BIOPROCESS DESIGN OF A PHB PRODUCTION PLANT

Universitat Autònoma de Barcelona

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Final Degree Project of Biotechnology (2018-2019)

Tutored by *Carles Solà i Ferrando*

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PART I: INTRODUCTION

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1. Abstract

During the last century we have created a plastic dependent lifestyle, consuming each year an increasingly quantity of it, leading to severe environmental issues. Over 270 million tons of plastic residues were generated in 2017. Plastic not only is a non-degradable material, but it also comes from petroleum, which is a non-renewable resource which reserves are shrinking day by day, so an alternative had to be proposed. There are many biological substitutes for plastic, from which polyhydroxybutyrate (PHB) has been chosen for this project due to their especially suitable properties and wide and well-developed production.

PHB is a biodegradable and renewable polymer with plastic properties, commonly produced by many bacteria species under specific conditions as a carbon storage. It can be synthesized from a wide variety of substrates ranging from pure glucose to many organic residues. Using residues as substrate constitutes a necessary strategy in order to achieve a profitable bioprocess in a very competitive scenario. Cheese whey residue has been the one proposed in this project, as it is an abundant residue from dairy industry and it has become a serious environmental problem over the years for its high organic load.

To summarise, this project aims at producing PHB using whey residue as substrate, thus helping with two environmental concerns while trying to make profit at the same time.

2. Materials and methods

The whole project, *Bioprocess design of a PHB production plant*, was developed using SuperPro Designer v8.5 (Intelligen, Inc) for the plant and bioprocess design and calculations, and MATLAB 2011 (MathWorks) was used in fermentation process mathematical modelling. Whole process Block Diagram in this work was developed with PowerPoint (Microsoft). Bioprocess idea and central fermentation development were taken from the reference work *Production of poly(3-hydroxybutyrate) from whey by cell recycle fed-batch culture of recombinant Escherichia coli*, developed by Ahn and colleagues [1].

The present work, *Part I: Introduction*, is a mainly bibliographic compilation gathering introductory information and concepts to the designed bioprocess and globally presenting the whole developed project.

3. Bioplastics, PHAs and PHB

Biopolyesters are organic polymeric compounds made up of biological monomers chained by ester links, and they are an important biomaterial family identified as bioplastics, as they show physico-chemical properties resembling petrochemical plastics [2]. Depending on its forming monomeric units, each type of biopolyester is differently synthesized and presents different characteristics, comprehending almost all the existing non-biological thermoplastics.

Polyhydroxyalkanoates (PHA) are the family of biopolyesters made up by esterified alkanoic acids, and they constitute the largest, best studied and more diverse and produced type of bioplastics, as well as the only biopolyester family completely synthesised by biological means [3]. PHAs are produced as a carbon and, in consequence, energy reserve by many microorganisms, which synthesize and store it in intracellular granules in response to exhaustion of a non-carbon limiting nutrient essential for bacterial growth [2], [4], [5].

Poly(3-Hydroxybutyrate) (P(3HB)) is one of the two existing isomers of PHB, and usually referred to simply as PHB as it is, by far, its most common form (in front of P(4HB)). PHB is the PHA which forming monomer is hydroxybutyric acid, its β isomer in case of P(3HB) (*Figure 1*) and its γ isomer in P(4HB); α-hydroxybutyric acid isomer does not form ester polymers. P(3HB) is the most naturally synthesised PHA and bioplastic, and also the most industrially produced, as it was the first discovered and therefore best characterized PHA regarding both its biological, chemical and physical properties, its biosynthetic pathways, its industrial production processes and also its wide range of developed applications. These are the reasons why P(3HB) has been the chosen polyester to be produced by the designed bioprocess and industrial plant.

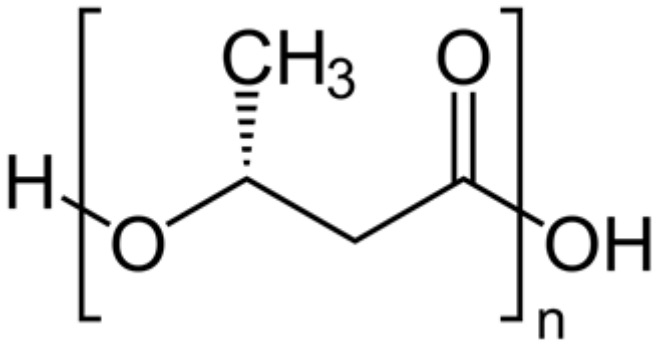

Figure 1. Molecular structure of P(3HB) monomer units [5].

3.1. Properties and characterization of P(3HB)

P(3HB) is a polyester chain made up of 3-hydroxybutyrate monomers (*Figure 1*), with chemical formula $(CH(CH_3)CH_2COO)n$, being *n* usually between 100 and 30,000 [2]. P(3HB) is a fully biodegradable polyester with optical activity, piezoelectric activity and good barrier properties. It is a good electrical insulator, with electrical resistivity between 1×10^{16} and 1×10^{18} μohm·cm and a dielectric constant around 4. It is a partially crystalline material with a high melting point (135 ºC), which makes it a thermoplastic material and a high degree of crystallinity. It has a density of 140 kg/m^3 [6] and a number-average molecular mass ranging from 0.5 MDa to 20 MDa, which seems decreased with time during the course of cellular accumulation [7]. Under specific and controlled culture conditions with modified *E. coli*, production of PHB with an extremely high molecular mass (20 MDa) has been reported [7].

As most of the bioplastics, P(3HB) shows also high biocompatibility, which made it suitable and specially interesting for biomedical applications such as construction of heart valves, tissue scaffolds or for their use as drug carriers, among many other applications [8].

3.2. Applications

Bioplastics in general and PHAs in particular, as the most diverse and resistant materials among bioplastics, have a large range of applications in different fields, such as agriculture, chemical industry, biomedicine or nanotechnology among others [2], [8], [9], especially due to their properties regarding biocompatibility, environmental sustainability and health safety. These are the main reasons why PHB and its copolymers are presented as an ideal replacement to conventional petroleum-based plastics.

Among all their possible applications, one of the most promising research areas lies in the field of biomedicine, where biological properties and advantages of PHB regarding petrochemical plastics are priority so its use is not restricted by its higher production costs. PHA copolymers have proved many potentials uses as antibacterial agents, drug carriers, biocontrol agents, tissue engineering scaffolds, biomedical devices, biodegradable implants, anti-osteoporosis effectors and memory enhancers. P(3HB) and its forming monomers are being used in the synthesis of novel polymers with proved features which make them potent drug carriers [8]. Furthermore, extracellular matrix and tissue scaffolds from P(3HB) and other PHAs can be also be created through the electrospinning technique [10]. The use of bioplastics in this field is especially interesting and promising due to their biodegradability and biocompatibility properties. It is important to remark that in this kind of applications it is usually required treatment with hydrophilic polymers, as PHAs are in general hydrophobic [10].

3.3. Plastics vs Bioplastics

P(3HB) is a biodegradable compound with thermoplastic properties and biological origin, and it constitutes a potential alternative to non-biodegradable and non-renewable petroleum-based plastics in many products manufacture. Despite chemical modifications after its synthesis are usual for many applications, PHB production by a chemical procedure instead of a biological one has not been reported, so its large-scale production remains exclusively biotechnological.

Contrarily to petroleum-based plastics, PHB shows biocompatibility and no biological toxicity, what makes it perfectly suitable for food packaging and medical applications [11]; it is also biodegradable, even anaerobically and beneath water, and can be produced from renewable resources, while regular plastics are produced using petroleum as a source, which is not only a pollutant but also is running out [11].

Despite all the advantages offered by PHAs, there are still important challenges faced by their industry at the current state [11], mainly concerning production costs, but also economic and environmental competitivity and industrial operativity and scale-up of the process. Fermentation carbon source, product yields on the selected carbon sources, process productivity, running cost of fermentation and downstream processing are major factors affecting the cost of production [12]. Nowadays, the still high price for PHAs production (4$/kg approx.) cannot yet compete with petroleum-based plastics prices, approximately a fourth. Great efforts are being done in order to use waste streams as raw materials and thereby reduce production costs and increase process competitivity, but complexity and variability of these kind of materials involve several difficulties in process operation and downstream processing.

Almost all these challenges and the approaches to overwhelm them are related to the used substrate for the biological process, as it is usually the main production cost [2], a great determinant of the fermentation development and productivity and also a natural resource which interests could compete with other human necessities, such as food. In order to increase economic and environmental competitivity and to avoid competition with human needs, use of residues as substrate becomes necessary. Purer substrates reduce operational complexity, and generally reports better productivity, so use of specific industrial organic residues, usually with low degree of heterogeneity and large-scale generated, had become the default option. Cheese whey, a rich residue hugely generated by food industry, has been the selected substrate for the designed plant, as it is deeply discussed in forward sections in this work (see *Whey* and *Upstream*).

3.4. PHB biosynthetic pathway

P(3HB) biosynthetic pathway consists of three sequential enzymatic reactions beginning from universal metabolite Acetyl-CoA; thus, bacterial ability to synthesize this storage material with plastic properties mainly relies on their genetical constitution possessing the three genes encoding for the three responsible enzymes on PHB biosynthesis. PHB is naturally produced by multiple bacteria, such as *Cupriavidus necator*, several species

of *Pseudomonas, Bacillus* and *Azotobacter*, and can also be produced by recombinant strains of non-natural producers expressing the PHB biosynthetic genes from some natural PHB producer [11]. Although the wide range of reported PHB-producing bacterial species, there are few which can accumulate it enough for large-scale production [9].

P(3HB) biosynthesis starts with the condensation of two acetyl-CoA molecules to form an acetoacetyl-CoA molecule, liberating a CoA molecule. This reaction is mediated by the enzyme β-ketothiolase. The acetoacetyl-CoA molecules are then reduced by the acetoacetyl-CoA reductase enzyme into 3-hydroxybutyryl-CoA using, NADPH as cofactor. Finally, the enzyme PHA synthase catalyses the esterification between the 3-hydroxybutyryl-CoA monomers to P(3HB), liberating CoA [13]–[15].

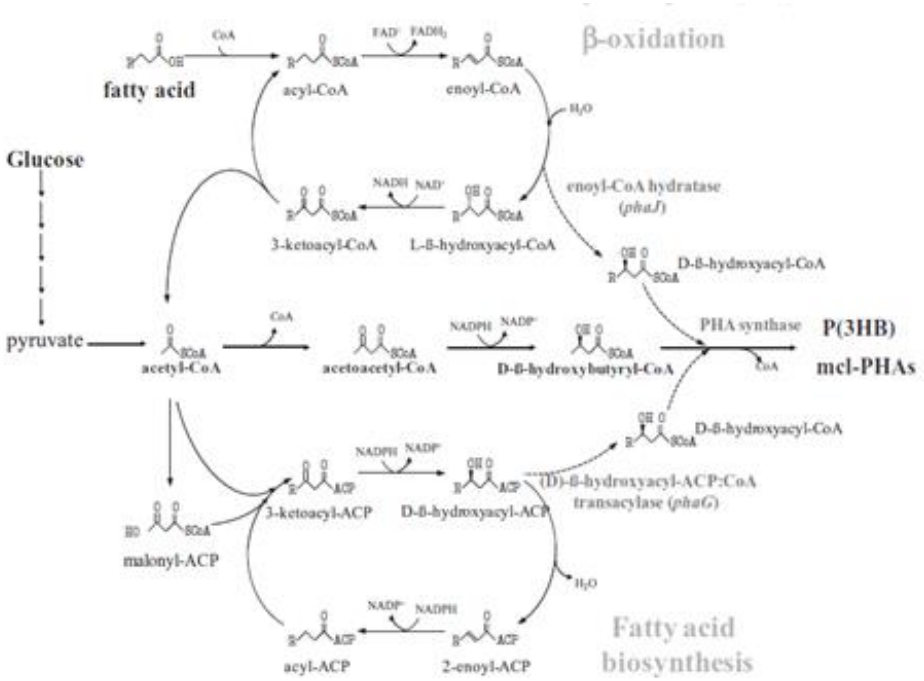

Figure 2. Metabolic pathways involved in P(3HB) biosynthesis [14].

Many carbon sources can be used for the biosynthesis of P(3HB), from sugars to lipids, depending on the used microorganism and its ability to metabolise each carbon source to acetyl-CoA, the origin molecule on the P(3HB) biosynthetic pathway.

Metabolic pathways and involved enzymes differ for each PHA regarding to its forming monomer. PHA diversity is consequence of the wide range of existing PHA synthases and the broad substrate range they exhibit. Different PHAs are synthesized depending also on the carbon source provided, the metabolic routes present to convert that carbon source into the hydroxyacyl-CoA monomers and the specificity of the PHA synthase of that particular organism [14].

4. Whey

Whey is an abundant by-product of dairy and cheese industries, consisting of the watery portion after the separation of fat and caseins from the whole milk [16]. Its current worldwide production is estimated at 180 million tons per year, mainly concentrated in Europe and USA (70% of total world whey), and it shows a global growth rate around 2% per year [17].

In cheese-making, generated whey represents around 90% of the processed milk volume, and it contains around 55% of milk's nutrients and almost its entire carbohydrates content, mostly lactose [17]. Therefore, similarly to milk, whey constitutes a rich food material with an important nutritional value, with high contents of carbohydrates, proteins, lipids and minerals, especially calcium [16]. That makes whey an interesting and complete substrate for many industrial applications and, at the same time, a severe environmental pollutant when treated as a waste.

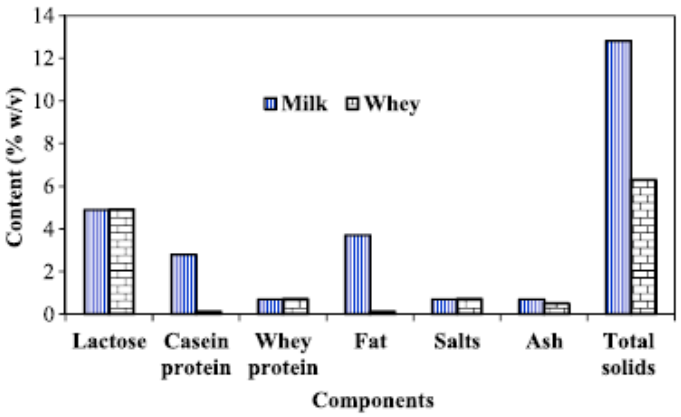

Figure 3. Comparison of the proximate analysis of bovine milk and its whey [17].

Nowadays, around 50% of total produced whey worldwide is used for animal feed or transformed into different value-added products, mainly food items and supplements [17], taking profit of its important content on residual milk nutrients. Despite these novels and increasing uses for whey, its generation largely exceed its usage, and huge amounts of whey surplus must be managed as waste. Whey residue disposal or treatment report important economic and environmental costs, derived from the large managed volumes and its high biochemical oxygen demand, which due to its high organic load basically in form of lactose content.

Table 1. Compositional characteristics of sweet whey and acid whey [17].

Constituents	Sweet whey (g/L)	Acid whey (g/L)
Total solids	63.0–70.0	63.0–70.0
Lactose	46.0–52.0	44.0–46.0
Protein	6.0–10.0	6.0–8.0
Fat	5.0	0.4
Lactate	2.0	6.4
Ash	5.0	8.0
Calcium	0.4–0.6	1.2–1.6
Phosphate	1.0–3.0	2.0–4.5
Chloride	1.1	1.1

A wide variety of biotechnological and physico-chemical processes can be applied on whey to use it as substrate for industrial transformation into multiple value-added products [17]. Physico-chemical procedures allow differential recovery of different whey fractions and components: lipids recovery by skimming, proteins recovery by precipitation or ultrafiltration, and carbohydrates recovery or concentration by drying or reverse osmosis [16]. Each one of these fractions or any of their combinations, as well as the original non-treated whey, are susceptible of being directly commercialised as well as further processed.

Microbial fermentation on whey or whey permeate (carbohydrate fraction) arises as a powerful means for producing a wide variety of value-added products from microorganisms using this surplus as carbon source or complete media. Direct fermentation is limited to lactose-consuming microorganisms, but enzymatic hydrolysis of this disaccharide into its forming monosaccharides (glucose and galactose) as well as genetic engineering increases even more the large number of usable microorganisms and obtainable products [17].

5. Production

5.1. Annual production

Bioplastics are a biotechnological bulk product, with very low added value and large-scale industrial production. Lower production costs and commercial price of petrochemical plastics still restricts its extensive use as substitutes to these non-renewable ubiquitous materials, but their biological attractive properties have led to a wide use in biomedical application, as a specialty product, and a great social acceptance, which together with a progressive environmental awareness are leading to promoting policies and industrial implementation.

Industrial by-products are largely available and homogeneous resources which must be costly treated as residues if not used by other processes. These characteristics make them especially appropriate and used substrates for bioplastics production processes, also for ours, as they increase bioprocess competitivity reducing economic costs and not

only preventing resources consumption but recycling a pollutant residue. As bioplastics price and commercial opportunities are nowadays limited by conventional plastics competitivity, an especially interesting strategy is to fit its trade production to the substrate availability as residue management demand, which may even permit to include avoided economic costs for residue treatment as additional benefits of the bioprocess. For this reason, the plant and bioprocess design has been dimensioned in order to be able to entirely treat the annual production of whey residue of a middle-sized Spanish cheese-maker enterprise (Central Quesera Montesinos, montesinos.es; data from wheypack.eu), which corresponds to approximately 200.000 m^3 of whey residue allowing a PHB annual production of 4.100 tons.

5.2. Bacterial strain

As already mentioned, there are many bacterial species with the natural capacity to produce different PHAs [11], but most of them shows limited industrial applicability compared to genetically modified strains of model organisms, such as *Escherichia coli*, *Saccharomyces cerevisiae* or *Pichia pastoris*. Even, use of recombinant *E. coli* strains reports higher cell concentration cultures (up to 200 g DCW / litre), cell growth rates and PHB content (up to 90% of CDW) than natural producer species [1], [4], [15], [18].

A lactose consuming microorganism is also needed in our process, in order to use residual whey as fermentation substrate and this disaccharide, its major component, as carbon source. This means a great limiting fact in order to select a natural producer and, consequently, to optimize fermentation productivity. Use of a recombinant *E. coli* strain again arises as the optimal choice, as in addition to a model organism, which industrial use is widely standardized and optimized, it is a natural lactose consuming, with a highly productive metabolism also on this substrate. Despite naturally not being a PHB producer, recombinant incorporation of PHB biosynthetic pathway into *E. coli* is a straightforward procedure, and various PHB-producing recombinant strains have been yet developed by several authors [16], [18].

The selected strain for our bioprocess design is a genetically modified *E. coli* which harbours the *Alcaligenes latus* PHA biosynthesis genes. This strain, named *E. coli* CGSC 4401, was developed in 1998 by Choi and colleagues [15], and it is to date the PHB producing strain with higher volumetric productivity (4.6 g of PHB / litre / h) reported in literature [16]. It was obtained by transforming into *E. coli* XL 1-Blue strain the recombinant plasmid pJC4, which was constructed by cloning the *A. latus* PHA biosynthetic operon into plasmid pSYL104, a stable high-copy-number plasmid. The insert consisted of a 5.3 kb *A. latus* genomic DNA fragment containing, in this order, a σ^{70}-dependent promoter and the genes $phaC_{Al}$, $phaCA_{Al}$ and $phaB_{Al}$, coding for PHA synthase, β-ketothiolase and acetoacetyl-CoA reductase respectively [15]. Prior to the development of this specific strain, other recombinant *E. coli* strains containing the *Ralstonia eutropha (R. eutropha)* genes had already been developed [15], [19], but obtained productivities were lower than the obtained with wild-type *A. latus* [15].

5.3. Global process

General PHA industrial production process presents the same main steps and characteristics which generally presents industrial production processes in chemical and biotechnological industries. This means upstream, fermentation and downstream as major sections dividing the global industrial bioprocess and grouping single-step activities. The main operations generally found in PHA production process includes bioreaction media preparation, fermentation, biomass recovery and concentration, cell disruption, PHA purification and product adaptation [3]. For each operation, several options according to number and specific procedures are possible, depending on the selected option in previous procedures and on the desired results. Our designed bioprocess, as the whole set of selected procedures to achieve our productive and operational purposes, is schematically presented in the block diagram presented below, in *Figure 5*.

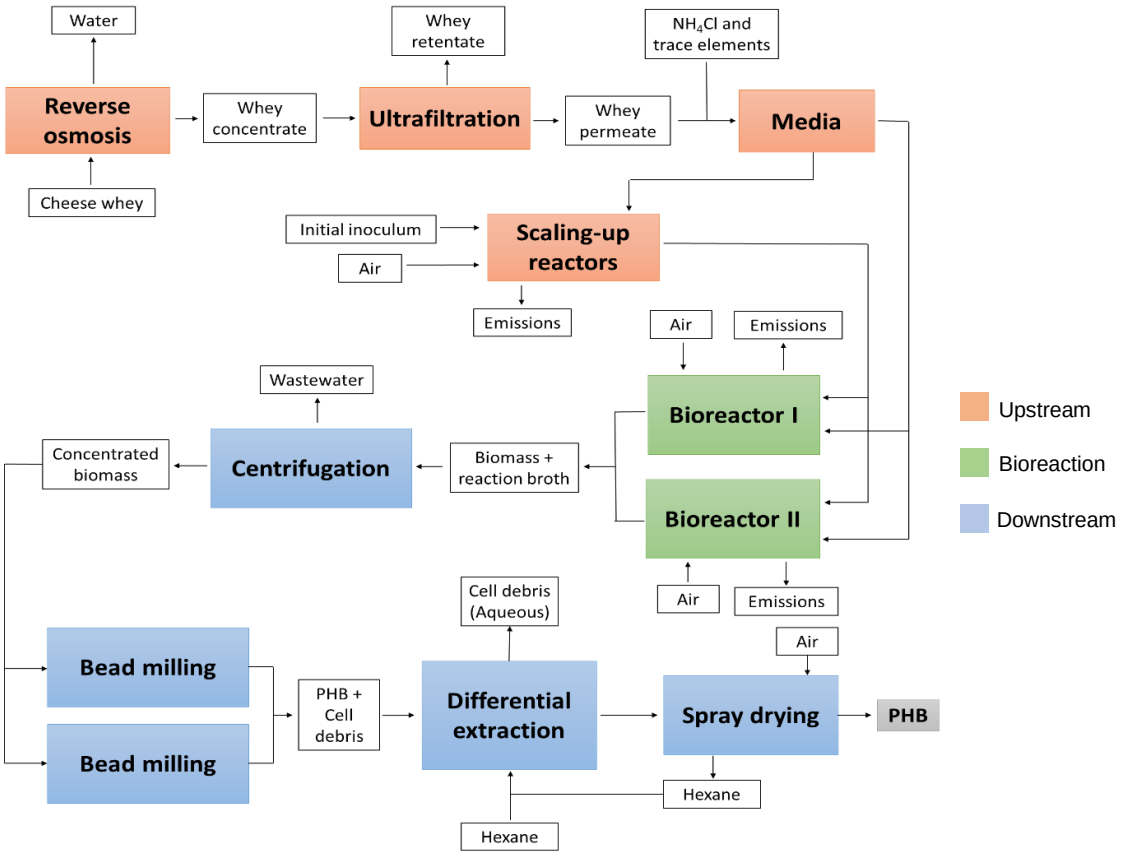

Figure 4. Process Block Diagram.

5.3.1. Upstream

The production plant should be located geographically near, associated if possible, to a cheesemaker industry, which would take profit of our activity in whey residue depuration while, at the same time, it would mean our substrate supplier. In a bulk production process context as ours, producing a low value added good with hard commercial

competitors, minimizing (or supressing if possible) substrate transportation is obligated. Considering our substrate nature, a liquid, aqueous and diluted residue, long transportation would mean prohibitive economic and environmental costs.

The plant arriving whey would need a physico-chemical pre-treatment, as previously mentioned, in order to separate several fractions and impurities for adequation to use as substrate. First step would consist in a whey lactose concentration through a reverse osmosis equipment, in order to remove a noteworthy volume of water from the entering stream, and in this way reducing volume and costs in later equipment and infrastructure and increasing process productivity and competitivity. Lactose concentration would be increased from approximately 47 g/L (average value in whey) to 280 g/L, in order to reproduce the fermentation conditions reported in our experimental fermentation reference.

Ultrafiltration would be next applied to previously concentred whey, in order to remove their present proteins (mainly α and β-lactoglobulin, [16]). In some cases, these proteins can be used as a nitrogen source enhancing microbial cultivation and fermentation [16], but its removal from whey concentrate permits to obtain a purer carbon source and a more standardisable medium. At the same time, these purified proteins present important trade and interesting applications in nutraceutical and pharmaceutical industries [17].

Whey is usually a nutrient rich residue which can be used alone as a complete growth media. Nonetheless, its specific and exact composition is not determined, as it lightly varies among the origin animal species, its feed, and also weather. Therefore, the concentrated and filtered carbon source should be analysed for our supplier, maybe periodically, and, if required, supplemented with commercial trace element solutions or ammonium chloride (NH4CL) as nitrogen source (most likely limiting non-carbon element), in order to optimize bacterial growth and fermentation productivity.

The prepared media would be used as feed for the fed-batch phase in the main fermentation. For the initial batch phase in main fermentation as well as for inoculum growth batches in the scaling-up, lactose concentration should be redressed to 60 g/L using water stream generated in initial reverse osmosis; this concentration value is a standardised and optimised value for sugar concentration in E. coli growth batches, which avoids osmotic stress or inhibition.

5.3.2. Bioreaction

The main fermentation in our bioprocess would take place in two bioreactors, each one disposing of 80 m^3 of operational volume, working alternatively, with 6 hours delay between first's and second's start, in order to optimise downstream equipment utilization (see *Part III* of this project). PHA producing fermentations are strictly aerobic, but microaerophilic conditions has showed higher productivity, PHA accumulation and enhanced microbial biosynthesis in many works [20], [21]. This is ostensibly due to Acetyl Co-A accumulation and redirecting to PHA biosynthetic pathways (see *PHB biosynthetic pathway* in this work) as a consequence of a lower consumption in TCA cycle, as it is downregulated by accumulation of their products in low oxygen availability conditions

[21]. By this reason, Dissolved Oxygen Concentration (DOC) is finely controlled during fermentation and lowered at final production phases. Bioreaction development is further discussed in *Part II* in this project.

5.3.3. Downstream

Downstream processing is one of the major factors affecting PHB production costs, as it is a long and complex process for a bulk product purification. Usually, it also includes using a dangerous solvent as differential extraction organic phase. Choosing this organic phase is a critical point of the global downstream process, as most of them show toxicity, explosion or environmental damage dangers. Our proposed solvent would be hexane, as PHB and cell debris show, respectively, high and almost null solubility in this solvent, so it reports high partition, purification and recovery values. It is also less environmental-harmful than chlorinated solvents, but it must be finely monitored as it is explosive.

Our downstream process would follow standard procedures and use optimized operation in PHB purification, in order to keep a reliable process maximizing recovery efficiency and obtained purity. Firstly, bioreactor exit stream would be centrifuged to concentrate total biomass and decrease the volume to be treated in further operations, thus decreasing operational costs and increasing recovery rates and efficiencies. Cell disruption would then take place through bead milling of concentred total biomass, in two parallel bead mills in order to optimize volume and time management. PHB purification and separation from cell debris would be achieved through differential extraction using hexane as organic phase; cell debris would be discarded within the aqueous stream, whilst PHB would be recovered within the organic solvent. PHB recovery from the organic phase would be achieved by spray drying, obtaining a dried, solid and marketable product by one side and an hexane rich gas stream by another. Hexane in this gas stream would be condensed and thereupon recycled to differential extraction column. Downstream processing is deeply discussed at *Part III* in this project.

6. Conclusions

Petrochemical plastics are nowadays an indispensable and basic product for most people worldwide, especially in developed societies. At the same time, their environmental accumulation and interference at all live levels is becoming a major global problem, as they are not biodegradable nor biocompatible. Also, their production is based on the use of a fossil non-renewable resource, petroleum. For all these reasons, developing a reliable bio-based alternative to conventional plastic materials means a major need and challenge for nowadays global society. Biopolyesters are well-known biological plastic materials arising as a promising solution to all these issues, albeit several shortcomings should be overcome in order to optimise their production process and commercial competitivity.

Substrate supplying for bacterial fermentation in bioplastics production usually means the main cost in the overall production process. Pure substrates are also primary resources which may compete with other human or environmental needs. Using organic residues as bioprocess substrate overcome both issues, not only avoiding resources consumption and reducing environmental impact in production fermentation, but also in these wastes' treatment. Whey is an abundant residue, especially in developed west countries, and it is a rich and nutritive material, which makes it a highly pollutant waste and a complete fermentation media at the same time, so then an especially interesting and advantageous substrate to use in biotechnological processes.

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BIOPROCESS DESIGN OF A PHB PRODUCTION PLANT
PART I: INTRODUCTION

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Final Project of Biotechnology Degree (2018-2019)

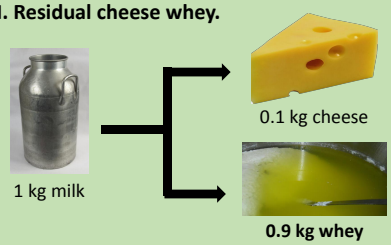
ABSTRACT: The aim of the project is to design an industrial PHB production plant using residual whey as bioreaction substrate. Whey is a largely produced by-product of cheese-making, and it has been proven to be harmful to the environment if disposed onto farmland or waterwaste. PHB is a biodegradable polymer which can be used in products ranging from plastic bags to cutting edge surgical stitches or even prothesis. This part aims to introduce the global project , presenting the treated problem, the used substrate, the obtained product and a general description of the production system.

Two problems, one solution

I. Plastics pollution.

270 millions tons/year of plastic waste.

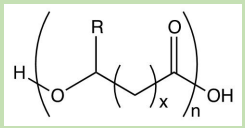
II. Residual cheese whey.



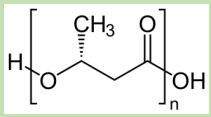
180 million tons per year.
Only 50% transformed into value-added products.
High organic load → 50 g/L lactose.
High Biochemical Oxygen Demand → 50 g/L BOD.
Serious environmental problems derived from its waste disposal or treatment.

Poly-3-hidroxybutyrate

Bioplastics → Bio polyesters with plastic properties.
PHA → Bio polyesters of hydroxyalkanoic acids.
PHB → Bio polyester of hydroxybutyric acid.
P3HB → Bio polyester of 3-hydroxybutyric acid.



X = 1
R = CH_3



PHA_s general structure. P3HB molecular structure.

Conventional thermoplastic properties + **Biodegradability, biocompatibility and nontoxicity**

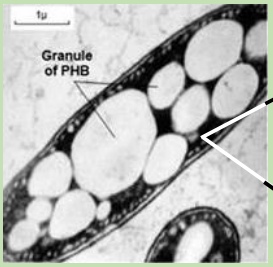
Applications

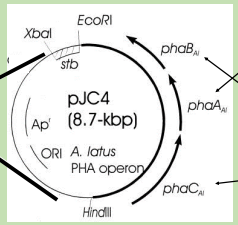
- **Biomedical applications**
  - Tissue engineering scaffolds.
  - Drug delivery carriers.
- **Eco friendly substitutes to conventional plastics**

Biosynthesis

Bacterial strain

- Recombinant *Escherichia coli* strain CGSC 4401 harboring *Alcaligenes latus* PHA biosynthesis genes in plasmid pJC4.
- Three enzymes operon constitutively expressed from the natural promoter.



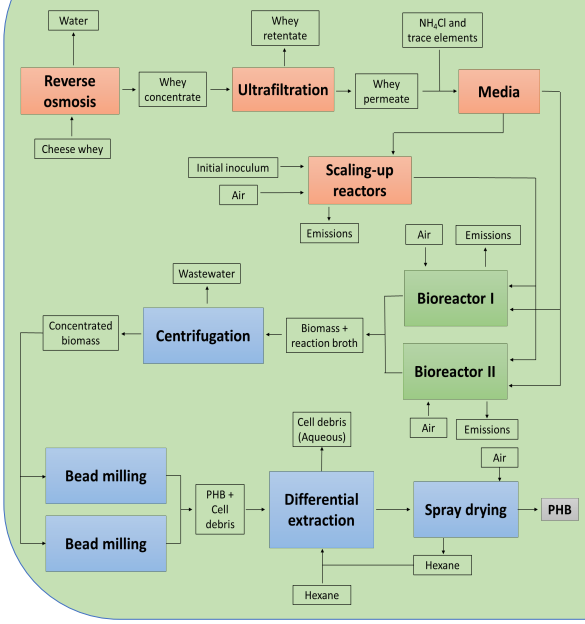


Biosynthetic pathway

Carbon source (carbohydrates or lipids)
↓ Bacterial metabolism
Acetyl-CoA ---- Krebs cycle
↓ β-ketothiolase
Acetoacetyl-CoA — O_2
↓ Acetoacetyl reductase
D-β-hydroxybutyril-CoA
↓ PHA synthase
PHB

Production

Block diagram



Upstream

- Media preparation.
  - Whey concentration by reverse osmosis.
  - Proteins removal by ultrafiltration.
  - N source and trace elements addition.
- Inoculum scaling-up.
  - *E. coli* CGSC 4401 strain.
  - 4 sequential growth batches, 10x volume.

Bioreaction

- Cell recycle fed-batch.
  - Fresh media feeding.
  - Exhausted media extraction through membrane with cells recirculation.
  - PHB biosynthesis promotion by limiting dissolved O_2.

Downstream

- Centrifugation → Biomass concentration.
- Bead milling → Biomass disruption.
- Differential extraction → PHB purification.
- Spray drying → Product adequation.

Annual throughput

Goal: Spanish middle-sized cheese producer residual whey treatment.

200.000 m^3 cheese whey/year

80 m^3/bioreactor
2 Bioreactors/batch
1 batch/2 days
164 batches/year

4.100 tons P(3HB)/year

1. Ahn *et al*. (2001). Production of poly(3-hydroxybutyrate) from whey by cell recycle fed-batch culture of recombinant *Escherichia coli*. *Biotechnology letters*, **23**, 235-240.
2. Choi *et al*. (1998). Cloning of the Alcaligenes latus Polyhydroxyalkanoate Biosynthesis Genes and Use of These Genes for Enhanced Production of Poly(3-hydroxybutyrate) in Escherichia coli. *Applied and Environmental Microbiology*, **64**, 4897-4903.
3. Chen, G.Q. (2010). Plastics from Bacteria: Natural Functions and Applications. In *Microbiology Monographs*. Volume 2. Ed A. Steinbüchel. Heidelberg, Germany: Spinger.
4. Yadav *et al*. (2015). Cheese whey: A potential resource to transform into bioprotein, functional/nutritional proteins and bioactive peptides. *Biotechnol. Adv.*, **33**, 756-774.
5. Koller et *al*. (2012). Whey Lactose as a Raw Material for Microbial Production of Biodegradable Polyesters. In *Polyester*. Ed. H.E.D. Saleh. InTech. DOI: 10.5772/48737.

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BIOPROCESS DESIGN OF A PHB PRODUCTION PLANT
PART II: SCALING UP AND BIOREACTION

Universitat Autònoma de Barcelona

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Final Degree Project of Biotechnology (2018-2019)

Tutored by *Carles Solà i Ferrando*

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1. Abstract

During the last century we have created a plastic dependent lifestyle, consuming each year an increasingly quantity of it, leading to severe environmental issues. Over 270 million tons of plastic residues were generated in 2017 *[1]*. Plastic not only is a non-degradable material, but it also comes from petroleum, which is a non-renewable resource which reserves are shrinking day by day, so an alternative had to be proposed. There are many biological substitutes for plastic, from which polyhydroxybutyrate (PHB) has been chosen for this project due to their special suitable properties and wide and well-developed production.

PHB is a biodegradable and renewable polymer with plastic properties, commonly produced by many bacteria species under specific conditions as a carbon storage. It can be synthesized from a wide variety of substrates ranging from pure glucose to many organic residues. Using a residue as a substrate constitutes a necessary strategy in order to achieve a profitable bioprocess in a very competitive scenario. Cheese whey residue has been the one proposed in this project, as it is an abundant residue from dairy industry, and it has become a serious environmental problem over the years for its high organic load.

To summarise, this project aims at producing PHB using whey residue as substrate, thus helping with two environmental concerns while trying to make profit at the same time. This part of the project (*Part II)* is focused on describing the strategies used to produce PHB based on the work done by *Ahn et al [2]*.

2. Materials and Methods

The whole work was developed with **SuperPro Designer v8.5** (*Intelligen, Inc) [3]*. The mathematical modelling was addressed with **MATLAB 2011** (*MathWorks*) and supported with **Excel 2016** (*Microsoft)* to modify and create the graphics *[4][5]*.

3. Scaling Up and Bioreaction

Bioreactors are wide-spread devices that provide a biologically active environment where microorganisms can grow comfortably. Lots of industries take advantage of this systems to produce a wide range of products which can go from biopharmaceutical ones to plastic ones *[6]*. In this particular case, PHB is produced on a recombinant *Escherichia coli* GSC 4401 as an **intracellular non-growth associated product** which will require and extensive downstream purification (*as addressed in Part III*) *[7]*.

In general terms, two bioreactors are fed by an upstream process. Each bioreactor has a total **operational volume** of 80m^3 (*see Annex 6.1*) and the final volume of inoculum is 8m^3 which splits into to flows that feed equally each bioreactor meaning the volume of inoculum per reactor is 4m^3. The bioreaction is divided into two stages: **growing phase** and **production phase.** The change from one stage to another is induced by a modification in the dissolved oxygen concentration. Both the scaling up process and the bioreaction use as substrate purified whey which comes from a reverse osmosis.

The full review of the process is addressed in the following sections. *Everything that will be discussed is in terms of operational volume*.

3.1 Scaling Up

Biomass inoculum is usually stablished at 10% of its operational volume. As the PHB production reaction at the final bioreactor would include an initial batch stage followed by a fed-batch and a semicontinuous perfusion, inoculum for the 80 m^3 bioreactor has been considered only as 10% of the 8m^3 volume used for the initial growth batch. Biomass concentration for the inoculum would be the same as the objective for the final batch, 30g/L, thus giving an initial biomass concentration of 3g/L, 10% of the finally achieved as it is the inoculum volume proportion respect that of the batch.

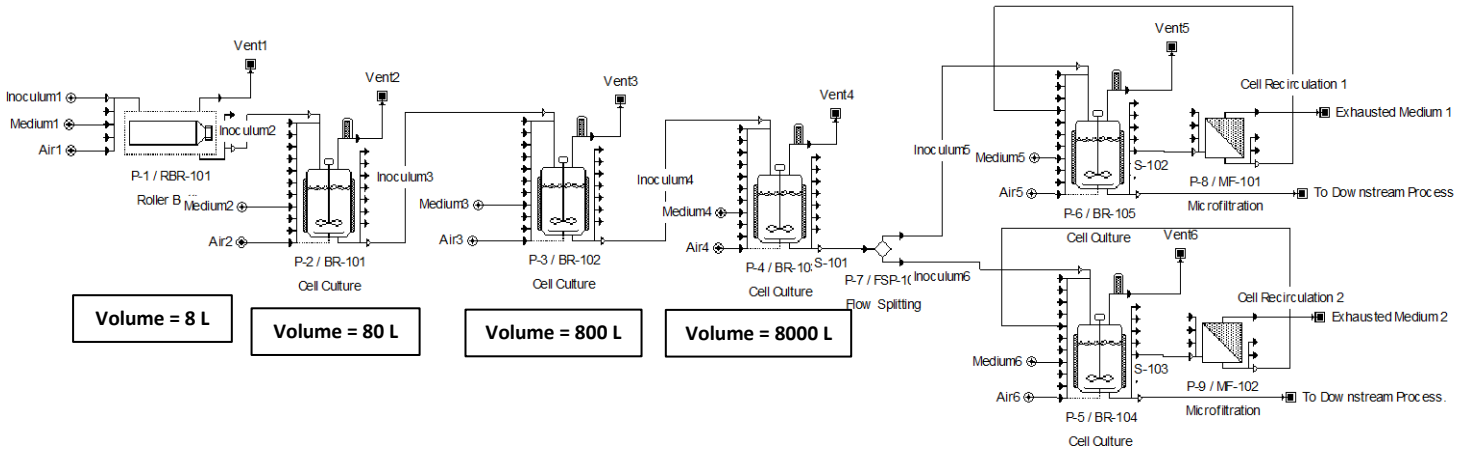

Figure 1. Scaling Up and Bioreaction Flow Process Diagram designed with SuperPro Designer v8.5.

As said, inoculum for the production bioreactors would consist on **8m^3 of 30g/L** which will split and feed equally each bioreactor with **4m^3**. The biomass inoculum would result from a batch growth reaction on 8000L of 30g/L biomass. This biomass inoculum would result from a batch growth reaction on a 800L bioreactor, which at the same time needs an inoculum of 10% of its volume at the final biomass concentration, giving an initial biomass concentration within the bioreactor of 10% of the final. This means that, if the process starts with an initial laboratory-scale inoculum of 8L with 30g/L biomass three more reactors (as shown in *Figure 1)* would be needed in order to achieve enough inoculum for the 80m^3 reactors. These reactors with same species concentrations (biomass, glucose, ammonium chloride and salts) would be initially charged with **40%** of its volume using the culture medium specified in *Part I* and achieving the **50%** of the final volume with the final inoculum (36m^3 of culture media and 4m^3 of inoculum). It has to be taken in count that there is **no PHB production** during the scaling up process giving the fact that there is no manipulation in the oxygen concentration.

3.2 Bioreaction

Preceded by the upstream section, there is the bioreaction during which the microorganism grows and produces the desired product. In this case, the bioreactors would be operated in three different modes: **batch**, **fed-batch with exponential addition** and **semicontinuous perfusion**. This methodology minimizes substrate inhibitions as well as reaching higher levels of biomass *[8]*. As said before, PHB is a non-associated product so the bioreaction is divided into two basic phases:

- **Growth phase:** It is the phase that comes immediately after the scaling up. It would start with a batch and when the substrate is nearly or totally consumed an exponential flow of whey would start feeding the biomass in order to achieve greater levels of it.
- **Production phase:** Consists of a semicontinuous perfusion. With the desired concentration of biomass, a change in the D.O. is induced causing intracellular PHB accumulation.

The bioreactors would be parallelly disposed and alternatively feed the downstream process, increasing the productivity giving the fact that dead-times are reduced (*Part III*).

3.2.1 Bioreaction: Set up and culture conditions

As said before, a recombinant *Escherichia coli* with a PHB production gene coming from *Alcaligenes latus* is used. The cultures would be carried out at **30ºC**. The culture would have a pH set point of **6.95** as well as a pH dead band of **0.1** and it would be regulated automatically by NaOH/HCl addition. The oxygen concentration would be maintained at **30%** *[2]*. Therefore, an oxygen enriched air would be needed at this point. Air (or oxygen enriched air if needed) would be used to aerate the reactors. *Due to technical problems with SuperPro Designer v8.5 it was not possible to represent those effects as it will be explained in the coming sections*.

Each of the scaling up reactors and the producing ones would be made of **concrete** except for the initial erlenmeyer which would be made of **glass**. Both concrete reactors and erlenmeyers could be reused by just cleaning and sterilizing them. The motives of this decisions are completely explained in *Part IV*.

3.2.2 Bioreaction: Kinetics model

The scaling up reactors and the production reactors follow the next growth kinetics *[2]*:

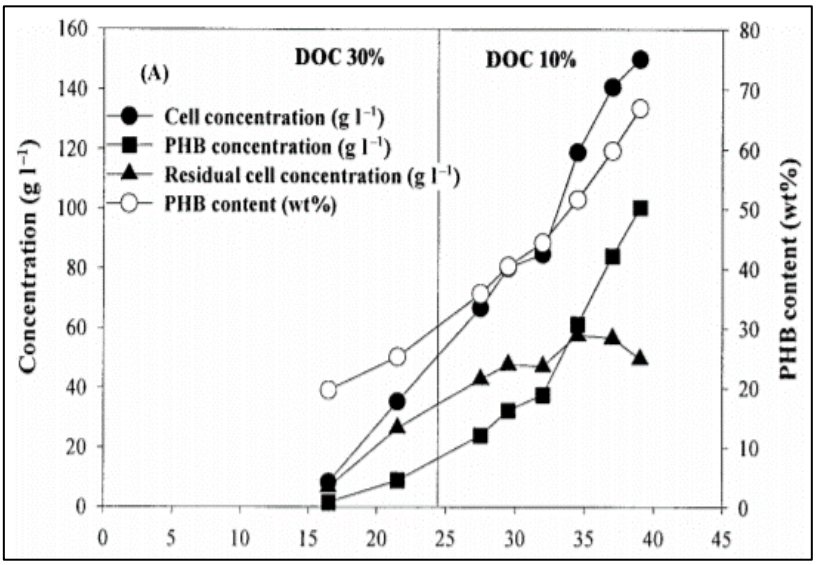

Figure 2. Growth kinetics of recombinant E.coli GSC 4401 and PHB accumulation.

The growth was represented as a **Monod-based model**:

$$= \frac{}{+} \qquad (1)$$

The maximum growth rate was extracted from the graphic by manually adjustment resulting in **0.8 h^{-1}**.

As it can be seen, there is a lag phase which lasts 16 hours approximately. After this period cells start to grow at an exponential rate. At this point, there is a change in oxygen concentration inducing metabolic changes in the **citric acid cycle (CAC)** and causing PHB accumulation *[9]*. PHB content starts to increase as pure cell biomass (Cell biomass-PHB biomass) starts to decrease. Cells focus their metabolism on PHB production and cell duplication is greatly reduced. That is reason why cell net weight (and in consequence the concentration) would increase at the same time there is little cell duplication. Using this basis, it was possible to simulate the production of PHB as explained below.

3.2.3 Bioreaction: Growth phase

This stage is ruled by the following stoichiometric equation (*see Annex 6.2)*:

. + . + . → + . + . ⁻ + . (2)

Where = 2.04 0.48 0.23 *[10]*

As said, the first step of the bioreaction is a pure growing batch which has an initial volume of **40m^3** (*see Annex 6.2.1)***.** The initial biomass concentration is **3g/L** and the initial concentration of whey is **60g/L** (standard recommended concentration). The biomass/substrate yield is **0.52g/g** *[2]* and the maximum growth rate is **0.8h^{-1}.** Finally, the constant which controls the affinity for the substrate ( ) was set **0.5 g/L** (recommended) obtaining the next profile:

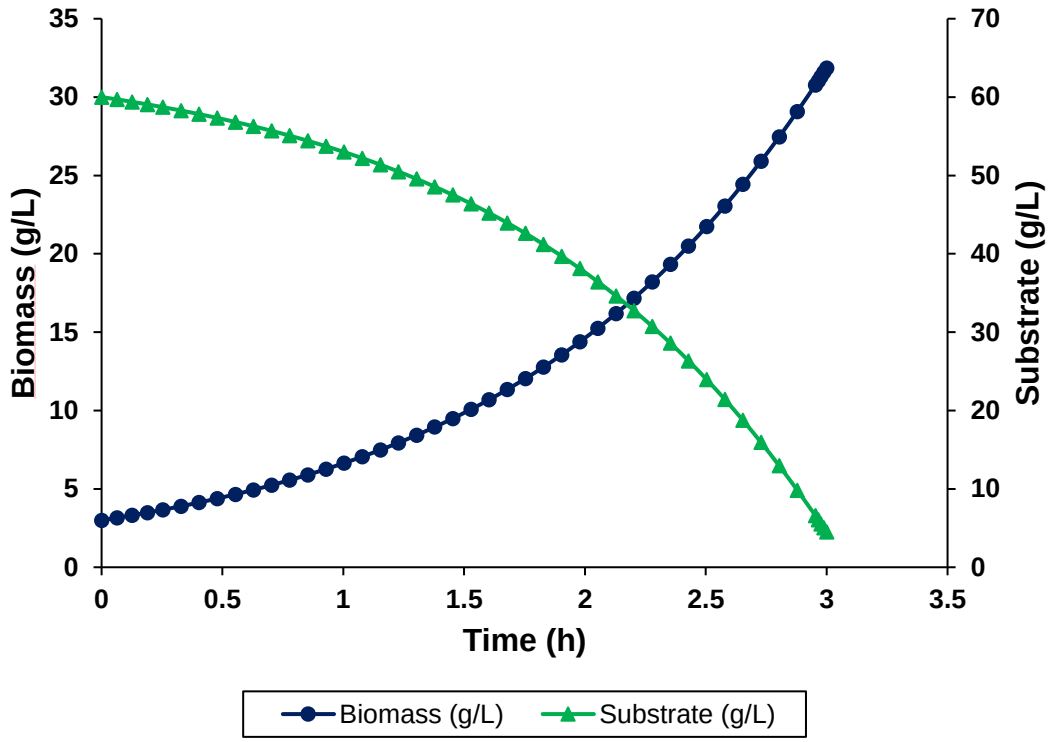

Figure 3. Estimated growth with MATLAB (MathWorks) of *E.coli* GSCC 4401in batch.

A final concentration of **30g/L** of biomass is achieved as well as consuming nearly all the substrate. At this point is when an exponential feeding flow is fed into the reactor. As the substrate inhibitions are minimized the concentration would be increased, following the work done by *Ahn et al*. At the same time the parameters that rule the bioreaction are adjusted. It is supposed that the yields are constant but the substrate concentration is increased up to **280g/L**. Maximum growth rate is reduced down to **0.3h^{-1}** and the reactor would operate at a constant growth rate of **0.22h^{-1},** exhibiting the next graphic (*see Annex 6.2.2)*:

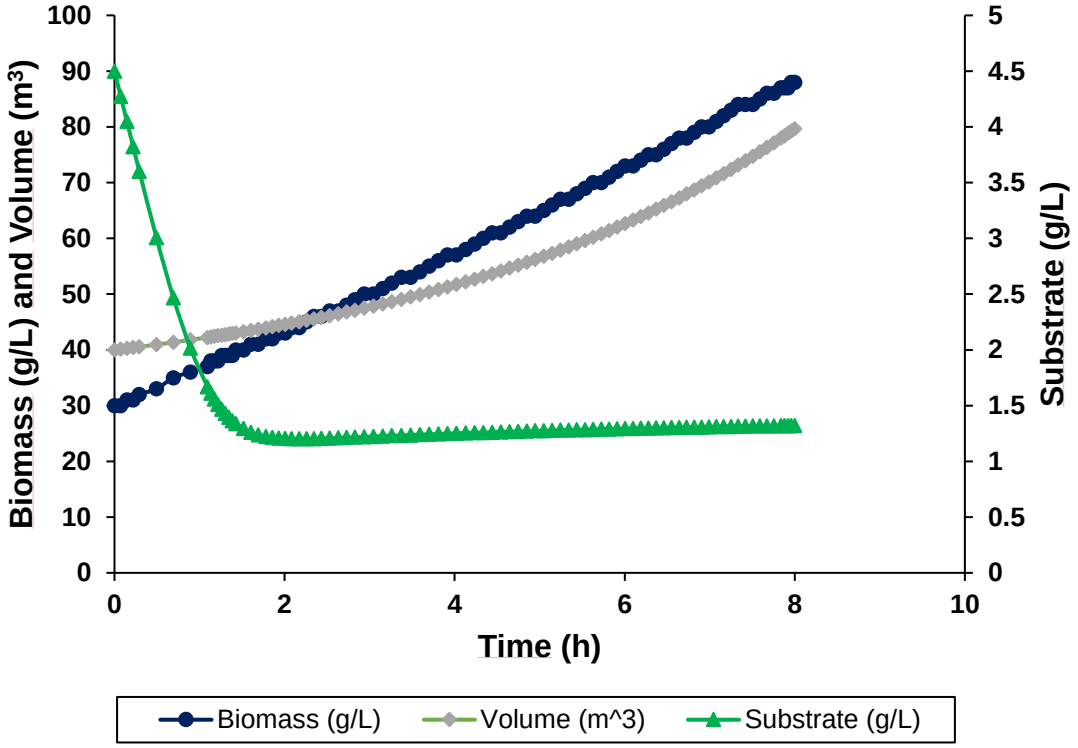

Figure 4. Estimated growth with MATLAB (MathWorks) of *E.coli* GSCC 4401in fed-batch.

Knowing it has an addition an no exit the volume increases exponentially to maintain a given growth rate. The substrate has an asymptote at 1.5g/L. Nevertheless, the volume and the biomass keep increasing until they reach **90g/L** and **80m^3** respectively. That would be the final operational volume.

3.2.4 Bioreaction: Production phase

The stoichiometric equation that rules this stage is shown (*see Annex 6.3)* :

. → . + . (3)

Where = 2.04 0.48 0.23

The coefficients of the reaction have been directly extracted from the biomass/substrate yield. It was supposed that there was no cell growth during this phase, the yield does not change, and it was all converted to PHB. In this last stage the reactor is operated in a **semicontinuous perfusion mode** which is represented in the next figure *[2]*:

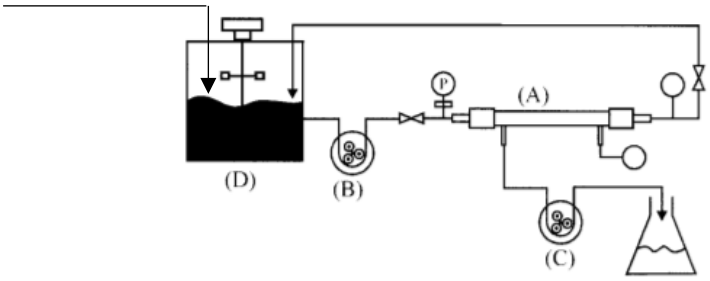

Figure 5. Perfusion system where (A) is a cell retaining filter, (B) is a pump, (C) is another pump and (D) is a bioreactor *[2]*.

A semicontinuous perfusion works the same way as a continuous perfusion. There is only one difference: there is no bleeding. It would be operated as:

1) A constant flow would enter the bioreactor at the same rate it is removed maintaining a constant volume inside the bioreactor.

2) Thanks to a cell retention filter no biomass is extracted from the bioreactor. So, only used-up media is extracted.
3) Cells keep growing until they achieve the desired concentration of biomass. At this point, the bioreaction ends and the content is transferred to the downstream process.

A continuous perfusion system operates in the same way, but it differs in one key aspect: when cells reach the desired **specific perfusion rate** (**CSPR**), a periodically bleeding process is started *[11]*. Using this strategy cells are maintained in **stationary phase** until the cell retaining filter is no longer usable. So, they are constantly producing at a given rate. It would not be possible to implement this strategy in a PHB production plant because it is amassed intracellularly so maintaining cells in stationary phase would not mean any improvement. In this phase is when there it would be a manipulation in the Dissolved Oxygen which would go from 30% to **10%**. There are three different ways by which the oxygen concentration can be reduced/increased and in consequence its mass coefficient (**K_La**) *[12]*:

- Reducing/increasing the air flow in.
- Reducing/increasing the percentage of oxygen that is contained in the air.
- Reducing/increasing the agitation.

The only way that it could be done in *SuperPro Designer v8.5* was varying VVMs (Volume of air under standard conditions per volume of liquid per minute). Supposing that VVMs are always proportional to the dissolved oxygen in the liquid, VVMs were reduced from 1 to 0.3 *[13]*. As reported, feeding should be done by pulses *[2]* but there was no way of reproducing that effect on *MATLAB 2011 (Mathworks)*. Those pulses increase with biomass so the more frequent they are the greater the biomass is. In fact, at some point pulses are that frequent that there is almost no time difference between them. Following that trend, it was decided that they would be modelled as a constant flow (*see Annex 6.3.1)*. The feeding flow would contain the same amount of whey as the fed-batch, **280g/L.** The initial biomass concentration would be **90g/L** and the yield would still be the same ($Y_{x/s}$=0.52). The maximum growth rate is reduced to **0.1**h^{-1} and the reactor would work with a growth rate of **0.065**h^{-1}. At the end of the bioreaction **87%** of the total biomass would be intracellular PHB. As it is an intracellular product and no kinetics are described, it was supposed that PHB increased proportionally to the biomass. Thus, the PHB final concentration is equal to the final concentration of biomass multiplied by a **factor of 0.87** *[2]*. Biomass increases, reaching a final value of **190g/L**. The entry flow is equal to exit flow, so the volume remains at **80**m^3**.** The substrate is consumed and reaching an asymptote of **0.9g/L**. After the whole process a concentration of **171g/L** of PHB is reached:

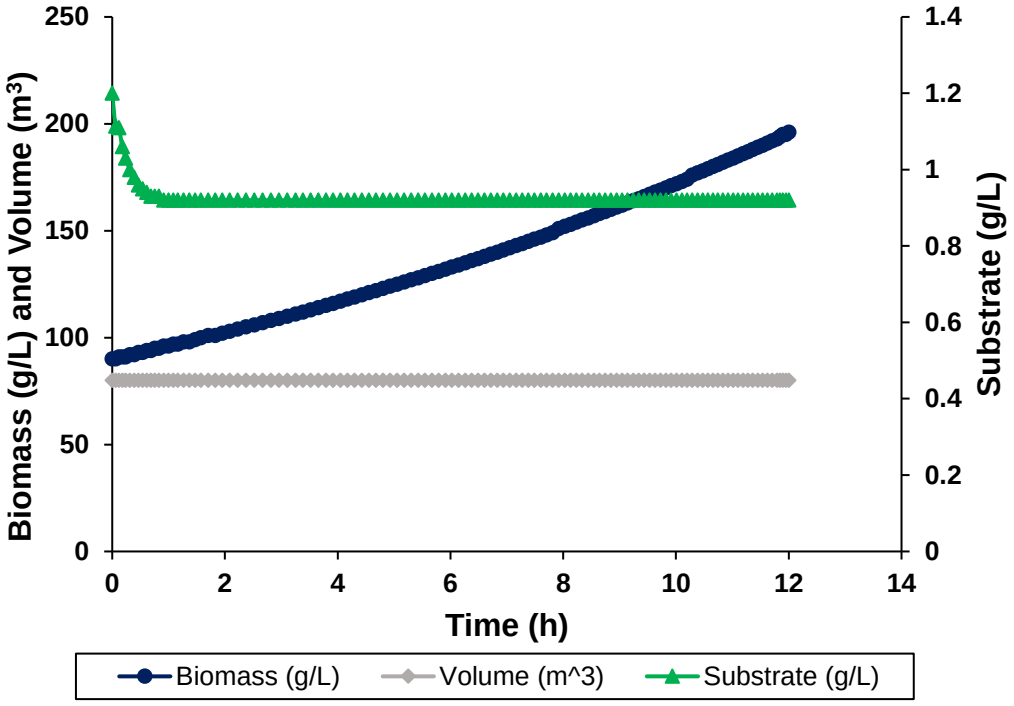

Figure 6. Estimated growth with MATLAB (MathWorks) of *E.coli* GSCC 4401in a semicontinuous perfusion.

3.2.4.1 Bioreaction: Cell retention system

The cell retention system is basically a **cross-flow microfiltration** (also known as **tangential flux filtration**). This mechanism of filtration consists of a central sample channel with a filtration mechanism separating adjacent filtrate channels. A constant turbulent flow along the membrane surface prevents the accumulation of matter on the surface itself lengthening its usable time. Usually, the membranes are contained on the inside wall of the tubes. The suspension flows inside the tubes. If the particles are larger than the pores only a continuous flux of medium gets through it. In fact, most industrial membrane processes are tangential filtrations *[14]*.

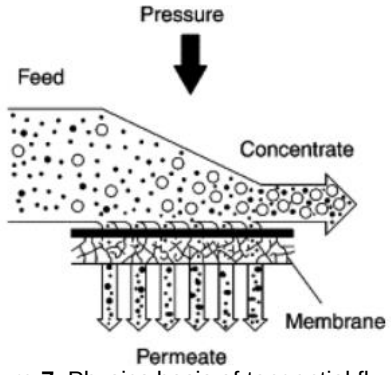

Figure 7. Physics basis of tangential flux filtration *[15]*.

The membrane would be externally located from the bioreactor and it would have a **cut off** of **500.000 Da** to retain the cells and let flow through the exhausted medium. The membrane would contain **polysulfone hollow fibers** which is an organic polymeric matrix. It has a **nominal pore size** of **0.1µm** and it is mechanical and thermally stable *[16]*. The total filtration area of each one of the filters would be **78m^2** (**156m^2** if we take in count both) and the total filtrated volume per unit of time would be set at: **0.61 m^3/h**.

Each m^2 of filter would have a cost of around **70-80€**. The lifespan would be around **10 years** and it would require just **1 extensive cleaning procedure each year** (using forward/backward flush and chemical cleaning processes) *[17]*. The information was kindly provided by *Suez Water Technologies & Solutions*.

3.2.5 Bioreaction: Control

Dissolved Oxygen is one of the most important parameters in this bioreaction as it defines the metabolic shift: from growth to production. Hence, having a precise tracking of the percentage of oxygen that is dissolved in the reactor is vital to minimize economic loss.

Concurrently, pH would also be fine-tuned since there is a dead band of 0.1 (meaning pH could vary from 6.85 to 7.05). Many ions intercede in the reactions that occur modifying the values. So, pH control would be implemented.

3.2.5.1 Control: D.O. control

There are two basic types of sensors: electrochemical ones and optical ones. Both are high performance probes but differ in one thing:

- Electrochemical sensors have a gas permeable membrane on their bottom part. Oxygen must be transferred from the liquid phase to the solid phase. Inside the membrane there is an electrode which gets oxidized when it contacts with O_2 generating a change in the voltage which turns into an electrical signal that is received by the D.C.U. That means there is a **lag time** of approximately **30 seconds** between the oxygen transference to the solid phase and its detection *[18]*. Whereas optical sensors have no lag time due to the absence of a membrane→ they have a higher cost.

Oxygen should have a real-time measurement even if it means a higher inversion so an **optical sensor would be more suitable** *[19]*.

When dissolved oxygen is not a vital parameter in a bioprocess a **P.I.** control is commonly implemented. High values of the **Proportional** part reduce dramatically excessive responses and the **Integrative** part contributes to get rid of the residual **error** but leaving an **oscillating** curve of D.O. Having said that, it would

be appropriate to implement a **P.I.D.** controller to reduce the **offset** value to 0 and to minimize the oscillating effect of the integrative part.

3.2.5.2 Control: pH control

As seen in the stoichiometric reaction, growing *E.coli GSC 4401* generates chloride ions (Cl^-). Those ions react with H^+ naturally contained in the water producing HCl which acidifies the culture medium. pH control should be implemented: **P.I.D.** controller. NaOH addition should be done periodically to keep the initial value of pH. A second auxiliary pump of HCl would be needed since chloride ions are only produced during the growth stage. Knowing there is a dead band of 0.1 there is no need of implementing an optical sensor. Using an **electrochemical** probe would be enough to regulate the pH.

3.3 Global bioreaction assessment

As for the upstream the general reactants and their consumption is shown in the next table:

Table 1. Total inlets and outlets of the upstream process.

	Total amount	
	Inlet (kg/batch)	**Outlet (kg/batch)**
Biomass	0.003	243.22
NH_4Cl	119.998	88.483
Whey	467.55	0.287

The outlet of NH_4Cl is in terms of chloride ions giving the fact that the amino group is consumed as a nitrogen source. As it can be seen, 243.22 kg of biomass at 30g/L concentration are produced which would be used to inoculate the producing reactors. Upstream processing lasts **27.1 hours**. Referring to the producing bioreactors:

Table 2. Total inlets and oultets of one producing bioreactor.

	Total amount/reactor	
	Inlet (kg/batch)	**Outlet (kg/batch)**
Biomass	121.61	15278.5
NH4Cl	80	92.896
Whey	139301.48	0

As shown, 15278.5 ———— would be produced. 87% of that biomass would be PHB so the total PHB produced by the bioreaction is: 13292.3 ————. As mentioned previously, the final biomass concentration is 190g/L which means that the final PHB concentration is 171g/L. The bioreactors have an operational time of **44.25 hours** and their **Cleaning in Place** would be set at **12 hours**. The whole scaling up and bioreactions last **64.75 hours**. Thus, two different productivities can be calculated:

1) **Productivity of the whole bioreaction** (Scaling up+Bioreaction+CIP):

$$\frac{171\ -}{64.75} = 2.64\ \frac{}{.}$$

2) **Productivity of the production bioreactors** (Considering the bioreaction itself):

$$\frac{171\ -}{44.25} = 3.86\ \frac{}{.}$$

4. Conclusions

In the last decades, plastic consumption is arising as a social problem. As well as reducing plastic consumption, and in consequence petroleum consumption, our PHB production plant would deal with the treatment of a common residue in countries like France, which is whey. There would not even be water consumption as the production plant would be strategically located near a cheese production plant and all the water would come from whey itself. Therefore, it is a sustainable operation since the substrate that would be used is an abundant and pollutant residue which has high costs of depuration on account of its organic load.

Quantitatively speaking, **30557 kg of PHB were produced in a time lapse of 64.75 hours** leading to a PHB concentration of **171g/L**. Considering just the producing bioreaction duration, a **productivity of 3.86** $\frac{g}{L \cdot h}$ would be achieved reaching our production goal stated in *Part I*. On top of that, the theoretical productivity would be amongst the highest that are described bibliographically which are about 4 $\frac{g}{L \cdot h}$ (all of them at laboratory scale) *[20]* .In the next table a summary of the whole bioreaction can be seen to have a global idea of this part of the design project:

Table 3. Summary table of the whole bioreaction estimated with MATLAB (Mathworks).

	Operation mode	**Reactor Volume$_0$ (L)**	**S_0 (g/L)**	**Biomass$_0$ (g/L)**	**Biomass$_F$ (g/L)**	**PHB$_F$ (g/L)**
Stage 1	Batch	40000	60	3	30	-
Stage 2	Fed-batch	40000	280	30	90	-
Stage 3	Semicontinuous perfusion	80000	280	90	190	171

Like it has been said several times, dissolved oxygen is the basic critical parameter of the bioreaction. Hence, smooth oxygen probes like the optical ones would be needed in order to monitor the bioprocess and to have a high-quality control over it→ P.I.D. controller. To complement that regulation, it would be required a pH probe that maintains stable the pH value in the given range contained in the dead band, adding NaOH/HCl when needed.

Lastly it is important to remark that PHB is an intracellular biopolymer. Consequently, a complex downstream process would be demanded. Since our PHB production plant would primary produce PHB for a high range of applications that go from plastic bags to medical material, a high purity must be reached. The economic parameters that determine whether it could turn into reality or remain as an idea are all discussed in *Part IV*.

5. References

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BIOPROCESS DESIGN OF A PHB PRODUCTION PLANT
PART II: SCALING UP AND BIOREACTION

Miquel Bistué Rovira, Carlos Martínez Martínez, Melisa Maurino Reyes & ***Pol Pérez Rubio***

Final Degree Project of Biotechnology (2018 – 2019)
Tutored by ***Carles Solà i Ferrando***

Abstract

The aim of this project is to design an industrial polyhydroxybutyrate (PHB) production plant using residual whey as bioreaction substrate. Whey is a largely produced by-product of cheese-making and it has been proven to be harmful to the environment if disposed onto farmland or waterwaste. PHB is a biodegradable polymer which can be used in products ranging from plastic bags to cutting edge surgical stitches or even prothesis. This part is focused on the strategies used to enhance PHB production based on the work done by ***Ahn et al.***[1]

Scaling Up and Bioreaction Flow Process Diagram

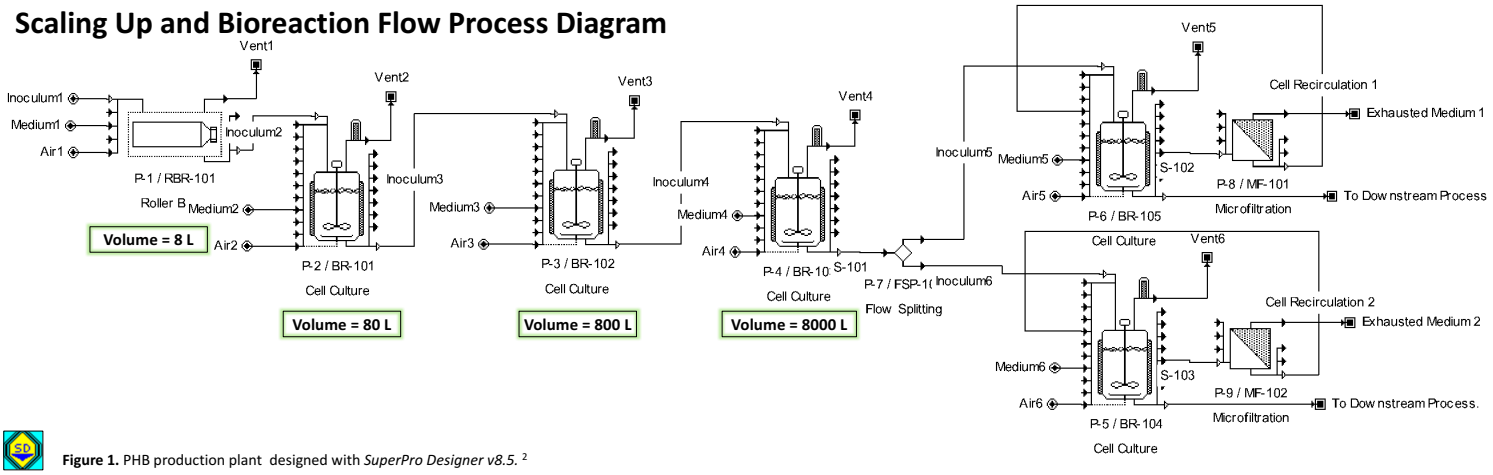

Figure 1. PHB production plant designed with *SuperPro Designer v8.5.* [2]

Bioreaction: Growing phase

$$0.23NH_4Cl + 1.68C_{12}H_{22}O_{11} + 0.64O_2 \rightarrow Biomass + 0.68CO_2 + 0.23Cl^- + 0.98H_2O$$

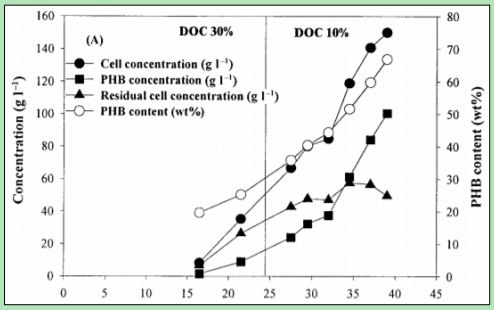

Figure 2. Growth kinetics of *Escherichia Coli* GCSC 4401 and intracellular PHB accumulation. [1]

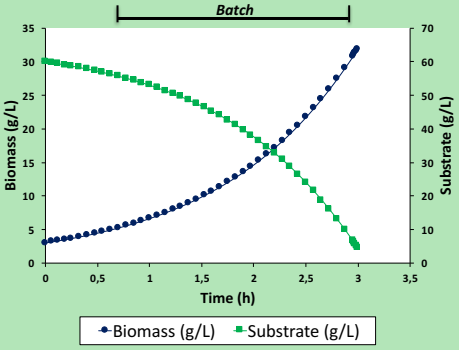

Figure 3. Estimated growth with *MATLAB (MathWorks)* of *E.coli* GSCC 4401 in batch. [3]

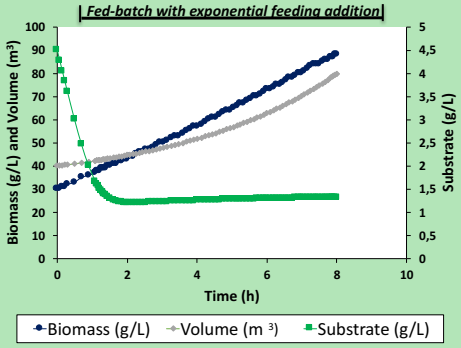

Figure 4. Estimated growth with *MATLAB (MathWorks)* of *E.coli* GSCC 4401 in fed-batch with exponential feeding. [3]

Bioreaction: Production phase

$$3.51C_{12}H_{22}O_{11} \rightarrow 2.09Biomass + 1.09CO_2$$

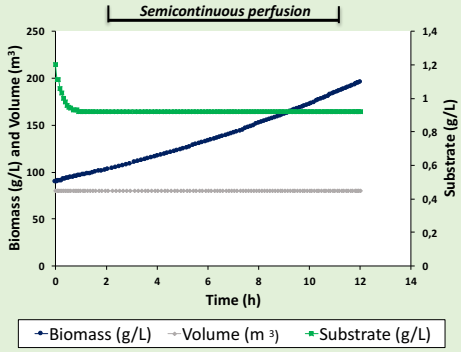

Figure 5. Estimated growth with *MATLAB (MathWorks)* of *E.coli* GSCC 4401in semicontinuous perfusion. [3]

- → Bioreactor feeding in the last stage is done by **pulses**.
- → Cells keep growing until the **oxygen concentration is manipulated** going from 30% to 10%. At this point cells start to generate PHB.
- → The filter cutoff is **500.000 Da.**
- → At the end of the bioprocess **87% of the biomass is PHB.**

Table 1. Summary table of the whole bioreaction.

	Operation mode	Reactor Volume$_0$ (L)	S_0 (g/L)	Biomass$_0$ (g/L)	Biomass$_F$ (g/L)	PHB$_F$ (g/L)
Stage 1	Batch	40000	60	3	30	-
Stage 2	Fed-Batch	40000	280	30	90	-
Stage 3	Semicontinuous perfusion	80000	280	90	190	**171**

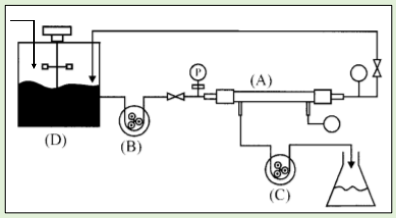

Figure 6. Perfusion system where (A) is a cell retaining filter, (B) is a pump, (C) is another pump and (D) is a bioreactor. [1]

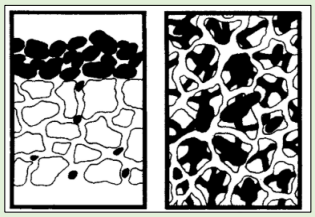

Figure 7. Cross section (left) and top section (right) of an schematic microfilter. [4]

Bioreaction control

- DO is the most important factor to monitorize in this bioprocess so there is a DO control implementation→ **P.I.D. controller.**
- The growth decreases the pH so NaOH has to be added →**P.I.D. controller.**

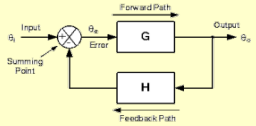

Figure 8. Closed control loop.

Conclusions

- ✓ The bioreaction produces 171g/L of PHB in 44 hours which leads to a **3.86** $\frac{g}{l \cdot h}$ productivity (one of the highest described).
- ✓ The critical point of this bioprocess is controlling the Dissolved Oxygen.
- ✓ PHB is an intracellular component so a complex downstream is needed in order to purify it.
- ✓ It is a sustainable operation giving the fact that the substrate is an abundant and pollutant residue → high cost of depuration.

References

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BIOPROCESS DESIGN OF A PHB PRODUCTION PLANT
PART III: DOWNSTREAM AND TIME MANAGEMENT

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Final Degree Project of Biotechnology (2018-2019)

Tutored by *Carles Solà i Ferrando*

TABLE OF CONTENTS

1. ABSTRACT

During the last century we have created a plastic dependent lifestyle, consuming each year an increasing quantity of it, leading to severe environmental issues. Over 270 million tons of plastic residues were generated in 2017. Plastic not only is a non-degradable material, but it also comes from petroleum, which is a non-renewable resource which reserves are shrinking day by day, so an alternative had to be proposed. There are many biological substitutes for plastic, from which polyhydroxybutyrate (PHB) has been chosen for this project due to their specially suitable properties and wide and well-developed production.

PHB is a biodegradable and renewable polymer with plastic properties, commonly produced by many bacteria species under specific conditions as carbon storage. It can be synthesized from a wide variety of substrates ranging from pure glucose to many organic residues. Using residues as substrate constitutes a necessary strategy in order to achieve a profitable bioprocess in a very competitive scenario. Cheese whey residue has been the one proposed in this project, as it is an abundant residue from the dairy industry and it has become a serious environmental problem over the years for its high organic load.

To summarise, this project aims at producing PHB using whey residue as a substrate, thus helping with two environmental concerns while trying to make a profit at the same time.

This part of the project will aim its focus on the purification process of the stream coming from the bioreaction where PHB is produced, the time management and equipment occupancy of the industrial plant, the layout design and the methodology for product analysis.

2. MATERIALS AND METHODS

A wide variety of programmes and software were used to develop the PHB production plant design.

To develop the downstream block flow diagram, draw.io from JGraph LTD was required. For most of this project, a programme called Superpro Designer v8.5 from Intelligen, Inc. was required not only to generate and design the flow diagram but also to calculate stream composition, economic evaluations, environmental evaluations and equipment occupancy. PowerPoint 2016 was the programme used to design the layout of the final production plant.

3. DOWNSTREAM PROCESS

3.1. How it is usually produced

Generally, PHA purification (and in consequence P(3HB) purification too) contains several steps, including precipitation, drying, powdering, extraction and centrifugation processes. In **Figure 1** it can be seen the general methodology used to purify a PHB stream.

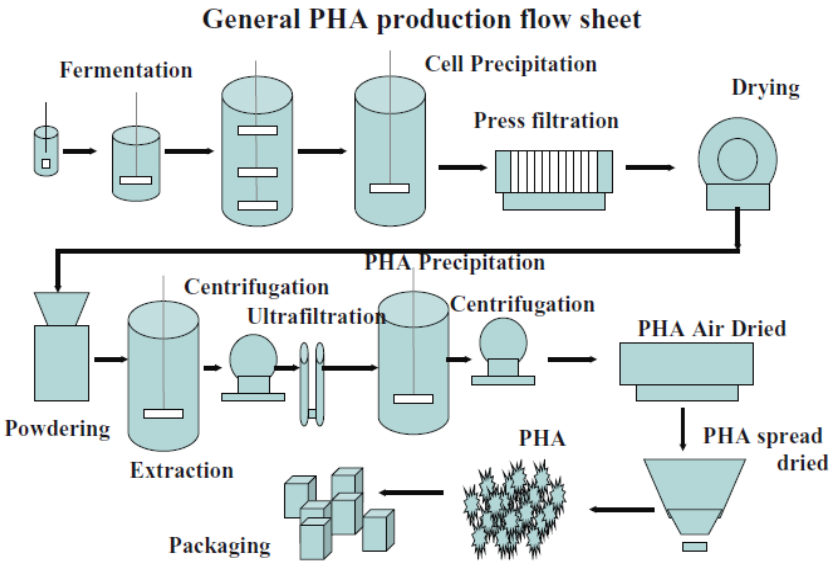

Figure 1 General polyhydroxyalkanoate production and extraction process.

3.2. Downstream block flow diagram

Figure 2 shows the block flow diagram of the downstream process for purifying P(3HB) that has been chosen in this project. It is a simplified version of the classic procedure, but it is expected to achieve high percentages of purity without sacrificing a high yield. It consists of four procedures; centrifugation, homogenization, differential extraction and drying.

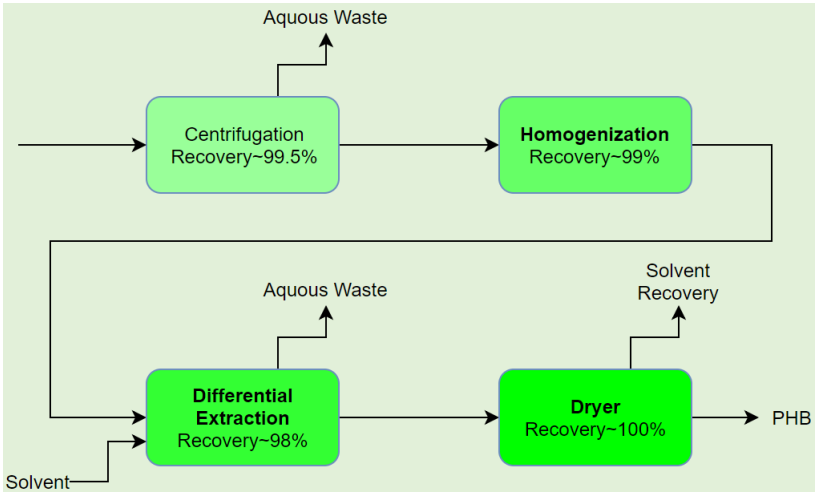

Figure 2. Downstream Process Block Diagram. (Own source)

3.3. Downstream equipment

Purification of PHB would work as a semi-continuous process meaning that when PHB coming from bioreactor nº1 had been purified, another batch from bioreactor nº2 would enter the same downstream process. Using the same downstream units for the two bioreactors would conclude in half the expenses regarding the purification process. This means that each process would have two batches of downstream procedure, meaning that the final production of PHB produced in the following process must be doubled in order to correctly show the real productivity of the whole procedure (as shown in the Gantt diagram in **Figure 7** below).

PHB purification process would specifically consist of a decanter centrifuge, a bead milling process, a liquid extraction process and a spray dryer.

3.3.1. Centrifuge

The centrifugation process refers to concentrating the PHB rich biomass using a decanter centrifuge. Centrifugation speed would be set at 5000G which has been proven to be safe for *Escherichia coli*. Setting a safe centrifugation speed must not be ignored regarding the fact that for *E.coli*, velocities surpassing 15000G have been proven to be harmful to cell integrity (Peterson *et al.*, 2012). In 3.5 hours of centrifugation period, 99.5% of biomass recovery can be achieved (Ling *et al.*, 1997). Chilled water is used to maintain the temperature at 39º and do not let it rise too much so that it can affect the PHB stability. The centrifuge would be made of carbon steel. By using this procedure, mass water content would be reduced from 66527 kg/batch to 15748 kg/batch.

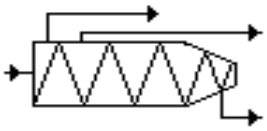

Figure 3. Centrifuge

3.3.2. Bead Mill

Bead milling is the process chosen to disrupt the biomass so that the intracellular PHB turns into extracellular PHB and it can be purified. This equipment has been chosen because even though it has been proven ineffective working with low concentrations of biomass (Tamer, Moo-Young and Chisti, 2002), it is remarkably effective with high concentrations of biomass (which is this bioprocess' case). Two bead mills working in parallel must be used in order to disrupt the amount of biomass present in the stream coming from the centrifuge. The processing time of each bead milling procedure would be 600 minutes and its efficiency in such time is about 99% (Tamer, Moo-Young and Chisti, 2002). Chilled water is used to drop the temperature down to 18ºC. Bead-Mills are made from carbon steel. At the end of this procedure, 13093 kg/batch of PHB would have been extracted from the biomass and 132 kg/batch would remain intracellular.

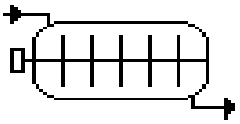

Figure 4. Bead Mill

3.3.3. Liquid extraction

By using differential extraction, it is expected to extract extracellular PHB from previous components such as water, biomass debris, residual whey or residual NH4Cl. Hexane will be the chosen organic solvent for its high solubility with PHB and low solubility with aqueous streams(Manangan and Shawaphun, 2010). It is also ideal because of its volatility, making it easier to evaporate once it has been used. Moreover, hexane will be used as an alternative to chlorate compounds such as chloroform which are toxic and far more harmful to the environment than hexane. The following procedure would focus on extracting the hexane stream and recirculate it onto the liquid extraction. Using that method would not only reduce the amount of hexane bought and in consequence its expenses, but also reduce the hexane emissions to a small residual stream. Since PHB is insoluble in water and highly soluble in hexane, the differential extraction process has an efficiency of 98%, being only 2% the amount of PHB remaining in the aqueous stream (Fei *et al.*, 2016). The processing time for this procedure is 200 minutes. Exit temperature is set at 25ºC. Extractor column is made from carbon steel. The result would be a stream containing 12832 kg/batch of extracellular PHB.

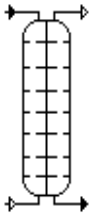

Figure 5. Liquid extraction

3.3.4. Spray Drying

This objective of this procedure would be to evaporate the most quantity of hexane possible. Since hexane is a volatile component, its efficiency is very high, being extracted almost 100% of the initial hexane in a processing time of 6 hours. Spray drying is an inexpensive and scalable process to produce dry powders from liquid streams by atomisation into a high-temperature gas, normally air. As stated before, this amount of hexane will be fed into the liquid extraction. It must be mentioned that, since it is generally a discontinuous process, in the first batch of all, the recirculation stream would be 0 and all the chloroform would have to be fed through the entrance feed. On the second batch and so on, the recirculation stream would be the 99% of the initial hexane entrance and the feed would downgrade to the 1% of its initial value (Barcelos *et al.*, 2014).

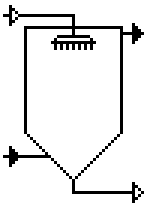

Figure 6. Spray Dryer

3.4. Downstream stream classification and characteristics

Once each process of the downstream was designed, a stream classification regarding the most important components was fabricated and the table is shown below. The quantities represent the amount corresponding to a whole production batch, which has two purification processes, and that is why the quantities of a single downstream process had to be doubled.

Table 1. Downstream quantities

Stream	PHB-rich Biomass [kg/batch]	free-PHB [kg/batch]	Cellular Debris [kg/batch]	Water [L/batch]	Hexane [kg/batch]
Input	30344	0	0	128000	0
Centrifuge Out	30192	0	0	31298	0
Bead-Mill Out	75	26004	3886	31298	0
Extraction Out	0	25485	0	0	31996
Output	0	25485	0	0	3

As it can be seen in **Table 1**, the downstream process is not only successful at purifying a highly cellular dense aqueous stream into a stream containing PHB almost completely pure (99%) but, as it will be calculated, it also provides a high recovery yield. The output stream contains 3 kg/batch of hexane solvent. Hexane is a volatile component and has a high explosion risk, for that reason the final product must be completely free of it. This amount of hexane has been decided to be burnt in an industrial flaming torch regarding the fact that it is covered by the current legislation to have this quantity of emissions. Moreover, this amount of hexane, when burnt, is capable of turning 4.4 kg of water at 25ºC into water vapour at 100ºC. For that reason, it has been proposed to use this flaming torch as a support to the boiler.

Knowing the process yield is important in order to adjust the volume of the different types of equipment for achieving the desired final production value. For doing so, one must know the reached yield in every step of the process and multiply them to know the final yield.

The final downstream process efficiency comes out at **96.5%**, which is high in an industrial process. That means that if we want to produce 1000 t/yr (for example purposes), we must produce approximately 1036 kg/yr per bioreaction process.

The operating time for the purification process takes **24 hours**, and **12.75 tons** of PHB are purified per downstream process which means that batch production comes at around **25.5 tons** of PHB.

Annual production would be set at around 4,182 tons/year, taken into consideration that 164 batches are made in a year and that the plant is working 330 days/year.

4. TIME MANAGEMENT

4.1. Gannt Diagram

As it can be seen in **Figure 7**, the PHB production process takes 3 days; 14 hours of which referring to the upstream, 37 hours to the bioreaction process and 34 hours to the downstream process. It must be considered that there is a superposition of procedure times, and that is the reason that their isolated procedure times take more than 3 days in total.
Even though the global bioreaction process takes 37 hours, each bioreactor has a duration of 31 hours. The second bioreactor starts 6 hours after the first one has started and that concludes in the global bioreaction duration.
Finally, the downstream process would be made from 2 batches procedures, each coming from a bioreactor, and having a duration of 24 hours.

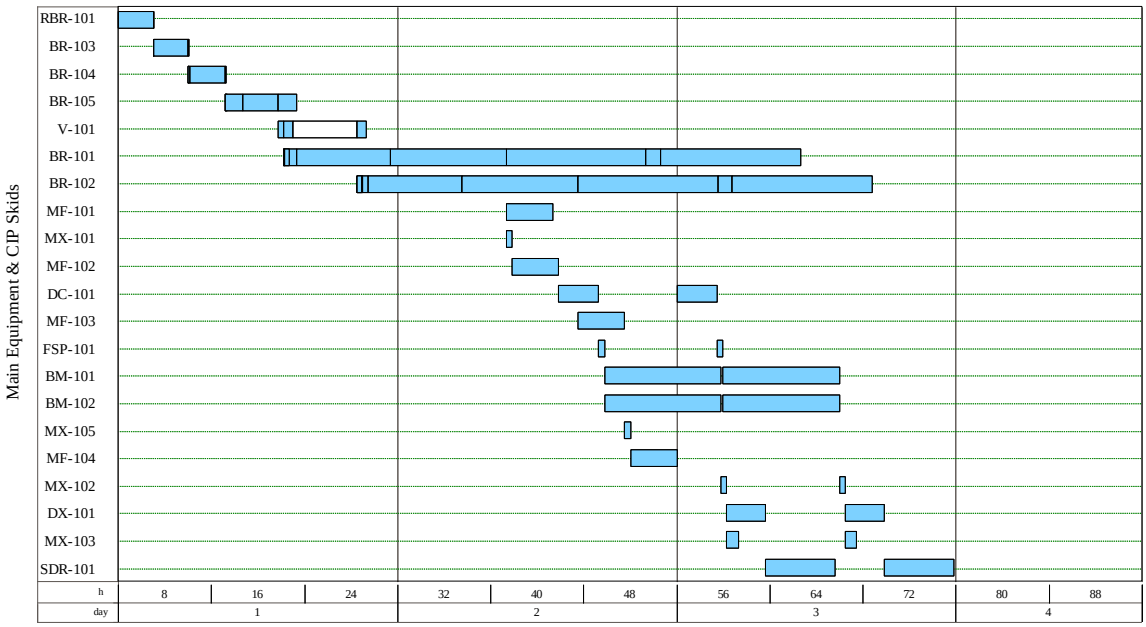

Figure 7. Gantt diagram

Even though the entire process has a duration of 3 days, if the downstream process of a previous batch and the upstream process of the following batch are done simultaneously, the whole process has a duration of 2 days. If 2 days are required to complete a batch and the production plant is working for 330 days, 164 batches are expected to be done in a year.

4.2. Time distribution

In a production process, it is of vital importance to analyse the time distribution and critical steps of the different phases of the process. The reason for it is that knowing the different processing times of each sub-process lets you know which part would benefit from a time optimisation and which part is already optimised to the maximum. In order to improve productivity and overall benefits, it is important to focus on improving the efficiency of those processed that take the longest, which will be called critical steps.

In **Figure 8**, it can be seen the time distribution of the PHB production process, and the critical step and the process that would benefit the most of an improvement is the fermentation phase. Following the fermentation process comes the downstream process. Reducing the time of those two processes would be very beneficial to the productivity of the plant and, because of that, future improvements will be focused on doing so.

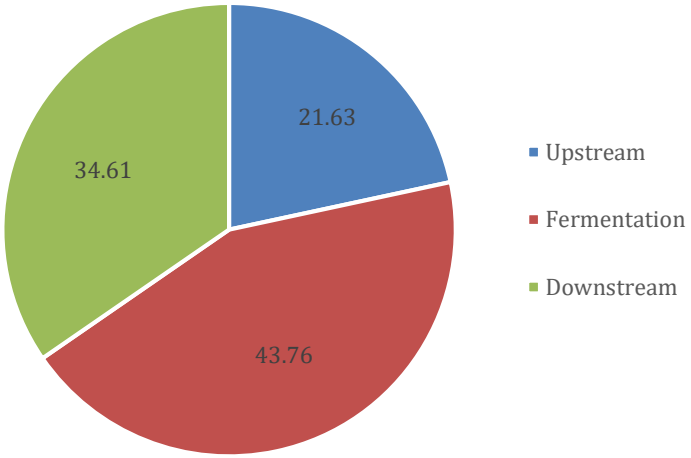

Figure 8. Time distribution for PHB production process

Finally, in **Figure 9**, it can be seen how the time distribution for the downstream process is in particular. It is clear that the homogenization process using a bead-mill is the longest out of all the downstream and consequentially the one that would benefit the most from improvements. For example, cell disruption by using a solvent is a method much faster to lyse the cells, and it could be analysed as an alternative for future improvements.

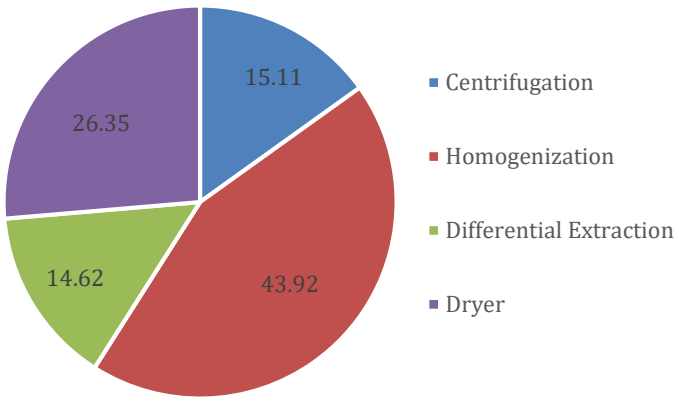

Figure 9. Time distribution for PHB downstream process

5. LAYOUT

The layout and design of a production plant are as important as the methodology and equipment since it can affect the well being of workers and the overall performance of the industrial process. It is vital to take into consideration factors like size and scaling, equipment distribution, special storage conditions for certain reactants, hypothetical future expansion work, staff comfort and much more.

In **Figure 10** it is shown the layout design which has been suggested in this project. It can be seen a raw whey entrance which is concentrated by a reverse osmosis process and stored in a special zone. There is another entrance for the other raw materials such as ammonia chloride and trace elements, and when these compounds get transferred to the zone where concentrated whey is, they get mixed up to generate the final medium for fermentation. The medium gets transported to the upstream area where the first inoculum is grown and scaling up occurs in 4 bioreactors of increasing size (8L, 80L, 800L, 8000L). Fermentation then occurs in the Bioreaction area and the Downstream area is required for the purification of PHB. Hexane must be stored in a separate container since it is inflammable, and it could be a hazard to the entire plant. Once PHB is pure, an area for product analysis is required in order to check if the experimental purity is the same as theoretical purity. Finally, PHB is stored in a special zone from which it can be easily distributed to consumers all around the world.

It has also been added a Research & Development laboratory where the base investigation would take place and an office and dressing room for the employees.

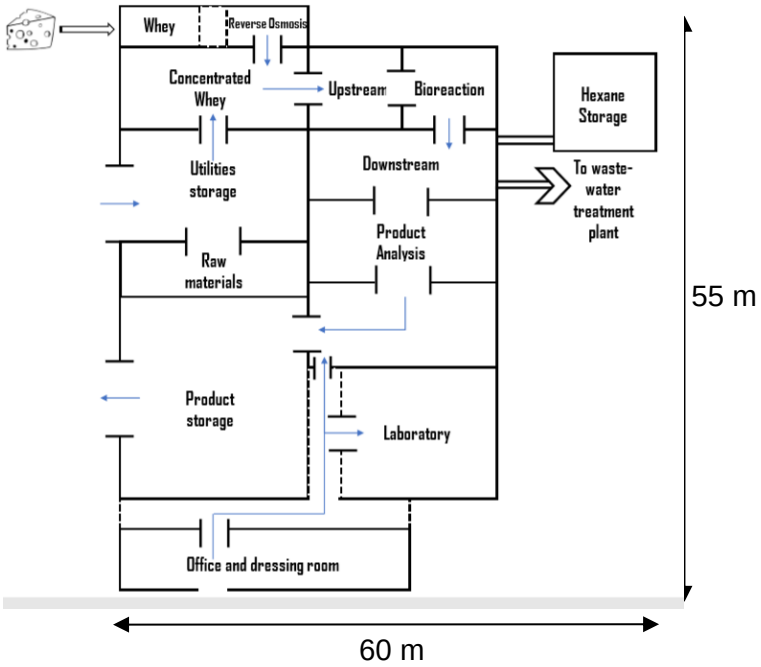

Figure 10. Layout of the PHB production plant

6. PRODUCT ANALYSIS

A quality assurance system must be developed in order to check whether the final product has the experimental characteristics that were predicted in the theoretical analysis. If the desired quality is achieved PHB would be then suitable for storing and distribution. If desired characteristics are not achieved, the production plant would then have to be stopped in order to find where the issue is and proceed to solve it.

There are a large number of methods to analyse, quantify and characterise PHB like staining reactions, spectrophotometric methods, infrared, FTIR spectroscopy, HPLC, gas chromatography, NMR spectroscopy, flow cytometry and spectrofluorometry. (Godbole, 2014)

Gas chromatography is the technique that has been chosen in this project for its robust and trustworthy behaviour. Gas chromatography is a type of chromatography that relies on vaporising compounds and make them run through a chromatographic column so that they interact with specifically with the solid phase. Analysing the resulting peaks of appearance provides a precise determination of the initial compound. (Juengert, Bresan and Jendrossek, 2018).

Gas chromatographers can be used to quantify PHB and to test its monomeric composition. PHB decomposes at temperatures below its boiling point. That is the reason why PHB must be converted into products that are stable and volatile at the temperature of the GC column. In order to do so, it is mandatory the conversion of PHB into volatile hydroxycarboxylic acid methyl esters by acidic methanolysis.

The methyl esters bond specifically with the solid phase, therefore, allowing separation of different hydroxyalkanoate methyl esters in case co-polyesters of several hydroxyalkanoates are being analysed. Measuring the time point of appearance and the area under the resulting compound peak of the detector signals in the chromatogram enables its quantitative and qualitative determination.

Among the advantages of Gas Chromatography, it has been found that gas chromatographers are relatively inexpensive to acquire and operate, they have low maintenance requirements and have high detection ranges for orgànic substances. Obviously, gas chromatography is limited to those compound that not only are volatile, but they are also thermally labile (Al-Bukhaiti *et al.*, 2017).

7. CONCLUSIONS

Bioplastics such as P(3HB) are able to improve the balance between the environmental benefits and the environmental impact of plastics. Life cycle analyses demonstrate that bioplastics can significantly reduce CO2 emissions compared to conventional plastics (depending on the material and application). What is more, the increasing utilisation of biomass in bioplastic applications has two clear advantages: renewability and availability. This could help to solve the problem of the improving levels of microplastic in the oceans,

which nowadays constitute an environmental and global health problem. In terms of health, microplastics could be introduced in the food chain, negatively affecting people and animal welfare.

The latest market data does not only demonstrate the contributions of the industry on moving towards a sustainable future with a reduced environmental impact. The forecast also predicts the budding bioplastics industry to unfold an immense economic potential over the coming decades. This is the reason why we think this project faces the nearest future and its development and implantation in real industries could be beneficial not only for the company but also for the environment.

Specifically, in this part of the project, it has been developed an effective and overall well-rounded downstream procedure which provides a high recovery yield with a high level of purity. But this downstream procedure is not only efficient, but it is also environmentally friendly. The reason being that hexane is the solvent chosen and it has not been proven to be toxic nor harmful to the environment if managed correctly. Hexane recirculation mechanism is also very efficient and less than 1% is not able to get recirculated and remains with the product. The remains of hexane in the final product are burnt in a flame torch, which might not seem environmentally safe, but it has to be taken into consideration that the amount is so small that not only is it permitted by the law, but it is also being used to support the boiler and decrease the total energy expenses.

A time management proposal has also been made and productivity increased by 12%.

Finally, gas chromatography has been chosen as a simple and trustworthy method to analyse product quality.

As optimised as this project might seem, it could benefit from some future improvements:

Time management proposals were analysed manually, which indicates that they could not be the optimal ones. If a time management software was used instead of analysing it manually, there could have been some better proposals that would increase productivity even further.

8. REFERENCES

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9. ANNEXES

Annexe 1: Stream classification and operation calculus

Stream compositions

It has to be taken into account that the following values correspond to one single downstream procedure and each batch would have two downstream procedures. Regarding that fact, all values must be doubled to have the stream quantities corresponding to 1 batch procedure.

Centrifuge in: 81828 litres/batch consisting of 15278 kg of PHB-rich biomass (186.7 g/L). It is all transported in an aqueous stream of 66526 litres of water.

Centrifuge out: 30414 litres/batch consisting on 15202 kg of biomass (500 g/L). The water content has been reduced to 15748 kg per batch.

Bead Mill out: 29563 litres/batch consisting of 1963 kg of cellular debris coming from the cellular disruption and 152 kg of unlysed biomass, 13093 kg (443 g/L) of extracellular PHB. The water content has not changed from the previous procedure.

Extraction out: In the extraction process, PHB has been extracted using hexane as a solvent. It is made of 12831 kg of extracellular PHB and almost 16000 kg/batch of hexane.

Spray Dryer out and final product: In the drying process, hexane is extracted and almost completely eliminated. The stream consists of 11824 litres/batch, the same quantity of PHB and biomass can be observed, and hexane is reduced to 1.6 kg.

Operation calculus

PHB recovery

To obtain the global PHB recovery, all partial recoveries for each downstream process must be multiplied:

99.5% · 99% · 98% · 100% = **96.5%** PHB recovery

PHB purity

$\frac{12831\ kg}{12831\ kg+1.6\ kg} \cdot 100$ **> 99 %** PHB purity

PHB production

330 working days · 1 batch/2 days = 164* batches/year.

* Each batch takes 3 days, but the upstream starts 1 day before the previous batch has ended and takes 1 day. That is the reason why every batch takes 2 days but the first one takes 3 days, and that is the reason why 164 batches are made in a year instead of 165.

2 downstream procedures/batch * 12.75 tons PHB/downstream procedure = 25.5 tons PHB/batch

25.5 tons PHB/batch * 164 batches/year = **4,182 tons PHB/year.**

Hexane waste heat energy

1500 g hexane/day · 7.7 KJ/g hexane = 11415 KJ/day → Heat produced by burning hexane.

2257 KJ/kg H2O → Heat required to boil 1 kg of water.

4.175 KJ/kg H2O → Heat required to increase 1ºC to liquid water.

11415 - X = m · 2257

X = m · 75 · 4.175

If we solve the system above, m= 4.4kg of water. 4.4 kg of liquid water at 25ºC can be turned into vapour at 100ºC every day with the heat coming from the hexane residue.

Time management productivity increase

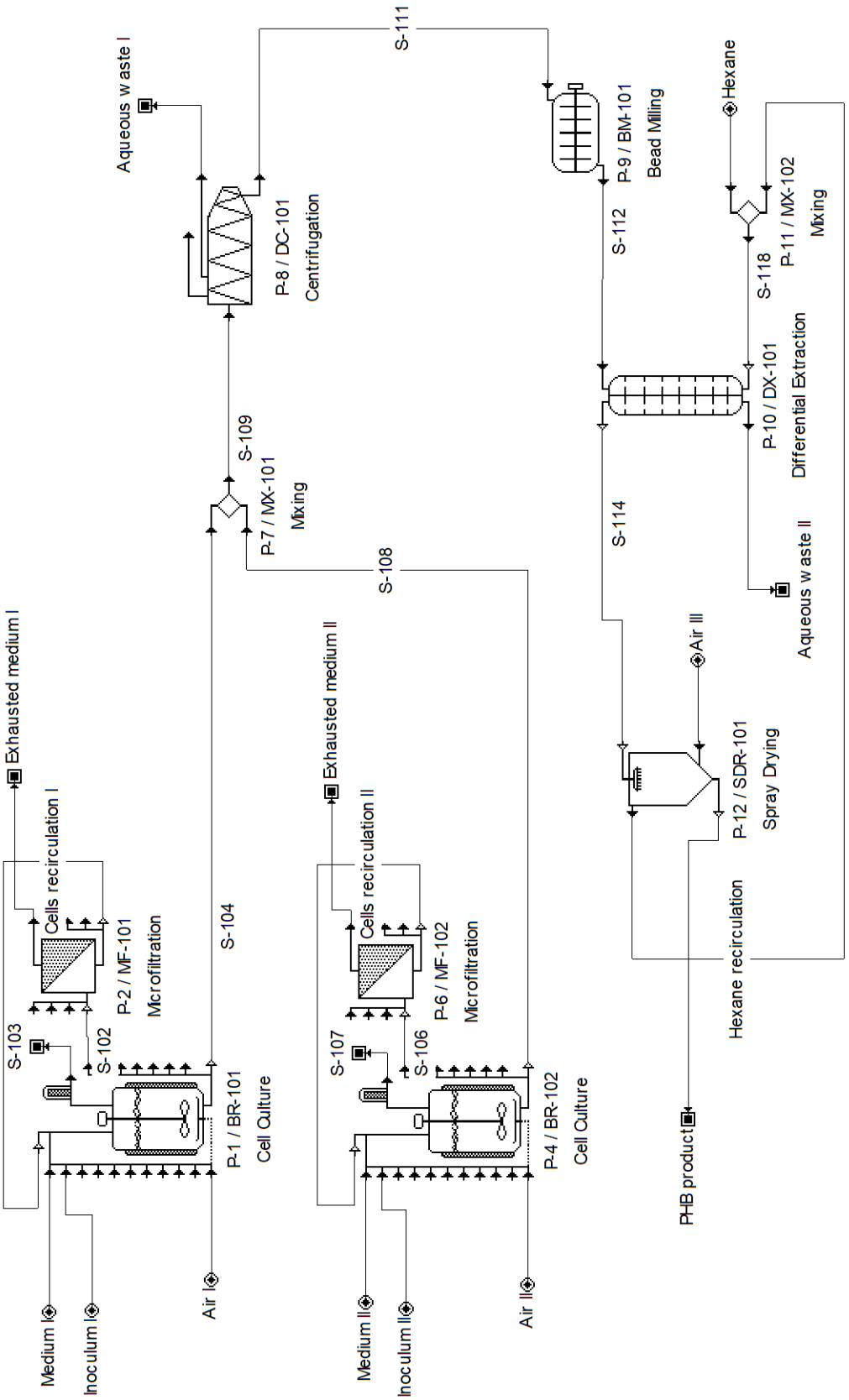
Before time management → At first, it as decided as an initial proposal that the second bioreactor started when the first was at half of the fermentation. This meant that each batch had a duration of 2.25 days.

330 working days · 1 batch/2.25 days = 146 batches/year.

25.5 tons PHB/batch * 146 batches/year = **3,723 tons PHB/year.**

Productivity increase = 4,182/3,723 = 1.12 → Productivity is increased by 12% with the new time management proposal.

Annexe 2: Process diagram



BIOPROCESS DESIGN OF A PHB PRODUCTION PLANT
PART III: DOWNSTREAM AND TIME MANAGEMENT

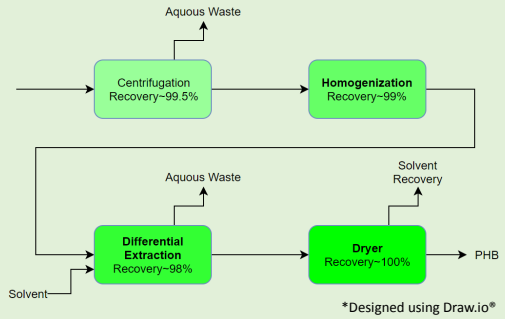
Miquel Bistué, **Carlos Martínez**, Melisa Maurino & Pol Pérez

Final Project of Biotechnology Degree (2018-2019)
Tutored by **Carles Solà i Ferrando**

Abstract

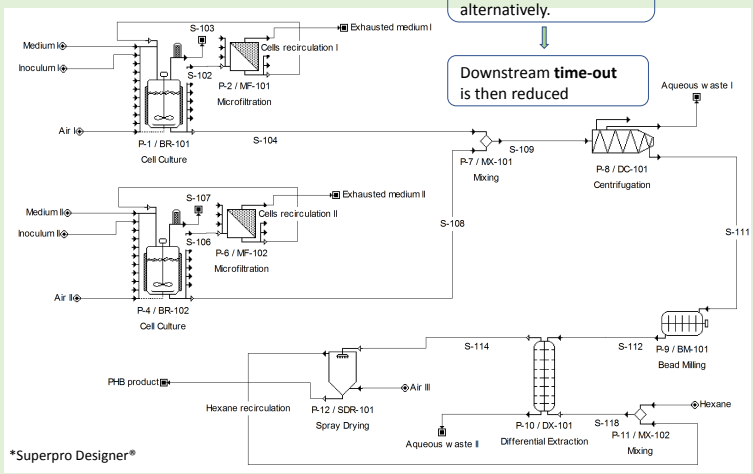
The aim of the project is to design an industrial PHB production plant using residual whey as bioreaction substrate. Whey is a largely produced by-product of cheese-making and it has been proven to be harmful to the environment if disposed onto farmland or waterwaste. PHB is a biodegradable polymer which can be used in products ranging from plastic bags to cutting edge surgical stitches or even prothesis. This part will be focused on the purification of the bioreaction stream containing PHB rich biomass into biomass-free PHB and the quality analysis of that stream so that it can be sold to third parties.

Downstream Block Diagram



*Designed using Draw.io®

Downstream Flux Diagram



*Superpro Designer®

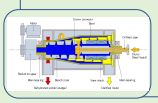
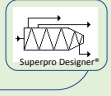
Both bioreactors feed the Downstream process alternatively.

Downstream **time-out** is then reduced

Downstream Equipment

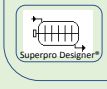
Centrifuge

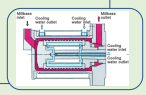
- ✓ Concentrate biomass
- ✓ Exit temperature: 39º C
- ✓ Op. Time: 3.5 hours
- ✓ ~99.5% Recovery Yield

Bead-Mill

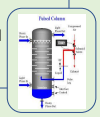
- ✓ Disrupt Biomass to free PHB
- ✓ Exit temperature: 18º C
- ✓ Op. Time: 10 hours
- ✓ ~99% Recovery Yield





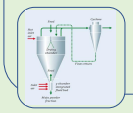
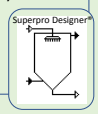
Extractor

- ✓ Separate PHB from water residue
- ✓ Solvent: Hexane
- ✓ Exit temperature: 25º C
- ✓ Op. Time: 3.3 hours
- ✓ ~98% Recovery Yield



Spray-Dryer

- ✓ Separate PHB from hexane
- ✓ Exit temperature: 60º C
- ✓ Op. Time: 6 hours
- ✓ ~100% Recovery Yield

Downstream Characteristics

Stream	PHB-rich Biomass [kg/batch]	free-PHB [kg/batch]	Cellular Debris [kg/batch]	Water [L/batch]	Hexane [kg/batch]
Input	30344	0	0	128000	0
Centrifuge Out	30192	0	0	31298	0
Bead-Mill Out	75	26004	3886	31298	0
Extraction Out	0	25485	0	0	31996
Output	0	25485	0	0	3

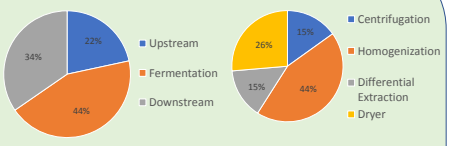
1.5 kg of hexane residue are burnt in a **gas torch** daily

- ✓PHB recovery → ~96.5%
- ✓PHB Purity → >99%
- ✓Operating Time → 24 hours
- ✓25.5 tons PHB/batch
- ✓164 batches/year
- ✓4,182 tons PHB/year

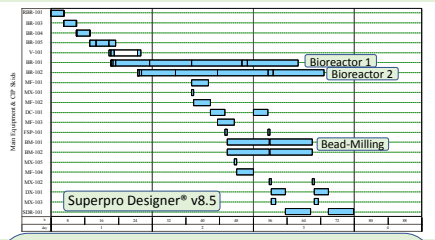
*Quantities reffered to the entire process (2 Downstream processes).

Capability to compete with current leading PHB-Producer companies.

Time Management

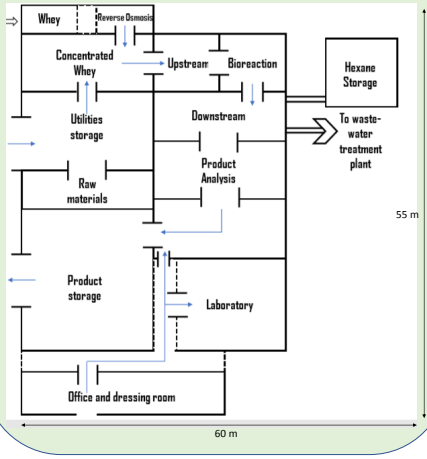


Downstream critical point → Cell homogenization
Global process critical point → Fermentation followed by downstream



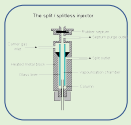
Objective: Attempt to maximize productivity by optimising equipment occupancy.
Problem: How much delay from start of Bioreactor nº1 and start of Bioreactor nº2 so Downstream time-outs are as low as possible?
Solution: If the second fermentation starts 6 hours after the first has started, the downstream process is optimised and productivity is increased.

Lay Out

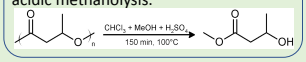


Product Analysis

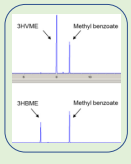
Gas Chromatography (FID) is used to quantify PHB and determine its monomeric composition.



PHB gets converted into volatile hydroxycarboxylic acid methyl esters by acidic methanolysis.



Methyl esters interact specifically with the solid phase



- ✓ Easy to operate and **robust method**
- ✓ **Low maintenance** requirements
- ✓ **High detection ranges** for organic substances

Conclusions

- Downstream process design proves to be effective since it provides **high recovery** and **purity**
- Highly efficient recirculation of solvent minimizes hexane loss
- A proposal for time optimization has been designed and PHB productivity has increased by **12%**
- **Gas Chromatography** has been proposed as a simple and trustworthy method to analyze product quality

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Spray Dryer diagram: GEA Group Aktiengesellschaft 2019 ©, *FSD Minor™ Spray Dryer [online].* Available at: https://www.gea.com/es/products/fsd-minor.jsp.
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Universitat Autònoma de Barcelona

BIOPROCESS DESIGN OF A PHB PRODUCTION PLANT
PART IV: SUSTAINABILITY ANALYSIS

Universitat Autònoma de Barcelona

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Final Degree Project of Biotechnology (2018-2019)

Tutored by *Carles Solà i Ferrando*

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Index.

Abstract.

During the last century we have created a plastic dependent lifestyle, consuming each year an increasingly quantity of it, leading to severe environmental issues. Over 270 million tons of plastic residues were generated in 2017. Plastic not only is a non-degradable material, but it also comes from petroleum, which is a non-renewable resource which reserves are shrinking day by day, so an alternative had to be proposed. There are many biological substitutes for plastic, from which polyhydroxybutyrate (PHB) has been chosen for this project due to their especially suitable properties and wide and well developed production.

PHB is a biodegradable and renewable polymer with plastic properties, commonly produced by many bacteria species under specific conditions as a carbon storage. It can be synthesized from a wide variety of substrates ranging from pure glucose to many organic residues. Using residues as substrate constitutes a necessary strategy in order to achieve a profitable bioprocess in a very competitive scenario. Cheese whey residue has been the one proposed in this project, as it is an abundant residue from dairy industry and it has become a serious environmental problem over the years for its high organic load.

To summarise, this project aims at producing PHB using whey residue as substrate, thus helping with two environmental concerns while trying to make profit at the same time.

Introduction.

One of the needs of society in light of the accelerating destruction of the ecosystem is to manufacture and dispose of products with the minimal impact on the environment. Is in this context where our project fits. Every year, thousands of whey tonnes are produced as a result of the milk-based products manufacturing such as cheese and dairy industry. This waste not only supposes a serious environmental problem because of its richness in nutrients and its bad disposal management, but also supposes a great economic issue to the companies because of the high cost of its treatment.

Therefore, the aim of this project is to combine these two environmental problems (fuel-based plastics and whey waste) to create a new product that allows reducing the damage caused to nature at the same time creating profit out of it. This product is the poly-3-hidroxybutyrate, which is a so-called “bioplastic” which places it as an alternative for products made by regular non-biodegradable plastics which are petroleum-based plastics

As we are talking about producing in a responsible way, the first word that jumps to our mind is sustainability. For a project to be sustainable, it has to be not only economic viable, but also social and environmental sustainable. This is the objective of this Bachelor’s Thesis, to analyse whether the proposed plant design for the production of P(3HB) is sustainable or not and if not, how could it be.

A sustainable project.

Economic analysis.

i. Annual cost of whey treatment for milk-based product industries.

For acknowledging the huge economic impact that has the whey disposal treatment for dairy industries we assume that treating 1kg of DQO in an independent sewage plant treatment costs 0.56$ (0.5€). Taking into consideration that all the whey is assumed to be chemical oxygen demand (COD) one can apply this data to the amount of whey that is used to produce P(3HB), which is 49,395,488 kg, it would cost to treat them 27,661,474$ (24,698,844€).

ii. **Economic analysis of the project**.

It is always necessary when designing a project plan to make an economic evaluation. For doing it, the estimated data provided by the software used to design the plant, SuperPro Designer v8.5, will be analysed and the economic evaluation parameters to be considered are the following:

- Year construction of analysis: 2018.
- Construction period: 30 months.
- Startup period: 4 months.
- Project lifetime: 15 years.
- Income taxes: 40%.
- NPV interest: 4%.

Parameters such as construction and startup period, income taxes and project lifetime were set by default. The interest rate was decided being conservative with the value and taking into consideration that nowadays we are experiencing an economic growth period and is therefore decreasing year by year and the current interest rate in Spain is 1.04% (“Long-term interest rate statistics for EU Member States,” 2019).

- **Executive Summary**.

The production capacity of the plan is estimated to be 4,542,422 kg/year of P(3HB) which has an estimated market value of 4.5$/kg (3.98€/kg). Taking into consideration all the parameters above mentioned, the next values stated in *Table 1* were estimated.

Table 1. Executive economic summary of the project.

Executive Summary	
Total Capital Investment (TCI)	61,566,000 $
Operating Cost	17,424,000 $/yr
Net Operating Cost	17,424,451 $/yr
Total Revenues	20,440,000 $/yr
Cost Basis Annual Rate	4,542,422 kg P(3HB)/yr
Unit production Cost	3.84 $/kg P(3HB)
Unit production Revenue	4.50 $/kg P(3HB)
Gross Margin	14.76%
Return on Investment	11.91%
Payback Time	8.4 years
IRR (After taxes)	3.98%
NPV (at 4.0% interest)	-99.87 $

The project has a high estimated capital investment and its annual operating cost represents a 28.3% of it. According to it and the production capacity, producing every kilo of P(3HB) costs 3.84$, which leads to a gross margin of the 14.76%. After applying taxes, the return on investment is little, so the payback time of the investment is 8.4 years.

- **Cash Flow Analysis. NPV and IRR.**

The Net Present Value (NPV) and the Internal Rate of Return (IRR) are both well-known economic parameters that are usually used to make a profitable analysis of projects. Because the NPV represents the total value of future net cash flows during the lifetime of a project, discounted to reflect the time value of money at the beginning of a project, if the NPV is not positive, the investment should not be undertaken (*SPD Manual SuperPro Designer*, n.d.). As shown in Figure 1, the NPV for this project is estimated to be -99.87 $, which indicates that taking into consideration the cash flow and the rate interest, the project should not be done. Although the NPV is negative, is close to zero and that relates to the IRR value. If the interest rate was below 3.98% (like the current rate interest), the NPV would be positive and carrying out the project would be a good investment. However, for this project one has not only to consider the economic results, but also the social and environment features. Therefore, even though the NPV is negative, the project future has to be studied from a sustainable point of view.

It also has to be considered that the data is an estimation and that the revenues and costs are actually not constant as shown in *Figure 1.* This cost and revenues estimation is based on the fact that the product failure rate is 0% and that every kg of P(3HB) produced is sold.

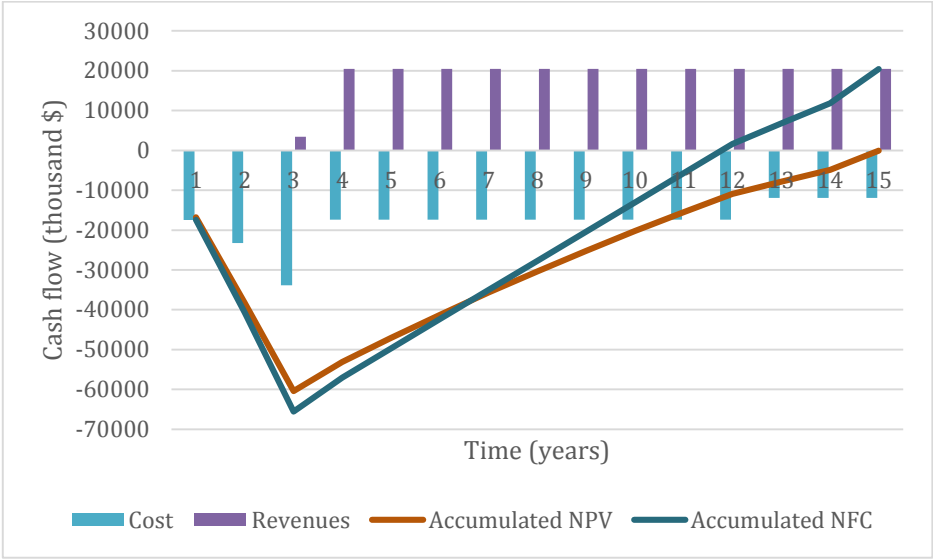
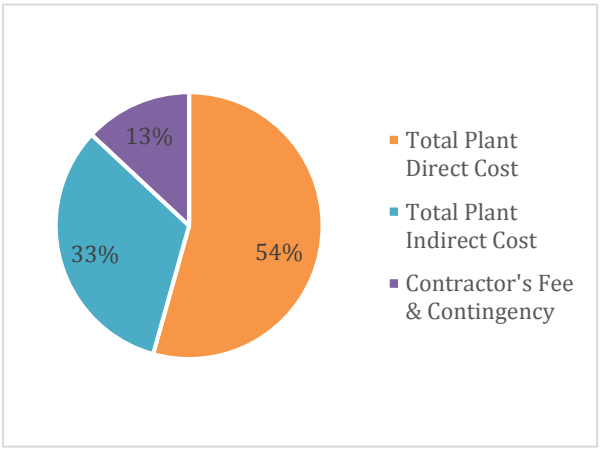
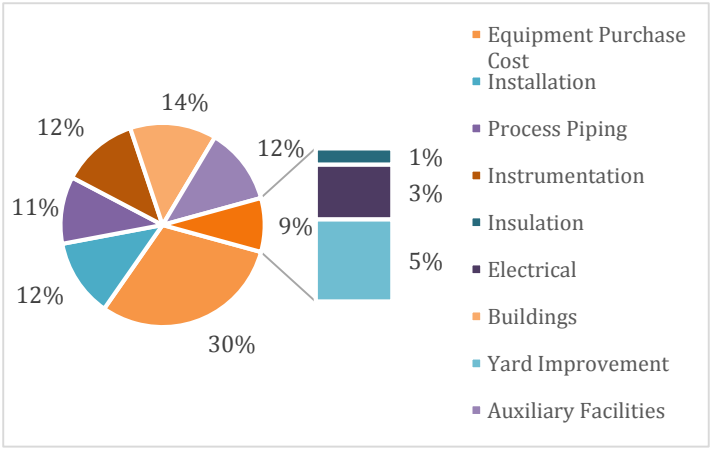


Figure 1. Cash flow analysis.

For making the project more attractive as an investment, costs should be reduced. Therefore, a study of how and in what the money is spent should be done. Starting with the fixed capital

- **Fixed Capital Estimation**.





Figures 2 and 3. Fixed Capital Estimation (left) and Total Plant Direct Cost (right)

As it can be seen in *Table 2* in Annexes, both the Total Plant Direct Cost (TPDC) and the Total Plant Indirect Cost (TPIC) are the major expenses. The TPDC is 31,576,000$, which represents a 54% of the TCI and the mayor charges are associated to the Equipment Purchase

Cost (*Table 3* in Annexes), the Buildings to shelter the plant and the Instrumentation and Installation cost. On the contrary, TPIC is high due to the high cost of Engineering and Construction and represents a 33% of the TCI. This project also has a high Contingency expense (an 8.6% of the Direct Fixed Capital Cost) to account for unexpected problems during constructions.

Most of the parameters are estimated using multipliers factors of the TPDC and TPDI, which are at the same time measured based on the cost of the Equipment Purchase. So reducing this cost would decrease the overall investment and would make the design more appealing for investment. Analysing this cost, it can be noticed that the mayor cost is associated with the two biggest bioreactors used (3,312,000$) and represents the 34.5% of the equipment charge. So decreasing volume of these reactors would help to diminish the cost. Moreover, this would not only help to decrease the cost of the equipment purchase cost, but also to reduce the cost of installation, piping, electrical, buildings and construction.

- **Annual Operating Cost (AOC).**

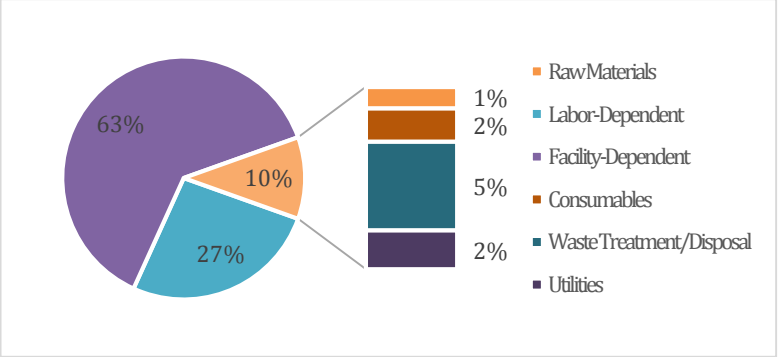


Figure 4. Annual Operating Cost of the Project.

The AOC is 17,424,000$/year and a 62.8% of it is again related to Facility-Dependent charges such as maintenance cost, depreciation of the fixed capital cost and other miscellaneous costs such as insurance or local taxes. Although only nine operators are needed per batch, the second biggest expense is the Labor-Dependent one (26.3% of the AOC) because the unit cost per hour and operator is estimated to be 69$ and a total of 66,496 hours are needed every year as shown in *Table 5* in Annexes.

Waste Treatment/Disposal Cost is considered a 5.3% of the AOC. This cost estimation is based on the fact that managing 1kg of DQO costs 0.56$ and how much organic waste are in every stream. The final waste treatment cost is 925,341$/year and represents a 3% of the estimated cost (27,661,474$) of managing the same amount of whey but in an independent sewage plant treatment.

The cost related to Raw Materials is not high due to the fact that the substances employed are cheap if buyed in bulk. For example, buying ammonium chloride cost 110$/ton (CO, n.d.), lactose cost is 19$/ton (CLAL, n.d.) and the price of the Whey (1.136$/ton) and Concentrated Whey (5.128$/ton) are estimated by SuperPro Designer software using the lactose price.

The Utilities Cost (*Table 4* in Annexes) is negligible compared to the Operating Cost because it only represents a 2.3% of this charge. Cost as Consumables are also not significant if they are compared to other expenses.

Environmental analysis.

For making the environmental analysis, the *Mass Index Analysis* was used. This analysis consists in studying all the input and output streams that are needed to produce the product. By doing this, one can analyse the mass index contribution of each component that is used and produced and whether if it is necessary to reduce one or more streams for reducing the environmental impact. In that sense, as shown in *Figure 5,* the major component used and produced is water. This water would have to be treated after its use in an independent sewage treatment plant, costing 925,341$/year. After water, the next major contributor to the production of P(3HB) is whey, followed by N_2.

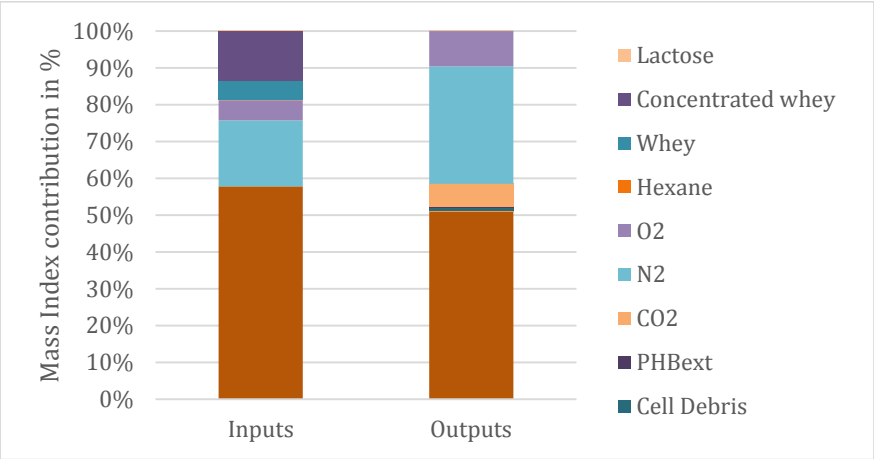


Figure 5. Mass Index Analysis of the project.

Most of the output streams are gases. N_2 and CO_2 are produced in great quantity. Also biomass and biomass debris. These last too can be disposed without treatment as the organism used belongs to the biosecurity level 1.

5,018,114 kg of CO_2 are produced every year. This amount can be set free to the atmosphere because it does not reach the maximum legal amount by law that you can liberate according to the Directive 2003/87/EC and the Commission Decision of 27th April of 2011 (Parliament, 2011).

Although it is true that hexane is a very inflammable component, its toxicity is low. The National Institute for Occupational Safety and Health (NIOSH) set a recommended exposure limit of 100 ppm over per day (The National Institute for Occupational Safety and Health (NIOSH), n.d.). For the little amount of hexane produced per batch, 3.2 kg, it has been de decided to burn it for its treatment. The CO_2 produced for this activity would be taken into account with the rest of the CO_2 emissions.

The microorganism used in the production of the P(3HB) is *Escherichia coli (E. coli*) CGSC 4401. This mesophilic proteobacteria is classified in the biosafety level number 1(BSL-1) by the Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (GmbH, n.d.). This means according to the Directive 2009/41/EC that for its use, containment and other protective measures have to be fulfilled. Because of its level, these measures and procedures are neither difficult to carry on or expensive. Most of the requirements are optional, as for example the "inactivation of bulk culture fluids before removal from closed system" or the fact that an "extract and input air from the controlled area should be HEPA filtered" (the European Parliament and the Council, 2009). As the GMO used has a BSL-1, there is no need for installation of all this features, which decreases the cost of production and facilities.

Social analysis.

Finally, for knowing if the proposed design is sustainable or not, a social analysis has to be done. Nowadays we are facing a terrible problem: mismanaged disposal of plastic.

It was estimated that in 2013 there were 268,950 tonnes of plastic in the ocean (Eriksen et al., 2014). Although the exactly amount accumulated cannot actually be known because it can be either digested by animals, incorporated to sediments or even be turned into microplastics. Depending on the country of origin (whether it is poor or in developing), disposal way, etc, plastic have several fates. Data from 2015 estimates that 30% of the primary plastic was still in use; 55% went straight to landfill or was discarded; 8 percent was incinerated; and 6 percent was recycle (Geyer, Jambeck, & Law, 2017).

Plastics are having a huge impact both in the wildlife and in human health. There are different pathways by which plastic debris can affect wildlife: it can constrict or encircle animals; they can cause obstructions or abrasions (Law, 2017); or they can be ingested, what can greatly reduce stomach capacity, leading to poor appetite and false sense of satiation (Day, Wehle, & Coleman, 1985).

In the case of microplastics the key concern is ingestion(Roser, 2019). Microplastic ingestion rarely causes mortality in any organisms. Although there is increasing evidence that its ingestion can affect the consumption of prey, leading to energy depletion, inhibited growth and fertility impacts. When organisms ingest microplastics, it can take up space in the gut and digestive system, leading to reductions in feeding signals (Roser, 2019).

There is, currently, very little evidence of the impact of microplastics in humans (Roser, 2019). Three possible toxic effects of plastic particle have been suggested: plastic particles themselves, the release of persistent organic pollutant adsorbed to the plastics, and leaching of plastic additives (Iñiguez, Conesa, & Fullana, 2017). However, there has not been conclusive evidence for neither of this three cases, so it is important to keep researching in this matter for a better understanding of the role of plastic within broader ecosystems and risk to human health (Roser, 2019).

Plastic not only affects the environmental, but also has a great negative economic impact. One of the most affected sector is the fishing industry, whose equipment can be damaged by obstruction. Also because of the diminishing sea-animal quantity, less and less fishes can be caught, causing economic loses to fishers.

Other industry sector that is being directly affected is tourism. Many touristic coast places rely their economy on the beauty and clearness of their water and they would experiment a decrease of visitors if the situation keeps getting worst.

PHAs in general have a large range of applications in different fields as agriculture, chemical industry, biomedicine or nanotechnology among others (Idris, Su Yean, & Kumar, 2018; Z. A., Abid, S. & Banat, 2018) due to its characteristics. Furthermore, this kind of bioplastics constitute a new raw material, which could be used for packaging instead of plastic bags or bottles, as it is environmentally friendly. This is the main reason why the PHB and its copolymers are presented as an ideal replacement for conventional petroleum-based plastics (S. & Kalia, 2017; Z. A., Abid, S. & Banat, 2018). Packaging is not only in the end the major industrial sector where plastic is used as a raw material (yearly produced 146 million tonnes of them (Geyer et al., 2017)), but also the sector that generates more plastic waste, around 141 million tonnes (Geyer et al., 2017).

Besides, P(3HB) could improve the lives of thousands of people due to its biomedicine applications. PHA copolymers have many potential uses as drug carriers, tissue engineering, biomedical devices and biodegradable implants (S. & Kalia, 2017).

Moreover, not only the project would help society by changing the population behaviour regarding the fuel-based plastic and delivering new products to the market, but also would create job positions that would have to be filled. This would contribute to the local economic growth by decreasing unemployment and also creating a new industrial hub where specialized professionals would be needed.

Improvements of the process, economics, environmental and social characteristics.

In order to improve the production and decrease the production cost of P(3HB) we propose these measures:

- Change of PHA synthase activity.

One of the most critical points in the metabolism for P(3HB) production is the reaction catalysed by the PHA synthase, which polymerizes the 3-hydroxybutyryl-CoA monomers to P(3HB), liberating CoA (Peña, Castillo, García, Millán, & Segura, 2014). The efficiency of this reaction depends on the specificity of the PHA synthase of the particular organism and the used carbon source.

There are several research investigations that have achieved an increase in this enzyme productivity. For example, a mutant that could produce twofold higher P(3HB) compared to the enzyme harbouring *Escherichia coli* strain was found (Taguchi, Nakamura, Hiraishi, Yamato, & Doi, 2002).

Therefore, by modifying the PHA synthase used in this project, a higher yield of P(3HB) production and its intracellular accumulation could be achieved. For finding the best mutant in the project case a further research investigation should be done.

- Other genetic modifications.

Biosynthesis of PHB in *E. coli* is not only a matter of pathway construction, but is also affected by many other factors. For example, acetyl-CoA is an essential central intermediate, which can directly increase 3-hydroxybutyryl-CoA formation and cell growth (Li, Zhang, & Qi, 2007). By inactivation of the *pta* gene, which encodes a phosphotransacetylase, *E. coli* will accumulate more PHB than wild type *E. coli* (Miyake, Miyamoto, Schnackenberg, Kurane, & Asada, 2000). Another important factor concerning this process is NADPH, which is necessary for PHB production. By knocking out the phosphoglucose isomerase (*pgi*) gene, more NADPH will be produced from pentose phosphate pathway (PP), and eventually the PHB production will be enhanced (Kabir & Shimizu, 2003). So more *E. coli* mutants could be tested in order to find the one that provides the best productivity.

- Construction of a sewage treatment plant.

The main component produced after the project is water. This water must be treated before being disposed and it represents the 6% of the annual operating cost because it is treated in an independent waste water treatment plant. The idea would be building a new sewage treatment plant within the facilities, making another investment, so in the future this cost would be reduced and amortized. Nonetheless, this new investment should be studied from an economic point of view in order to determinate if, indeed, it would represent a decrease in the cost production.

- Application of the process using new raw materials.

There has been described several microorganisms that produce PHB from different carbon sources. Some of them are also raw materials with what we have some problems disposing them. For example, using molasses as a carbon source and *Azotobacter vinelandii* (Page, 1992) as the microorganism, a productivity of 1.4 g of P(3HB)/L*h was achieved or using plant oils and Ralstonia *eutropha* (Kahar, Tsuge, Taguchi, & Doi, 2004) a productivity of 0.96 g/L*h was reached. Therefore, it would be an improvement if this project design could be applied to other waste from other industries that could help solve both the environmental an economic problem that they cause.

Future perspectives of the bioplastic industry.

Today, there is a bioplastic alternative for almost every conventional plastic material and corresponding application. Bioplastics have the same properties as conventional plastics and offer additional advantages, such as a reduced carbon footprint or additional waste management options such as composting. The current market for bioplastics is characterized by a dynamic growth rate and a strong diversification as shown in *Figure 6:*

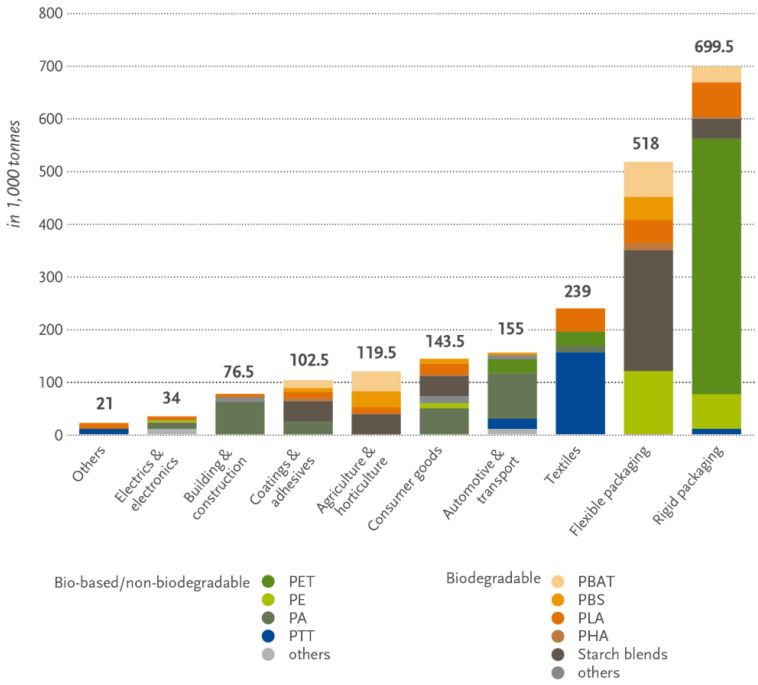


Figure 6. Global Production Capacities of Bioplastics 2018 (Goldsberry, 2017).

The increasing availability of raw renewable materials and increasing demand and use of biodegradable polymers for bio-medical, packaging, and food applications along with favourable green procurement policies are expected to benefit PHA market growth. According to a recent report, published in 2017, the global PHA market is expected to reach US$ 93.5 million by 2021, characterized by a compound annual growth rate (CAGR) of 4.88% (Bioplastics, 2018). On the other hand, the global market for plastic products is growing at about 3% year on year, according to research from the Business Research Co. (Philadelphia) in its report, *Plastics Product Manufacturing Global Market 2017*. It was worth $1.06 trillion in 2016 and will grow to $1.175 trillion by 2020 (Goldsberry, 2017).

If we attend to production numbers only, the world plastic production in 2015 was 322 Mtons and in 2021 is expected to be 370 Mtons (Z. A., Abid, S. & Banat, 2018). But world production of bioplastics in 2021 is expected to be 6.11 Mton of which only a 4.1% would be

PHA. If one consider European bioplastic production, by the year 2021 is expected to be a 26% of the total plastic production (Idris et al., 2018), although the production of bio-based plastics currently comprises ca. 1% of total plastics production (van den Oever, Molenveld, van der Zee, & Bos, 2017).

The factors driving market development are both internal and external. External factors make bioplastics the attractive choice. This is reflected in the high rate of consumer acceptance. Moreover, the extensively published effects of climate change, price increases of fossil materials, and the increasing dependence on fossil resources also contribute to bioplastics being viewed favourably. From an internal perspective, bioplastics are efficient and technologically mature materials ("Market drivers and development," 2018).

Regarding government policies, most of them are aimed to reduce single-use plastics as plastic bags and straws. The first policy adopted regarding plastic was in 1991 in Germany and since then, more policies have been developed. The latest great policy has been made by the EU, the European Commission declared "all plastic packaging on the EU market will be recyclable by 2030, the consumption of single-use plastics will be reduced and the intentional use of microplastics will be restricted"(European Commission, 2018). So every time, both governments and citizens are becoming more aware of the current situation with plastics.

Conclusions.

The current design of the project is not economically attractive enough for investment, but if changes like above mentioned are carried out, this project could be economically sustainable and even more, profitable. In the section of Economic Analysis, it has been stated that the project's NPV is negative, but almost zero, so it would not be good as an investment but it would be from the environmental and social point of view. So, taking into consideration that the NPV is 0, this would be a sustainable project.

Every year thousands of species are extinguished due to human behaviour against nature. One of the most important problems is plastic. Using new materials as P(3HB) would help not only to reduce contamination and gain health, but also to save money from being spent in future projects to recover wildlife.

Bioplastics such as P(3HB) are able to improve the balance between the environmental benefits and the environmental impact of plastics. Life cycle analyses demonstrate that bioplastics can significantly reduce CO_2 emissions compared to conventional plastics. What is more, the increasing utilisation of biomass in bioplastic applications has two clear advantages: renewability and availability ("Market drivers and development," 2018). This could help to solve the problem of the increasing levels of microplastic in the oceans, which nowadays constitute both an environmental and a global health problem.

The latest market data does not only demonstrate the contributions of the industry on moving towards a sustainable future with a reduced environmental impact. The forecast also predicts the budding bioplastics industry to unfold an immense economic potential over the coming decades ("Market drivers and development," 2018).This is the reason why we think this project faces the most nearest future and its development and implantation in real industries could be beneficial not only for the company, but also for the environment.

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Annexes.

The next tables are provided by the Economic Evaluation Report by the SuperPro Designer v8.5 program.

Table 2. Fixed Capital Estimate Summary (2018 price in $)

3A. Total Plant Direct Cost (TPDC) (physical cost)	
1. Equipment Purchase Cost	9,610,000
2. Installation	3,899,000
3. Process Piping	3,364,000
4. Instrumentation	3,844,000
5. Insulation	288,000
6. Electrical	961,000
7. Buildings	4,325,000
8. Yard Improvement	1,442,000
9. Auxiliary Facilities	3,844,000
TPDC	31,576,000
3B. Total Plant Indirect Cost (TPIC)	
10. Engineering	7,894,000
11. Construction	11,052,000
TPIC	18,946,000
3C. Total Plant Cost (TPC = TPDC+TPIC)	
TPC	50,522,000
3D. Contractor's Fee & Contingency (CFC)	
12. Contractor's Fee	2,526,000
13. Contingency	5,052,000
CFC = 12+13	7,578,000
3E. Direct Fixed Capital Cost (DFC = TPC+CFC)	
DFC	58,100,000

Table 3. Major Equipment Specification and Fob Cost (2018 prices).

Quantity/ Standby/ Staggered	Name	Description	Unit Cost ($)	Cost ($)
1 / 0 / 0	BR-101	Bioreactor Vessel Volume = 99921.16 L	1,656,000	1,656,000
1 / 0 / 0	BR-102	Bioreactor Vessel Volume = 99921.16 L	1,656,000	1,656,000
1 / 0 / 0	DC-101	Decanter Centrifuge Throughput = 45957.96 L/h	312,000	312,000
1 / 0 / 0	BM-101	Bead Mill Bead Volume = 168.97 L	92,000	92,000
1 / 0 / 0	DX-101	Differential Extractor Extractor Volume = 33943.33 L	114,000	114,000
1 / 0 / 0	BM-102	Bead Mill Bead Volume = 168.97 L	92,000	92,000
1 / 0 / 0	BR-103	Bioreactor Vessel Volume = 100.66 L	135,000	135,000
1 / 0 / 0	BR-104	Bioreactor Vessel Volume = 1006.64 L	426,000	426,000
1 / 0 / 0	BR-105	Bioreactor Vessel Volume = 10066.43 L	562,000	562,000
1 / 0 / 0	V-101	Blending Tank Vessel Volume = 8947.92 L	93,000	93,000
4 / 0 / 0	MF-101	Microfilter Membrane Area = 78.01 m2	158,000	632,000
4 / 0 / 0	MF-102	Microfilter Membrane Area = 72.12 m2	151,000	604,000
4 / 0 / 0	MF-103	Microfilter Membrane Area = 78.01 m2	158,000	632,000
4 / 0 / 0	MF-104	Microfilter Membrane Area = 72.12 m2	151,000	604,000
1 / 0 / 0	SDR-101	Spray Dryer Dryer Volume = 26671.08 L	78,000	78,000
		Unlisted Equipment		1,922,000
			TOTAL	9,610,000

Table 4. Utilities Cost (2018 prices) - Process Summary.

Utility	Unit Cost ($)	Annual Amount	Ref. Units	Annual Cost ($)	%
Std Power	0.100	2,250,157	kW-h	225,016	55.72
Steam	12.000	12,363	MT	148,359	36.74
Chilled Water	0.400	76,066	MT	30,426	7.53
TOTAL				403,801	100.00

Table 5. Labor Cost – Process Summary.

Labor Type	Unit Cost ($/h)	Annual Amount (h)	Annual Cost ($)	%
Operator	69.00	66,496	4,588,206	100.00
TOTAL		66,496	4,588,206	100.00

Table 6. Material Cost – Process Summary.

Bulk Material	Unit Cost ($)	Annual Amount		Annual Cost ($)	%
Ammonium Chlori	110.000	55	ton	6,009	2.68
Whey	1.136	15,693	ton	17,821	7.95
Air	0.000	67,428	ton	0	0.00
Whey concentrat	5.128	38,665	ton	198,282	88.49
Water	0.000	124,130	ton	0	0.00
Hexane	0.400	566	kg	227	0.10
Lactose	19.000	91	ton	1,733	0.77
Biomass	0.000	1	kg	0	0.00
TOTAL				224,073	100.00

NOTE: Bulk material consumption amount includes material used as:
- Raw Material
- Cleaning Agent
- Heat Transfer Agent (if utilities are included in the operating cost)

Table 7. Annual Operating Cost (2018 prices) – Process Summary.

Cost Item	$	%
Raw Materials	224,000	1.29
Labor-Dependent	4,588,000	26.33
Facility-Dependent	10,936,000	62.76
Consumables	347,000	1.99
Waste Treatment/Disposal	925,000	5.31
Utilities	404,000	2.32
Transportation	0	0.00
Miscellaneous	0	0.00
Advertising/Selling	0	0.00
Running Royalties	0	0.00
Failed Product Disposal	0	0.00
TOTAL	17,424,000	100.00

BIOPROCESS DESIGN OF A PHB PRODUCTION PLANT
PART IV: SUSTAINABILITY ANALYSIS

Miquel Bistué, Carlos Martínez, **Melisa Maurino** & Pol Pérez

Final Project of Biotechnology Degree (2018-2019)
Directed by **Carles Solà i Ferrando**

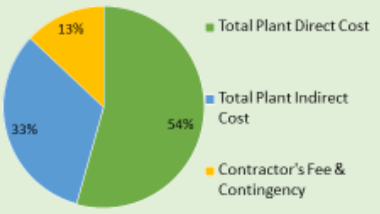
Abstract

The aim of the project is to design an industrial PHB production plant using residual whey as bioreaction substrate. Whey is a largely produced by-product of cheese-making and it has been proven to be harmful to the environment if disposed onto farmland or waterwaste. PHB is a biodegradable polymer which can be used in products ranging from plastic bags to cutting edge surgical stitches or even prothesis. This part will be focused on the sustainability analysis of the project in economic, environmental and social terms. In this part it is also found the conclusions and future perspectives of the project.

Economic Analysis

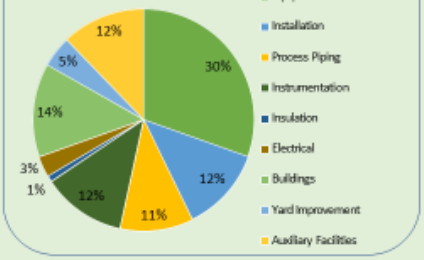
Executive Summary	
Total Capital Investment	61,566,000 $
Operating Cost	17,424,000 $/yr
Net Operating Cost	17,424,451 $/yr
Total Revenues	20,440,000 $/yr
Cost Basis Annual Rate	4,542,422 kg P(3HB)/yr
Unit production Cost	3.84 $/kg P(3HB)
Unit production Revenue	4.50 $/kg P(3HB)
Gross Margin	14.76 %
Return on Investment	11.91 %
Payback Time	8.4 years
IRR (After taxes)	3.98 %
NPV (at 4.0% interest)	-99.87 $

Fixed Capital Estimate Summary



Annual Operating Cost

Total Plant Direct Cost



Treating the whey in an external sewage treatment plant

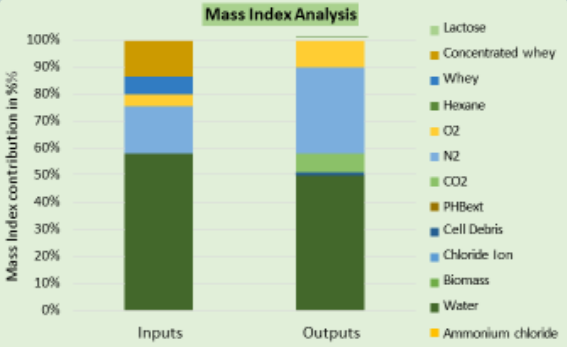
⇩

28,000,000 $/year

- High initial investment.
- Long period for recovering the investment: 8.4 years.
- Overall negative NPV ➡ almost zero ➡ the **investment should not be undertaken unless environmental and social considerations.**
- High value of revenue.
- Major part of the cost is associated with the equipment and necessary facilities.

Environmental Analysis

Mass Index Analysis



- Water is the major component both as input and output in the production of P(3HB). Aqueous waste would be treated in an external sewage treatment plant.
- Production of 5,000,000 kg of CO2/year.

GMO's Biosecurity Level 1 ➡ **Directive 2009/41/EC** ➡ **Reduced cost of production and facilities**

Social Analysis

Major environmental problem 2)

⇩

268,959 tonnes of plastic in the ocean

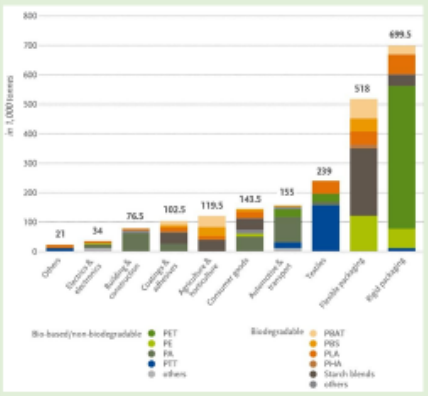
⇩

42% plastic production is for packaging

3)

- **Impacts on the environment and health**:
  - Interaction by animals with plastics: **ingestion, collisions, obstructions or abrasions.**
  - **Destruction** of entire **ecosystems.**
  - **Microplastics**: its ingestion can affect the consumption of prey, leading to **energy depletion, inhibited growth and fertility impacts.**
  - Very little evidence of microplastics' impact in humans.
- **Impacts on the economy**:
  - Fishing industry.
  - Tourism.
- New **solution for whey residual management.**
- Creation of jobs ➡ **contribution to local economic growth.**

Market Analysis and future perspectives



1)

- There is a **bioplastic alternative for almost every conventional plastic material** and corresponding application.
- **Market** for bioplastics is characterized by a **dynamic growth rate** and a strong diversification.
- World production of bioplastics in 2021 is expected to be 6.11 Mton of which only a **4.1% would be PHA.**

Factors driving market development:

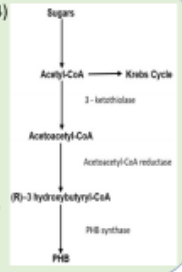
➡ **External**:
- Political **policies from governments.**
- High rate of consumer acceptance.
- Published effects of climate change.
- Price increases of fossil materials.
- Increasing dependence on fossil resources.

➡ **Internal**:
- Efficient and technologically mature materials.

Future Improvements

- Improvement affinity of **PHA synthase** for D-β-hydroxybutytyl-CoA.
- Being able to apply the process to **other waste residues** from other industries.
- A further Life Cycle Analysis is needed for knowing if the process is "eco-friendly".
- **Apply the process to every dairy industry** for managing all whey residues.
- Management of residual waste in an **own sewage treatment plant** ➡ decrease in cost of waste treatment (6% of the annual operating cost).

4)



Conclusions

Sustainable Process

- Current plant design is **economic viable and a sustainable process** if final NPV is considered 0.
- This project design solves both problems: plastic and whey pollution.
- Bioplastics such as P(3HB) are able to improve the balance between the environmental benefits and the environmental impact of plastics.
- The increasing utilisation of biomass in bioplastic applications has two clear advantages: renewability and availability.
- For changing the world current situation related to plastic, not only consumers have to accept new type of products, but also governments need to change their policies.

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