshall and Swift inflation index (1536.5 for 2011, the most recent available for free); *D* is the maximum tray diameter in ft; n_t is the number of trays (18 trays); h_t is the distance between trays (2 ft); *A* is the area of the exchanger, in ft^2; all the equipment are made by carbon steel. The cost so calculated corresponds to so called "grass-root module", which considers the whole installation cost (including piping and instrumentation) and the indirect expenses.
The results for the cost estimation of the solvent recovery section are listed in table 4.9.

Table 4.9. *Cost summary for the solvent recovery section*

Annual shell cost x1	1 036 857 $
Annual condenser cost x1	1 313 154 $
Annual reboiler cost x1	921 168 $
Fixed capital investment x4	**13 090 236 $**
Yearly cooling water cost	1 313 050$/year
Yearly steam cost	6 362 670$/year
Total utility cost x1	7 675 720$/year
Total utility cost x4	**30 702 880$/year**

Solvent hold-up

The solvent hold-up is a useful datum to quantify the first solvent investment to run the entire plant. This quantity can be calculated only at advanced stages of design: it is impossible to know the volumes of the facilities with a conceptual estimation. However a simple account can help to have an order of magnitude of the solvents volume in circle. It can be assumed that the bottom hold-up for each column is 1m high, giving 17.35m^3 . The total volume of liquid cyclohexanone (with very low amounts of ethanol) stored in the column is 69.4 m^3 . Considering also the hold-up of the 43 m^3 tank of the dissolver (as better explained in paragraph 4.3.2), the required volume of cyclohexanone is 112.4 m^3, corresponding to 107ton. Considering the cost of cyclohexanone, provided by the on-line resource guidechem.com ([20]), of 2215 $/ton the total cost is 237 000$. The amount of solvent inside the piping, the thickener and all the other devices has not considered yet in this calculation, but even if it is as much (225 m^3, rough approximation), the total cost for the purchase of cyclohexanone would not be higher than 480 000$. About ethanol, the same considerations are still valid: the volumetric rates are 4 times the cyclohexanone ones, but there are no static hold-ups, so the volumes of ethanol could be possibly lower. Price of ethanol is lower ([21]), 607.6 $/m3, so the final solvent investment, assuming for simplicity the same requirement of cyclohexanone, would be about 150 000$.
This first estimation gave hence 630 000$ for the solvents holdup, probably in high excess, which is only the 0.5% compared to the overall investment (paragraph 4.3). It is reasonable to ignore this contribution.

4.3.2 Debris and polymer removal

The block diagram of figure 4.9 shows the material balances on the solid phase entering and leaving the second part of the process.

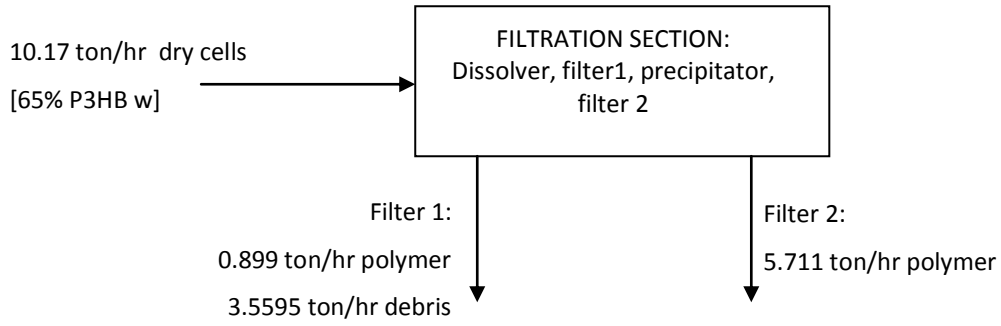


Figure 4.9. *Block flow diagram with overall material balance of the solid phase through the second section of the plant.*

The dry cells, coming from the spray dryer, are dissolved in cyclohexanone (concentration of 60g/L) in the first stirred tank "ST1" (Appendix 4). Heating is not required, but a standard insulation could ensure the reduction of energy loss and avoid the increase of solution viscosity. To give a contact time of at least 15 minutes, the time determined at lab scale, the tank should have a volume of 43m^3 (volumetric flowrate 172.24 m^3/h). in this case it could be necessary to provide the same volume in more smaller tanks in parallel, due to mixing issues. However, as the mixing tank can be considered as a minor equipment, its cost was not considered in the conceptual design cost estimation.

The first filtration is performed right after the dissolution and was assumed to separate the 100% of the cell debris and 13,6% of the polymer (on the base of an overall extraction yield of 86,4%). The hypothesis of complete removal of the solid phase by the filter should be checked when selecting the piece of equipment.

The clear solution of P3HB and cyclohexanone, coming from the first filtration, is then mixed to the stream of ethanol in the precipitator. Due to the antisolvent effect, solid polymer flocks are formed, suspended in the liquid phase. Also in this case the equipment can be a simple stirred tank, so its minor economic contribution was neglected.

The second filter, DF2, has the task of recovering the solid polymer: the percentage of recovery was assumed 100% also in this case.

The appropriate type of equipment filtration depends strongly on information that unfortunately are not available yet, so a kind of equipment was selected that allows

performing a filtration comparable to the steps at the lab scale. Among the wide offer of industrial filters, the rotary vacuum drum filter (with precoating, bottom fed) seems to be the most appropriate one: a sketch of this type of filter is reported in figure 4.10.

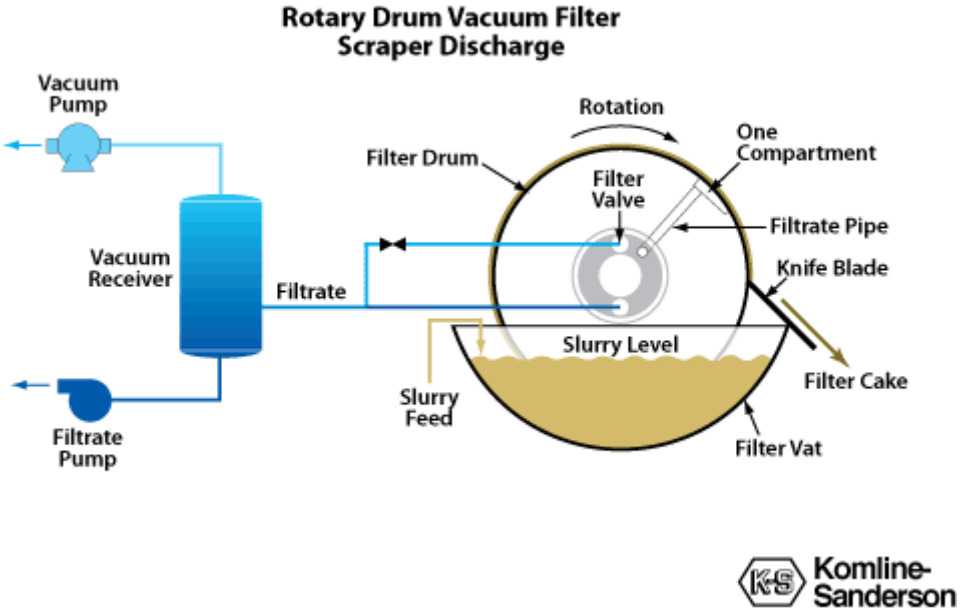


Figure 4.10. *Layout of a vacuum rotary drum filter of Komline- Sanderson ([30]), this structure is the standard for the most of the producers.*

The slurry is pumped to the tank at the bottom, which is equipped with a slow stirring to avoid the suspension sedimentation. The cylinder is partially immersed in the tank and a negative pressure is applied, which lifts the liquid carrying the particles suspended. These particles remain on the porous surface of the cylinder, forming the cake, which enhances the filtration performances. To reduce the minimum filterable size of the particles down to 1µm the cylinder can be pre-coated with diatomaceous earth, but in the case of cell debris and P3HB flocks a simple monofilament cloth on the drum surface could be enough. The cylinder rotates slowly, offering new clear surface to the liquid and continuing the process of cake formation: rotational speed is a function of the required cake thickness, of the suction energy and the flowrate to process. Outside the tank the cake is dried and cut away from the drum by a system of automated knives.

According to the results of the solvent recovery simulation, the flowrates for the two steps of filtering are too big to be treated directly by the filter: the stream containing the debris resulted to be 170m^3/h (5.89% w suspension), while the stream containing the flocculated polymer was of 854m3/h (0.8% w suspension). A pre-thickening step should be introduced upstream. Thickening can be achieved with a simple static-thickener, i.e. an inclined plate

settlers or lamella separator (Sutherland 2005), whose cost can be neglected due to the simpleness of the device. It was assumed that the flowrates coming out from the pre-thickener to process in the filters are of 80m^3/h, a reasonable amount according to the settler average performances.

Unfortunately, with the available information about the slurry properties, the dimensioning of the vacuum filter was impossible: to obtain an order-of-magnitude cost estimation, some other assumptions have to be made. Filtration area should be calculated knowing the flux velocity through the filter, which is a function of the properties of the cake of filtration. These properties can be determined through specific lab scale experimental campaigns (Perry and Green 2008; Tarleton and Wakeman 2011), but at the present they still have to be made. Therefore, standard data were used (particulate of diameter of 5μm, cake porosity 0.5, vacuum 0.5 bar, cake thickness 2mm) which gave a velocity of 0.00125 m/s, corresponding to 4.5m/h. Considering the 80m^3/h the required filtration area is 18m^2.

The purchase cost for a 20m^2 drum, estimated with literature correlations (Peters and Timmerhaus 1991; Peters *et al.* 2004), is of the order of 150 000$, which gives a grass roots cost of 664 125$. The equipment consumes around 4 kW (average consumption for 20m^2 drum filters from different producer catalogues, e.g. MecKey engineering [22]) for rotation of the cylinder and agitation of the bottom tank. The vacuum can be provided with a liquid ring vacuum pump of 4 kW (Vectra SX of Nash pumps, 0.5bar, 150m^3/h [23]). Hence the total power is 8kW, which gives 70 080 kWh/year consumption , which contributing to the cost with only 3504$/year (0.05 $/kWh). The costs are summarized in table 4.10.

Table 4.10. *Cost summary for the filtration section.*

Fixed capital investment (2units)	**1 328 000 $**
Total Utility cost	**7000 $/year**

It is clear that even if the assumptions lead to an underestimation of the costs, the filtration contribution does not affect much the overall result. Anyway further analysis should be performed to estimate properly the best alternatives and the costs of this plant section.

Other possible solutions for filtration are horizontal vacuum belt filters, which allow higher flowrates and even lower costs, or disc filters or centrifuges.

4.4 Conclusions

The process design and simulation of the new cyclohexanone extraction technique allowed to estimate the grass root capital investment for the plant and the annual operating cost. In spite of the strong approximations that the conceptual design procedure introduces, the results are interesting and can give useful indications for the future developments of P3HB technology.
Table 4.11 summarizes the costs evaluated for the different sections of the process.

Table 4.11. *Summary of the cost contributions of the different sections of P3HB recovery plant based on cyclohexanone dissolution.*

	Fixed capital investment	Annual utility cost
Evaporation	27 125 251 $	899 629 $/year
Spray drying	73 982 457 $	4 861 066 $/year
Filtering	1 328 000 $	7000 $/year
Solvent recovery	13 090 236 $	30 702 880 $/year
TOTAL	**115 525 894 $**	**36 469 761 $/kg**

A first remark regards the relative contributions of each section to the overall operating cost (utility cost): figure 4.11 shows how that solvent recovery is by far the major cost. Hence, the first intervention for price reduction should be the optimization of the solvent distillation.

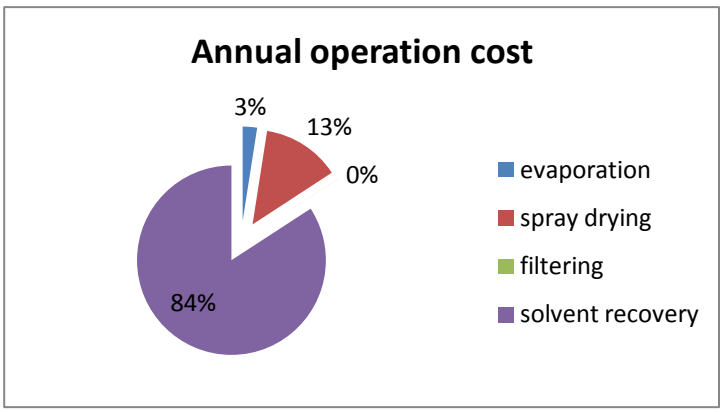


Figure 4.11. *Pie diagram comparing the single sections contributions to the overall operating costs of the plant (utilities).*

To estimate the total production cost, or better, the contribution of downstream process to the P3HB cost, it is possible to use a fast simplified approach proposed by Douglas(1988). Accordingly, the total product cost is divided into two main contributions: *general expenses* (SARE costs: sale, administration, research, engineering) and *production cost*, which considers direct production costs, fixed charges and plant overheads (i.e. Capital investment and operating costs). Using cost allocation indexes derived by industrial experience, Douglas obtained the following relation:

$$TPC\ [\$/year] = \frac{1.03VPC + 0.186FC}{0.65} \tag{4.21}$$

Where *VPC* are the variable production costs [$/year] and *FC* is the fixed capital investment [$]. Hence, the result of the equation gives 90 848 723 $/year of total production cost, which can be scaled with the assumed productivity of 50 Mton/year, giving a downstream cost of 1,817 $/kg of polymer P3HB. According to the literature (Jacquel *et al.* 2007) between the 60 and the 80% of the commercial price of P3HB is due to the extraction expenses: assuming 70% as an average, the total product cost (considering also the upstream) of the so extracted P3HB could be around 1.817/0.7= 2.596 $/kg. Table 4.12 summarizes the cost contributions calculated.

Table 4.12. *Summary of the cost contributions of the downstream process*

Total Fixed Capital Investment (FC)	**115 525 894 $**
Total Operating cost (VPC)	**36 469 761 $/year**
Calculated Production Cost (TPC)	**90 848 723 $/year**
Polymer downstream cost	**1.817 $/kg P3HB**

The calculated production cost for the polymer has to be compared to the reference-plant of this analysis, i.e. Telles facility of Clinton, Iowa (US). The commercial price of their product Mirel® (PHB plastic) is 2.50$/lb ([2]), corresponding to 5.51$/kg. Commercial price however includes taxation and complex financial evaluations that cannot be made at this level of study (such as profitability analysis and interest calculation). However the ICIS blogger and journalist Doris De Guzman wrote on 13/01/2012 ([1]) that the real product cost could be estimated for 1.50$/lb, that is 3.31$/kg. This value is close to the one obtained from the simulation (2.596 $/kg), in spite of the many approximations.
This is an indirect proof that the layout proposed for the plant could be taken into consideration for a large scale plant design.

Furthermore, the estimated cost for the polymer extracted with cyclohexanone appears to be lower than Telles one. With respect to chloroform, the amount of cyclohexanone is reduced to one quarter and thus the amount of ethanol, putting down the expenses for solvent recovery. Moreover, the additional economical advantage of cyclohexanone is that no chlorinated solvents are used, without their large safety and disposal cost associated. Clearly, the use of cyclohexanone improves P3HB extraction.

It has to be remembered, finally, that the process design and the cost estimation were performed with a conservative approach, leaving large space for further optimizations and improvements. This means that the real cost of the P3HB could be lowered, as soon as the studies and the experimentations will advance.
For example, the following points could contribute much to the cost reduction:

- the significant cost contribution of spray drying can be virtually removed if other alternatives (such as the filter press) are proven to be enough for the dewatering. Roughly, the extraction costs could be lowered by 10% and the initial investment by more than the 50%, without the spray dryer;
- the amount of ethanol for precipitation considered in the process design (4 times the volume) derives from a laboratory consuetude that grants the "far excess" of the anti-solvent. Probably the excess can be lowered, reducing sensibly the holdups, the size of the columns and the energy costs in particular;
- the energy consumption can be optimized, integrating the devices of the second part and the first part of the process (e.g. the same steam production facilities can feed both the MVR and the columns, reducing electricity costs of the first)

These are only some of the possible improvements for the process, but this points should be studied after giving an answer to the open issues presented in paragraph 4.1, which are of primary relevance for the development of a P3HB extraction process both efficient and eco-friendly.

Conclusions

The work carried out in this thesis addressed the industrial production of the biodegradable polymer poly 3 hydroxy-butyrate, with the aim to develop extraction techniques without chlorinated solvents.

In the first experimental part, a new method was tested to combine the two techniques of aqueous alkali digestion of membranes, and cell disruption through surfactants. The method proved to be effective raising the polymer purity from 65% to 98%, with yields equal to the reference of chloroform extraction. Unfortunately, the residual cell debris in the crystals of the polymer are the probable cause of an unexpected mechanism of oxidation-carbonization occurring during polymer melting. Hence, this extraction technique was abandoned. However, as the mechanism of degradation is still little understood, aqueous extraction could be suitable for low crystalline PHA extraction, such as the co-polymers P3HB-co-HV, exploiting the advantages of this technique, i.e. the need of low cost and safe chemicals, without any complex unit operation.

The recovery of P3HB was then investigated using new solvents, selected to be effective, inexpensive and with good environmental performances. The Solvent Selection Guide provided by GSK, allowed to choose the best among 110 solvents, evaluated according their environmental footprint and Life Cycle Assessment. Solubility of P3HB was then estimated with an empirical predictive equation; anisole, cyclohexanone, phenetole and diphenyl-ether gave the highest predicted solubility, which was then experimentally tested. Compared to the standard extraction performances with chloroform (at 4°C), anisole and cyclohexanone proved to be far better: the extraction reached the same purity with higher yields and higher chain length, the consumption of solvents was reduced to one quarter and the extraction time was reduced from 36h to only 15 minutes. Phenetole was abandoned due to lower solvation properties and diphenyl-ether due to bad workability (i.e. evaporation temperature higher than 250°C).

The economical evaluation of the downstream process with cyclohexanone was assessed with respect to a large scale process for 50 000 ton/year of P3HB, using the process simulator Pro/II, economic empirical equations from the literature, and equipment quotations directly from the producers. It was found that the total production cost (calculated with Douglas simplified method, including both fixed capital investment and operating expenses) is lower than the one of the commercial P3HB produced by Telles (USA), even if the estimation was made with conservative bases of calculation.

Further studies on the industrialization of cyclohexanone extraction are needed, so to optimize and improve the plant layout proposed and reduce further the costs.
However, according to the results, cyclohexanone is a good candidate to substitute chloroform successfully.

APPENDIX 1

The micro organism Burkholderia sacchari and the upstream process.

The name Burkholderia sacchari was proposed by Steinbuchel et al(2001) who identified as a new species of the genus Burkholderia the Gram-negative strain IPT101^{T}, isolated from the soil of a sugar cane plantation in Brazil. This bacterium caught immediately the attention of the scientific community because able to accumulate P3HB homo-polymer up to 68% of cell dry weight and, with different carbon sources, co-polymer P3HB-*co*-3HV (poly 3hydroxybutyrate-3hydroxyvalerate) up to 69%(Silva *et al.* 2000). But the greatest advantages of this strain is the ability to metabolize many different carbon sources, not suitable for other bacteria. Though with higher yields, many of the P3HB producer require expensive carbon sources like glucose (Lee 1996), while B. sacchari grows and accumulates polymer also in presence of xylose and arabinose, showing tolerance to many inhibitory compounds such as furfural. Hence, B. sacchari became a good candidate to produce a renewable biodegradable plastic which does not consume food resources, as many other renewable polymers do (for example mater B is produced by corn starch, [25]).
With the support of Bugworkers project, prof. Maria Manuela Regalo da Fonseca and her research group of Istituto Superior Tecnico (Lisbon) applied *B. sacchari* to produce homo and co-polymer starting from a lingo-cellulosic feedstock, an hydrolysate of wheat straw supplied by Biorefinery.de Gmbh (Cesário *et al.* 2012).

Figure A1. *One of the two STR of 2 liters of IBB/CEBQ laboratories in IST Lisbon. Inside, the fermentation broth in which B. sacchari accumulates PHA.*

These are the main topics focused inside the laboratories of IBB/CEBQ of IST in Lisbon:

- Characterization of P3HB productivity of B. sacchari using commercial sugars as C source
- Characterization of productivity using the wheat straw hydrolizate and definition of the best feeding procedure.
- Analysis of the proper fermentation conditions to induce co-polymer production with defined chain length and composition
- Characterization of the produced polymer
- Determination of eco-friendly and high yield procedures for polymer extraction and purification.

The experiments of fermentation and cell growth, performed at small scale with 500mL baffled shake flask and then with 2 liters STR (figure A1) showed that commercial sugar allow to obtain a final broth with high concentrations of suspended cells (up to 131.2g/L), containing 71% dry weight of P3HB. As predictable, hydrolysate gives lower performances, but considering the lower value of the carbon sources, large scale economics considerations could eventually elect *B. sacchari* as the best producer of PHA plastics.

The study of higher capacity plants and the fermentation scale-up are carried on by Biotrend SA, a private company partner of the project Bugworkers which works in collaboration with IST university.

APPENDIX 2

The Bugworkers project

Bugworkers (figure A2) is the name of an international collaborative project targeted to small and medium enterprises, promoted by European Community through the 7th Framework Programme. The full title of the project is "New tailor-made PHB-based nanocomposites for high performance applications produced from environmentally friendly production routes" and started in July 2010 , finishing in June 2014.

Figure A2:*Official Logo of bugworkers project, grant agreement 246449 of FP7/2012-2013*

Bugworkers objectives are many:

- To develop new cost-competitive nanoparticles from lignocelluloses by product providing antibacterial and antifungal properties.
- To study and improve PHB production techniques to obtain polymer from industrial/agricultural by-products using non-food sugars as feedstock, reducing its cost in at least 70%, improving its mechanical and chemical-resistance properties.
- To develop a compounding process for the new nanoparticles with PHB plastics, without alteration of the materials improving mechanical properties.
- To develop multilayer structures in order to obtain composites suitable to replace engineering materials, reducing a weight by more than 10% without mechanical and thermal properties loss, 100% made from biomass.

These new materials should become real alternatives to existing oil-derived materials such as PA and ABS in household appliances and communication devices.

The project is carried out by a consortium of 15 partners of 9 different countries, involving 9 SMEs, 2 large companies and 4 research institutions. The research leading to these results has received funding from FP7/2012-2013 under grant agreement 246449, with a overall budget of 4,5M€. More information and a detailed list of the companies and institutions involved in the project can be found in the official web-site ([26,27]).

APPENDIX 3

Cost analysis indexes and utilities price.

Fuel and electricity cost

Fuel 3.788$/GJ or 4$/MBtu

Electricity: 0.05 $/kWh

These values were provided by the Center of Energy Efficiency and Renewable Energy of University of Massachusetts Amherst, in the IAC (Industrial Asssessment Center) website ([24]).

These values are to be considered as rules of thumb, near to the real values which change daily according to the market fluctuations.

Steam cost

The equation to estimate the cost of steam (A1) derives directly from the equation of fuel consumption, with a correction to include other costs of the reboiler (e.g. feed water supply and treatment, pumping, emission control,..).

$$C_{steam}\ [\$/klb] = a_f \left(\frac{H_s - h_w}{1000 \cdot \eta_B} \right) \cdot (1 + 0.3) \qquad \text{(A1)}$$

Where a_f is the fuel cost ($/MBtu), H_s is the steam enthalpy (Btu/lb) and h_s is the liquid water enthalpy (Btu/lb). η_b is the reboiler efficiency, between 80% and 85. With the mean value of 4$/MBtu for the fuel cost the calculation gave a steam cost of 5.59 $/klb, which corresponds to 0.01232$/kg of steam.

Cooling water cost

The cost of cooling water, supposed from a closed circuit with evaporating towers, was calculated according to the relations of (Ulrich and Vasudevan 2006): the cost of 4$/MBtu for the fuel and 568.3 for CEPCI inflation index (2013) were used in the equation. The final result for the cooling water was 0.05156 $/m^3, a conservative value as the used volumes are higher than the ones for the correlation.

Total module cost estimation

Peters (2004) provided a list of cost factors that allow to calculate the overall grass roots cost factor starting from the purchase cost. These values are based on data collected from existing plants and can give a reasonable approximation of those costs that are properly calculated only at very advanced stages of process design.

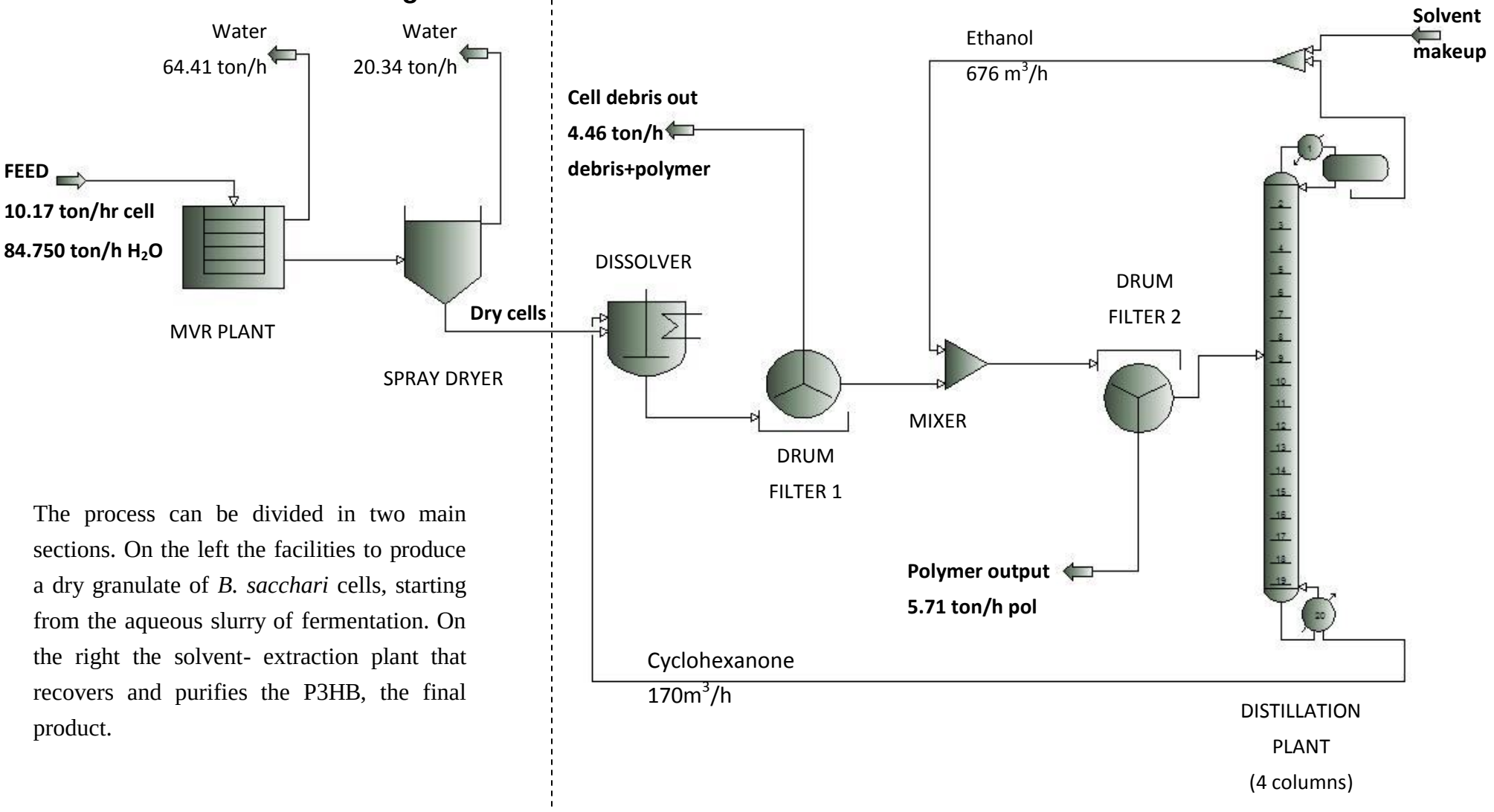
Table A1. *Cost factors determining the grass root cost of a purchased equipment(Peters et al. 2004), specific for solid-liquid facilities*.

Purchased cost PC	PC
Delivered cost DC	110%PC
Piping	31% PC
Yards improvements	10% PC
Service Facilities	55% PC
Buildings	47% PC
Installation	25% DC
Electrical system	15% DC
Instrumentation and control	26% DC
Engineering and supervision	30% DC
Legal consultancy	4% DC
Construction	34% DC
Constructor's fee	1.5%DC
Contingencies	37% DC

$$TMC = (0.31 + 0.47 + 0.1 + 0.55) \cdot PC + 1.1 \cdot PC \cdot (1 + 0.25 + 0.15 + 0.26 + 0.3 + 0.04 + \\ + 0.34 + 0.015 + 0.37) = 4.4275 \cdot PC \quad \text{(A2)}$$

Where TMC is the total module cost and PC is the purchase cost.
The overall cost factor is 4.4275.

APPENDIX 4 Process flow diagram



The process can be divided in two main sections. On the left the facilities to produce a dry granulate of *B. sacchari* cells, starting from the aqueous slurry of fermentation. On the right the solvent- extraction plant that recovers and purifies the P3HB, the final product.

Notation

Symbols

A_{cond}	=	exchange area condenser
A_{reb}	=	exchange area reboiler
C	=	cost
$Cost_x$	=	cost of the scaled-up equipment
$C_{purchase}$	=	cost of purchase
c_{pw}	=	specific heat of water
E	=	electric power consumption
H_s	=	steam enthalpy
h_w	=	liquid water enthalpy
m_{cell}	=	amount of cell before extraction
m_{evap}	=	mass flowrate of water to evaporate
$m_{recovered}$	=	mass of solid material recovered from extraction
m_{solid}	=	mass flowrate of solid phase
Mw	=	molecular weight
m_{Win}	=	outlet mass flowrate of water
m_{Wout}	=	inlet mass flowrate of water
P_{cell}	=	mass rate of cell
Q_c	=	cooling energy
Q_h	=	heating energy
r	=	solubility radius
s	=	solubility
S	=	steam consumption
U	=	overall energy transfer coefficient
X	=	purity of recovered polymer
x	=	polymer content inside the cells
Y	=	extraction yield
δ_d	=	Hansen parameter for dispersion component
δ_h	=	hansen parameter for hydrogen bonding
δ_p	=	hansen parameter for polar interaction
ΔT	=	temperature difference
η_B	=	reboiler efficiency
λ_s	=	latent heat of vaporization

Abbreviations

CMC	=	Critical Micellar Concentration
DC	=	Delivered Cost
FC	=	Fixed Capital
HV	=	Hydroxy Valerate
M&S	=	Marshall & Swift
MVR/TVR	=	Mechanical Vapour Recompression/Thermal Vapour Recompression
P3HB	=	Poly 3-Hydroxy Butyrate
PC	=	Purchased Cost
PHA	=	Poly Hydroxy Alkanoates
SDS	=	Sodium Dodecyl Sulfate
TMC	=	Total Module Cost
TPC	=	Total Production Cost
TS	=	Total Solid
VPC	=	Variable Production Cost
w/v	=	weight over volume

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