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PhD Thesis

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DTU Chemical Engineering
Department of Chemical and Biochemical Engineering

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DTU

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PhD Thesis

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November 2016

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Abstract

Natural products constitute an extensive family of organic molecules with more than 200,000 compounds discovered in several natural sources (plants and microbes). Most of these compounds have a very complex structure, multiple chiral centers and can have different biological activities. These biological activities can be of interest for use in different sectors of chemical industry, in particular pharmaceutical industry where several drugs are derived or inspired by natural products structure.

However, the large scale production of natural products is hindered by its relatively poor abundance in nature, which makes extraction from natural sources an economically unfeasible technology in most of the cases. Chemical synthesis is also very difficult given the structural complexity and chirality of the molecules.

Synthetic biology offers very promising tools for production of natural products. Genetic engineering allows expressing the genes responsible for the biosynthesis of these natural products and to insert them into fermentable organisms like yeast or bacteria. And protein engineering offers the possibility to engineer the enzymes that perform the natural products' biosynthesis, which allows the production of these complex molecules in single or multi-steps biocatalytic reactions.

In this thesis, a systematic approach for route selection and screening of the different process options to manufacture a natural product is presented. This methodological approach includes a set of evaluation tools to assess processes both from an economic and environmental perspectives and it is demonstrated with two case-studies. For each case-study different tools are used to evaluate the process.

The first case-study consists of the bioconversion of (*R*)-limonene to (*R*)-perillic acid by *Pseudomonas putida* GS1. (*R*)-perillic acid is a monoterpenoic acid with antimicrobial properties. It has a strong inhibitory effect on bacteria and fungus, which makes it an attractive compound to be used as a preservative for instance in cosmetic industry, but on the other hand makes the biosynthesis a complicated process to develop. An environmental assessment of the different synthetic routes for (*R*)-perillic acid production showed that biosynthesis represents the most promising option. This process was further explored through an economic assessment and process modelling including a sensitivity analysis on key process metrics, which allowed the identification of the main process bottlenecks. Product inhibition and substrate loss were identified as some of the main process limitations and strategies for improving them were suggested.

The second case-study describes the production of a recombinant protein, brazzein, by fermentation of the yeast *Pichia pastoris*. Brazzein is a natural sweetener found in the fruits of the African plant *Pentadiplandra*

brazzena Baillon, which is sweeter than regular sucrose by several orders of magnitude. Here different tools were applied to evaluate the environmental profile of the process in comparison with other sweeteners.

The main benefit of this early-stage evaluation is putting the biosynthesis of natural products into context in relation to demands of an industrially feasible chemical process. Moreover, it can give very meaningful insight into process development and provides a good overview of the whole reaction and process. The proposed *in silico* approach can guide research and development and ultimately contribute to the implementation of more bioprocesses for the production of natural products.

Resumé

Naturlige produkter er en stor familie af organisk molekyler der indeholder mere end 200.000 forbindelser opdaget i natur (planter og mikroorganismer). De fleste af disse forbindelser har en kompleks struktur og flere kiralecentre og kan have forskellige biologiske aktiviteter. Disse biologiske aktiviteter kan være interessante for forskellige sektorer af kemi industri, især for den farmaceutiske industri hvor mange lægemidler er afledt eller inspireret as naturlige produkters struktur.

Industriel produktion af naturlige produkter er begrænset af deres lave koncentrationer i naturen, der gør ekstraktion en økonomisk umulig teknologi. Kemisk syntese er også en meget vanskelig opgave på grund af den strukturelle kompleksitet og den molekylære asymmetri.

Syntetisk biologi tilbyder mange lovende værktøjer til produktion af naturlige produkter. Genteknologi gør det muligt at udtrykke generne der er ansvarlig for biosyntese af disse naturlige produkter og indsætter dem i fermenterbare mikroorganismer, som gær og bakterier. Og proteinoptimering gør det også muligt at syntetisere naturlige produkter i enkelt- eller flertrins biokatalytiske reaktioner.

Arbejdet i denne afhandling præsenterer en systematisk metode til udvælgelse af ruter og processer til produktion af naturlige stoffer. Denne metodiske fremgangsmåde inkuderer værktøjer for økonomisk og miljømæssig evaluering af bioprocesser og er blevet valideret gennem to forskellige eksempler.

Det første eksempel er produktionen af (*R*)-perillic acid fra (*R*)-limonene ved brug af *Pseudomonas putida* GS1. (*R*)-perillic acid er en monoterpen syre med antibakteriel aktivitet. Det er interessant at bruge (*R*)-perillic acid som en konserveringsmiddel i den kosmetiske industri, men dens biosyntese er en vanskelig opgave. En miljømæssig evaluering af forskellige processer til produktion af (*R*)-perillic acid viste at biosyntesen er det mest lovende alternativ. De økonomiske aspekter af processen blev også undersøgt baseret på en procesmodel der inkluderer en sensitivitetsanalyse af de væsentlige parametre. Dette muliggjorde identifikationen af de primære flaskehalse i processen. Produktinhibering og substrattab blev identificeret som de største begrænsninger for processen, og en række strategier blev foreslået for at udbedre disse.

Det andet eksempel er produktionen af det rekombinante protein, brazzein, ved fermentering af gæren *Pichia pastoris*. Brazzein er et naturligt sødemiddel som findes i frugterne af den afrikansk plante *Pentadiplandra brazzena* Baillon, der er adskillige gange sødere end sukrose. I dette eksempel blev en miljømæssig evaluering anvendt til at sammenligne forskellige processer for sødemiddel.

Den største fordel ved denne type evaluering er at kunne sætte kravene for en industriel biosyntese af naturlige produkter i perspektiv. Den kan også give meningsfuld indsigt i procesudviklingen og et overblik over reaktionssystemet og processen. Denne *in silico* fremgangsmåde kan guide procesudviklingen og bidrage til introduktionen af flere bioprocesser i produktionen af naturlige produkter.

Preface

The work presented in this thesis was conducted in CAPEC-PROCESS research center at the Department of Chemical and Biochemical Engineering from the Technical University of Denmark as partial fulfillment of the requirements for acquiring a PhD degree in engineering. The work was developed in the period from December 2013 until November 2016 and was supervised by Professor John M. Woodley (DTU Chemical Engineering) and Dr. Mathias Nordblad (DTU Chemical Engineering) and Dr. Jessica Rehdorf (BRAIN AG) as co-supervisors.

The work has been performed within the strategic alliance NatLifE 2020 coordinated by BRAIN (Zwingenberg, Germany) and funded by the German Federal Ministry of Education and Research (BMBF) (grant no. 031A206-B).

Kgs. Lyngby

November 2016

Catarina Seita

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Last but not least, thank you to my family, friends and to João for all the support and patience throughout the project.

List of abbreviations

Nomenclature	Description
ACS	American Chemical Society
API	Active pharmaceutical ingredient
CCM	Corn cob mix
ChemSec	International Chemical Secretariat
DMAc	N,N-dimethylacetamide
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
D-PheOMe	D-phenylalanine methyl ester
DSP	Downstream processing
EHS	Environmental, health and safety
FCI	Fixed-capital investment
GCIPR	Green Chemistry Institute's Pharmaceutical Roundtable
GMO	Genetically modified organism
GSH	Globally harmonized system of classification and labelling of chemicals
HPLC	High-performance liquid chromatography
ISPR	*In situ* product removal
ISSS	*In situ* substrate supply
KREDs	Ketoreductases
LCA	Life cycle assessment
LCI	Life cycle inventory
LCIA	Life cycle impact assessment
L-PheOMe	L-phenylalanine methyl ester
MTBE	Methyl *tert*-butyl ether
NMP	N-methylpyrrolidinone
PM	Phenylalanine methyl ester
RAPEX	Rapid alert system for dangerous non-food products
REACH	Registration, evaluation, authorization and restriction of chemicals
SE	Sucrose equivalent
SIN	Substitute it now
STY	Space-time yield
TB	Terrific broth
UPS	Upstream processing
ZA	N-benzyloxycarbonyl-aspartic acid
WFI	Water for injection

List of nomenclature

Nomenclature	Description	Unit
AP	Acidification potential	g SO_2-eq
AE	Atom economy	%
CAPEX	Capital cost	€
cdw	Cell dry weight	g
CE	Carbon efficiency	%
ee	Enantiomeric excess	%
E-factor	Environmental factor	kg waste/kg product
EMY	Effective mass yield	%
EP	Nutrient enrichment potential	g PO_4^{3-}-eq
ETP	Eco-toxicity potential	PAF.m^3.day
GWP	Global warming potential	g CO_2-eq
HTP	Human-toxicity potential	CTUh
HWP	Hazardous waste potential	g waste
MW	Molecular weight	g/mol
OD_{600nm}	Optical density at 600 nm	Dimensionless
OPEX	Operational cost	€
pKa	Acid disassociation constant	Dimensionless
PMI	Process mass intensity	kg materials/kg product
POCP	Photochemical ozone creation potential	g C_2H_4-eq
RME	Reaction mass efficiency	%
SI	Solvent intensity	kg solvents/kg product
SOP	Stratospheric ozone depletion potential	g CF11-eq
V	Volume	L
WI	Water intensity	kg water/kg product
Y_{SX}	Yield coefficient of biomass on substrate	kg biomass/kg substrate

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1. Introduction

This thesis attempts to create a bridge between science, engineering and economy and apply different concepts of these areas to the development of processes for the biosynthesis of natural products. Thus, different methods for the assessment of the sustainability of bioprocesses at early stages of development, both from an economic and environmental perspectives, as well as technologies for its intensification are explored throughout the thesis.

In the following sections some background information on the type of processes and products under study is given.

1.1 Background

Over the past years, there has been a generalized perception of the emergence and expansion of a so-called bioeconomy. Government policies and industry sustainability programs are driving a bio-based economy all over the world, supported by a growing concern about sustainability from consumers.

But what is this bioeconomy? According to European Commission, the bioeconomy encompasses the production of renewable biological resources and their conversion into food, feed, bio-based chemical products and bioenergy. It thus includes agriculture, forestry, fisheries and food production as well as parts of the chemical, biotechnological and energy industries. Bioeconomy sectors are expected to have a strong innovation potential, using a wide range of sciences (life sciences, agronomy, social sciences, etc.) and technologies (biotechnology, nanotechnology, information and communication technologies) (European Commission, 2014).

The EU estimates that the sectors that comprise the bioeconomy account for 22 million jobs, which is approximately 9% of the EU's workforce (European Commission, 2013). One of the goals of the European commission on research, science and innovation is to move from a fossil-based economy to a bioeconomy. And bio-based chemicals are expected to constitute the largest segment of potential growth for industrial bio-based products. The bio-based chemical sector achieved a market value of $3.6 billion in 2011 and it is estimated that by 2021 the global market for bio-based chemicals will have increased to $12.2 billion. While this represents a significant increase, there is the potential to produce two-thirds of the total volume of

chemicals from bio-based material, which represents over $1 trillion annual global market (Golden and Handfield, 2014). Such bio-based chemicals can be produced either by bioprocesses or by conventional chemical processes using a bio-based feedstock. Therefore, there are good opportunities for the development of novel bioprocesses for the production of chemicals in the coming years with an increasing number successfully applied at an industrial scale. Different types of bioprocesses and examples of bioprocesses that have been successfully applied in industry are given in section 1.2.

The increasing interest in bio-based products and the definition of a bioeconomy strategy by European Commission is explained by the many challenges that Europe and the world is nowadays facing, such as an increasing world population; depletion of many natural resources, namely fossil fuels; the impacts of increasing environmental pressures and climate change (European Commission, 2013). But it is clear that one driver for the expansion of bioeconomy is also the consumers demand for product transparency. Governments are pushing for sustainability labeling of products, for instance, the Grenelle Law II in France covers the quantification of environmental impacts and the communication of the environmental footprint of the products to the consumers via labeling. And major multinational retailers and brands are also undertaking life cycle assessments of their entire supply chain (Golden and Handfield, 2014). Hence, improved environmental performance of industrial and consumer goods is a significant driver of the development of bio-based chemicals.

However, there is also a need for more effective quantification of how the expansion of bioeconomy might impact the environment, namely land use and water quantity and quality. A study by Weiss and co-workers compared 44 LCA studies of bio-based materials and showed that bio-based materials saves, relative to conventional materials, 55±34 gigajoules of primary energy and 3±1 CO_2 equivalents of greenhouse gases. However, bio-based materials may also increase eutrophication by 5±7 kilograms of phosphate equivalents and stratospheric ozone depletion by 1.9±1.8 kg of nitrous oxide equivalents. The significant errors associated to this values, as well as the variability in the results of LCA studies stresses difficulties in drawing general conclusions but in any way it is important to take these environmental aspects into account when developing processes for production of bio-based chemicals (Weiss et al., 2012). Different tools for assessment of sustainability of bioprocesses are further described in Chapter 2.

1.2 Bioprocesses

Bioprocesses include both fermentation and biocatalytic processes and in some cases it might not be straightforward to distinguish them.

In the context of this thesis biocatalytic processes are those applying enzymes as catalysts in the conversion of one main substrate to a product in one or more steps. The biocatalyst can be used in two different forms: i) isolated enzymes, or ii) whole-cells, which can be growing or resting. These two forms of biocatalysts can then be used in different formulations - liquid formulations or immobilized on or into larger particles of inert support materials.

Fermentation processes on the other hand correspond to the production of products associated with cell growth, which are produced by primary or secondary metabolic pathways. Primary metabolic pathways produce compounds with low molecular weight, such as alcohols, aldehydes, ketones and carboxylic acids. Examples of products of secondary metabolic pathways are amino acids, proteins or antibiotics (Villadsen, 2015).

Examples of both types of bioprocesses are given in the following sections.

1.2.1 Biocatalytic processes

Biocatalytic processes are those that apply enzymes, which can be isolated or used as whole-cells, as catalysts for the synthesis of chemical products.

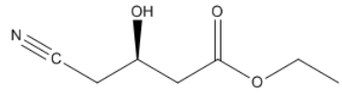
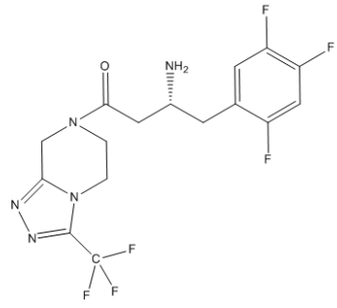
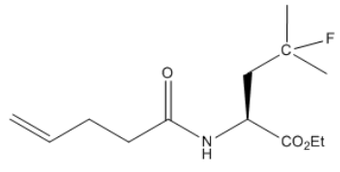
Enzymes have an exquisite advantage over chemical catalysts, - they have a very high selectivity (enantio-, regio- and chemoselectivity). As a result of this excellent selectivity, enzymes can be used in complex reactions without the need of blocking and deblocking steps that are common in conventional organic synthesis. This further results in fewer by-products and consequently reduced waste production (Schmid et al., 2001).

Biocatalytic processes also operate at milder reactions conditions, with moderate temperatures, pH and pressures and generally run in aqueous media, preventing large consumption of organic solvents. Unlike many of the metal and organometallic catalysts conventionally used in chemical industry, enzymes are non-

toxic catalysts and are produced by fermentation from renewable feedstocks. All these features can make biocatalysis an environmentally friendly alternative to conventional chemical catalysis.

But the most promising advantage of biocatalysis over conventional chemical catalysis is the possibility of modifying the catalyst and improve it to have a set of desired properties. When using a biocatalyst there is the freedom to improve the selectivity, stability and activity of the enzyme, and therefore improve reaction rate and reaction yield. The initial phase of enzyme discovery involves screening available microbial culture collections or libraries of enzymes from a variety of sources in order to identify an enzyme with activity towards the substrate of interest. In some cases the wild-type enzyme may have the required activity and selectivity to be applied in a reaction system, but the most likely scenario is that it will require some improvements in catalytic activity, selectivity and stability under process conditions that are required for practical applications. Directed evolution of enzymes by iterative cycles of random mutagenesis, protein expression and screening or selection of the desired activity emerged as a powerful technology that allows significant improvements of an existing enzyme (Turner, 2009). Enzyme improvement and optimization is however a time consuming process and it can take between 3 to 15 months to develop a new biocatalyst employing a team of skilled scientists (Tufvesson et al., 2010). Still, in many cases improvement of the biocatalyst is essential to ensure an industrial application of the biocatalyst and there are several examples where this has been successfully achieved, particularly in pharmaceutical industry (Bornscheuer et al., 2012). Table 1.1 shows some examples of biocatalytic processes that have been successfully developed and applied in industry.

Table 1.1 Some examples of industrial biocatalytic processes.

Product	Enzyme Class	Technology	Reference
 (S)-ethyl-4-chloro-3-hydroxybutyrate, an intermediate for atorvastatin (API of the cholesterol lowering drug Lipitor®) manufacture.	Ketoreductases (KREDs) EC 1.1.1.2	Various protein engineering approaches, including directed evolution, to increase activity, stability and coenzyme specificity. The volumetric productivity per biocatalyst loading was improved 2500 fold, resulting in a 14-fold reduction in reaction time, a 7-fold increase in substrate loading, 25-fold reduction in enzyme use and 50% improvement in isolated yield.	(Ma et al., 2010)
 The product is sitagliptin, an antidiabetic drug.	Transaminases EC 2.6.1.X	Protein engineering to develop enzyme with initial activity, followed by activity improvement in increasing DMSO, substrate and isopropylamine, as well as increased thermostability.	(Savile et al., 2010)
 L-fluoro-leucine, an intermediate in odanacatib manufacture. Odanacatib is a cathepsin K inhibitor, which is an important class of antiresorptive agents that help prevent bone loss.	Hydrolases EC 3.X	Enzyme immobilization and reaction engineering increased stability and activity under process conditions. The process has been demonstrated several times at 100 kg scale (>90% yield and 88% ee).	(Truppo and Hughes, 2011)

Biocatalysis also shows an exquisite advantage over fermentation processes, which is the possibility to run a reaction with unlimited space-time yield. Typically in aerobic fermentations, space-time yield is limited by the oxygen consumption required for cell growth. But by disassociating the desired reaction from cell growth it is possible to achieve a much higher space-time yield.

1.2.2 Fermentation processes

Fermentation processes involve *de novo* production of a molecule from a carbon and energy source, such as glucose via primary metabolism, rather than conversion of a precursor molecule to the desired product by enzymes or whole cells (Straathof et al., 2002).

Fermentation processes can be used to produce a wide range of products in all sectors of chemical industry – bulk, fine and pharmaceutical chemicals, with varying production prices and volumes and some examples are given in Table 1.2.

Table 1.2 Examples of industrial products produced by fermentation (adapted from (Heinzle et al., 2008)).

Product	Annual production (tons/year)	Price range (€/kg)
Ethanol	99,000,000	0.2
Citric acid	1,700,000	1
Proteases	100,000	3
Aspartame	10,000	5
Tetracyclines	5000	55
Insulin	8	120,000

According to their size, fermentation products can be sub-divided into small molecules and large molecules. Small molecules have a molecular weight of 30-600 Da and a radius smaller than 1 nm. Examples of small molecules are sugars, amino acids, organic acids or vitamins. Large molecules include proteins, nucleic acids and polysaccharides and have a molecular weight of 10^3-10^{10} Da and a radius typically larger than 1 nm. Among small molecules, a distinction can also be made between primary and secondary metabolites. Primary metabolites, e.g. organic alcohols and acids are produced in the primary growth phase of the microorganisms, while secondary metabolites are formed at or near the stationary phase. One classic example of secondary metabolites are antibiotics. However, in many cases the distinction between primary and secondary metabolites is not straightforward (Heinzle et al., 2008).

The microorganisms used as production hosts for fermentative production of chemicals can also be numerous, being filamentous fungi, the yeast *Saccharomyces cerevisiae* or the bacteria *E. coli* some of the most common ones.

Some fermentation processes, such as the production of citric acid or ethanol show remarkably high yields, productivities and product concentrations and have become the conventional method for production of these chemicals, overcoming chemical production methods. Some successful examples of fermentation processes are shown in Table 1.3.

Table 1.3 Some examples of commodity chemicals produced by fermentation technology. The achieved yield, product concentration and space-time yield correspond to the highest values that have been reported in scientific literature (adapted from (Straathof, 2014)).

Product	Production organism	Production by fermentation (million tons/year)	Theoretical yield (g/g)	Achieved yield (g/g)	Product concentration (g/L)	Space-time yield (g/(L.h))
Citric acid	*Aspergillus niger*	1.7	1.07	0.88	240	1.4
Ethanol	*Saccharomyces cerevisiae*	99	0.51	0.50	170	82
Lactic acid	*Lactobacillus sp.*	0.37	1.00	1.00	231	150

Developing a commercially relevant fermentation process is a challenging task, especially if the product is a bulk chemical. Space-time yield, product concentration and the yield of product on carbon source determine the economy of these processes.

One of the main difficulties when developing a fermentation process is to be able to transfer the required amount of oxygen for cell growth. The rate of cells growth is often limited by the rate of dissolution of oxygen from air bubbles into the liquid and this topic has been subject of research for decades (Tsao and Lee, 1975). The oxygen transfer limitations is translated in the differences in space-time yield between aerobic and anaerobic fermentations. Ethanol and lactic acid production are good examples of anaerobic fermentation processes, where the space-time yield that is achieved is extremely high (see Table 1.3). On the other hand, citric acid is a typical example of an aerobic fermentation where space-time yield does not exceed 2 g/(L.h).

Further limitations in the development of fermentation processes are the cost and availability of feedstocks, and a lot of research has been done in the development of fermentation processes from alternative

feedstocks, e.g. lignocellulosic materials; and metabolic engineering of the cells so that more products can be produced from renewable bio-based materials by fermentation (Woodley et al., 2013).

1.3 Natural products

Natural products constitute an extensive family of diverse organic molecules with more than 200,000 compounds discovered and extracted from a variety of natural sources. (Pearsall et al., 2015).

Natural products can be sub-divided into two categories – primary and secondary metabolites. Primary metabolites are essential for all types of living cells (plants, microbial and animal) and include sugars, fatty acids, amino acids, nucleic acids as well as all the chemicals considered ubiquitous for plants growth and development, such as regulators, cell wall components and metabolites unique to photosynthetic organelles like chloroplasts. Secondary natural products are structurally and chemically much more diverse than primary natural products, they outnumber primary natural products by orders of magnitude and are named secondary because they have no established role in growth and development (Wu and Chappell, 2008). There are several different classes of natural products, that can be either primary or secondary metabolites: polyketides; non-ribosomal peptides; alkaloids; phenylpropanoids, which include flavonoids, coumarins, lignans and stilbenes; fatty acids and terpenoids, also known as isoprenoids (Pearsall et al., 2015).

Natural products are compounds that are structurally very complex, have multiple chiral centers and can have different biological activities. The vast number of secondary natural products is thought to be a result of evolution, for instance, for development of plant defense responses to predators, parasites and diseases. For example, capsidiol is an antimicrobial sesquiterpene biosynthesized by tobacco cells and tissues in response to microbial threats (Wu and Chappell, 2008)**.**

Natural products can have several interesting properties and they have been particularly important for pharmaceutical industry, delivering novel antibiotics, hormones and antitumor agents to the list of therapeutic drugs. Over the last 25 years, nearly two-thirds of all newly approved drugs (between 1981 and 2012) were derived or inspired by natural products structure (Newman et al., 2003) (Newman and Cragg, 2007).

The richness of natural products lies within the structural complexity associated with these compounds. The possible benefits of natural products to modern society are however hindered by their relatively poor abundance in nature, which restricts their use in research and development as extraction from natural

sources has very low yields. Besides the low concentration of natural products in plants, they tend to accumulate over long growth periods, often as mixtures of hundreds of different compounds, and the quantity and quality of natural products also varies with geographical location and climate conditions (Wu and Chappell, 2008). All these features make the extraction of natural products economically unfeasible in most cases.

Moreover, the structural complexity of these compounds presents challenges for chemical synthesis and most conventional organic chemistry methods are impractical for the synthesis of natural products (Pearsall et al., 2015). Synthetic chemists have developed means to synthesize some natural products, but often with relatively low yields and resulting in racemic mixtures at each step in multistep synthesis. Coupling together multiple synthetic routes of such complexity results in very low yields.

Expressing the genes responsible for the biosynthesis of these natural products and inserting them into fermentable organisms like yeast and bacteria represent an interesting alternative to meet these production limitations. Thus metabolic engineering is one of the areas where great success on the biosynthesis of natural products has been achieved - namely the production of terpenoids, carotenoids, vitamin E and flavonoids (Ajikumar et al., 2010) (Martin et al., 2003) (Anthony et al., 2009) (Pfeifer et al., 2001) (Katsuyama et al., 2007) (Hawkins and Smolke, 2008) (Minami et al., 2008) (Lee and Schmidt-Dannert, 2003) (Das et al., 2007). Enhancement of the production of natural products may involve the import of heterologous genes and overexpression of required native enzymes; increasing the cell uptake of essential substrates; optimizing pathways by minimizing alternative product formation; increasing cofactor availability and augmenting substrate usage; optimizing enzyme activity and specificity by protein engineering or directed evolution of enzymes and optimizing access to the final products by secretion or cell lysis methods. Examples of tools that are typically used to tackle these aspects include overexpression of enzymes mediating rate-limiting reactions, deregulation of gene expression, introduction of regulatory transcription factors, heterologous pathway construction, optimizing codons for efficient protein expression, use of feedback-resistant enzymes, exclusion of opposing pathways to minimize by-product formation and prevention of product degradation (Yadav et al., 2012).

The three possible routes for production of natural products are represented in Figure 1.1.

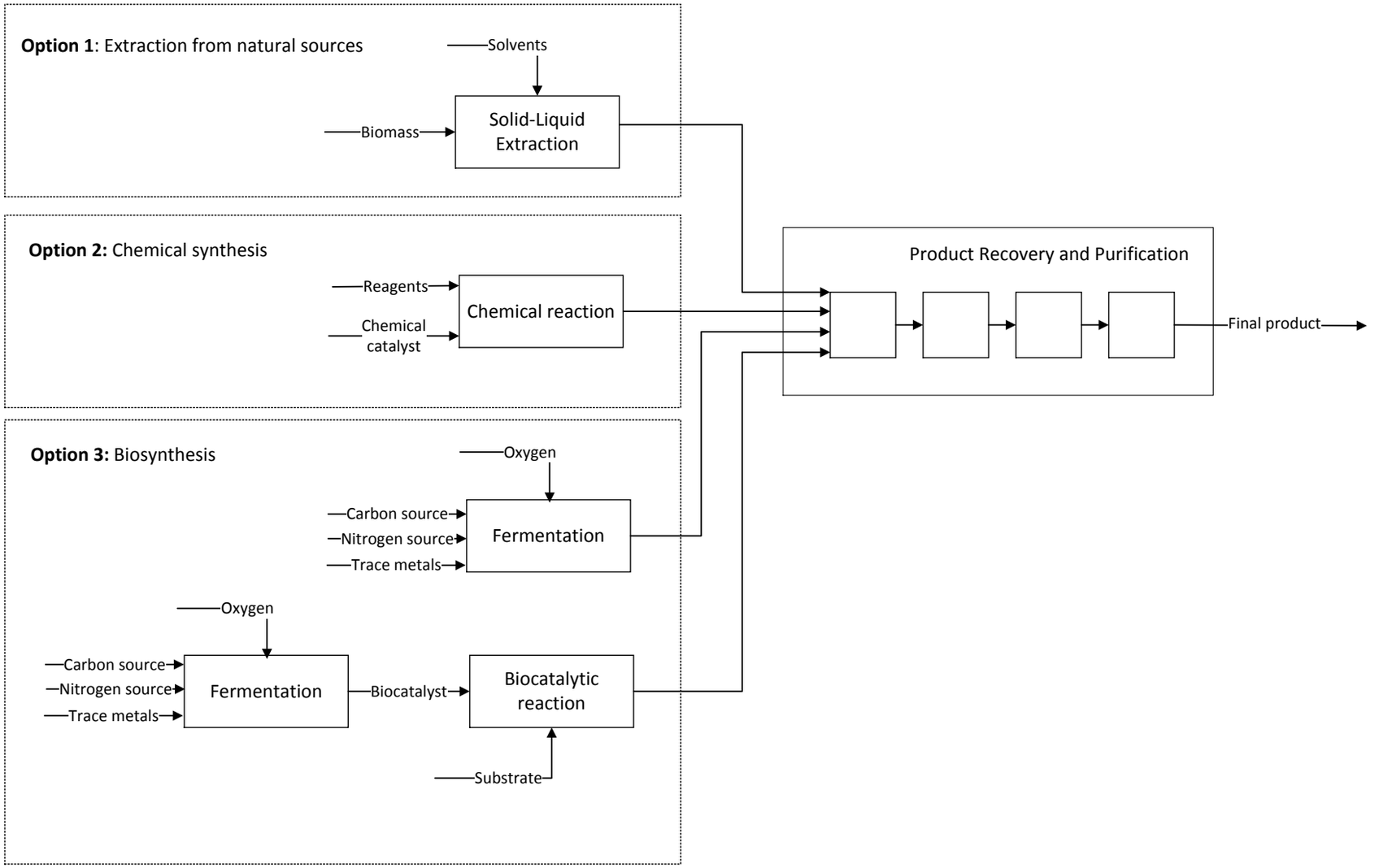


Figure 1.1 Schematic representation of all the possible routes for natural products manufacturing.

One particular class of natural products where there has been a lot of focus on research are the isoprenoids or terpenoids. This is the largest class of natural products with more than 40,000 different molecules identified. Many terpenoids are commercially interesting because they can be used as flavors or fragrances in foods and cosmetics, e.g. menthol; they can have medicinal properties, e.g. paclitaxel, an anti-cancer drug; or have agricultural significance, e.g. cucurbitacins have shown to be involved in insect resistance (Aharoni et al., 2005).

Two examples of successful natural products production by heterologous gene expression are i) the production of the antimalarial drug artemisinin; and ii) the production of paclitaxel, a powerful anti-cancer drug.

Starting with paclitaxel, also known as Taxol, this is a natural diterpenoid alkaloid that was first isolated from the bark of the pacific yew tree *Taxus brevifolia*. It is a powerful anti-cancer drug that was first prescribed for the treatment of ovarian and breast cancer, but later on was found to be effective in the treatment of other types of cancer. It is nowadays commercialized by Bristol-Myers Squibb under the name Taxol®. As for most natural products, extraction from the natural sources is not a viable option – the extraction yield is only 1 gram of paclitaxel per 10 kg of dry barks (Fatima et al., 2014). Taxol's structural complexity limited its chemical synthesis. It was first synthesized in 1994 by Nicolaou and co-workers in 51 steps but with a final yield of 0.002% (Nicolaou et al., 1994). Later on a method for plant-cell based culture has been developed and *Taxus* plant cell cultures have been scaled-up up to 7,500 liters and are currently commercially employed by the company Phyton Biotech (Germany). However, *Taxus* plant cell cultures are still limited for large-scale commercial use because of the low and unstable yield of paclitaxel, high production cost and low selectivity over unwanted by-products. Moreover, plant cell cultures display a large degree of heterogeneity in secondary metabolite production capabilities (Frense, 2007). Recent developments in metabolic engineering and synthetic biology allowed the expression of the metabolic pathway for Taxol precursor, taxadiene, in yeast (Engels et al., 2008) and in *E.coli*, the last with a very promising titer of approximately 1 g/L (Ajikumar et al., 2010).

Another interesting progress made in the production of natural products is the biosynthesis of artemisinin, an antimalarial drug. Artemisinin is naturally produced by the plant *Artemisia annua*, which has a long history of use in traditional Chinese medicine. The antimalarial properties of *A. annua* extracts were rediscovered in the 1970s and later on the active compound artemisinin was identified as well as its structure. Artemisinin derivatives were recognized as anti-malarial drugs by World Health Organization in 2002, but artemisinin extraction from the plants showed several complications, namely fluctuations in artemisinin availability and price which is a major concern given the fact that malaria is predominantly a disease of the developing world.

Thus, the Semi-synthetic artemisinin project was born as a partnership between the University of California (Berkeley, USA), Amyris Inc. and the Institute for OneWorld Health and funded by Bill and Melinda Gates Foundation. The project has been able to successfully engineer both *E. coli* and yeast *Saccharomyces cerevisiae* to produce artemisinic acid, which can later on be converted to artemisinin by conventional organic chemistry conversion. The engineered yeast was selected as the production host and is able to produce 25 g/L of artemisinic acid. Industrial production of semi-synthetic artemisinin (semi-synthetic because the last steps are accomplished by organic chemistry) by the company Sanofi has already successfully started (Paddon and Keasling, 2014).

The biosynthesis of the antimalarial drug artemisinin is a very successful case of heterologous gene expression for microbial production of natural products. However, the number of natural products that are commercially available is still very limited and more efforts are required for the development of synthetic approaches for scaling up the production of bioactive natural products (Fatima et al., 2014).

1.4 Scope of the thesis

Even though there is an enormous potential in natural products and a big interest in their commercialization, the task of developing an industrial process for manufacturing of natural products is very challenging. This thesis aims at defining some tools for benchmarking of processes for natural products production, as well as guidelines for the development of biosynthetic processes for a successful intensification and implementation at industrial scale.

The set of tools established in this thesis can be used to evaluate processes for natural products production in an early stage of development, both from an economic and an environmental point of view. These evaluation tools allow benchmarking of different routes and process screening of the different alternative processes for production of a given natural product, as well as determination of the main process bottlenecks which can provide guidance for research and development.

This systematic evaluation approach is illustrated with two different case-studies that involve the production of two different natural products – i) the bioconversion of (*R*)-limonene to (*R*)-perillic acid by a whole-cell biocatalyst, and ii) the fermentation of *Pichia pastoris* for brazzein production.

The main components of this thesis are schematically represented in Figure 1.2.

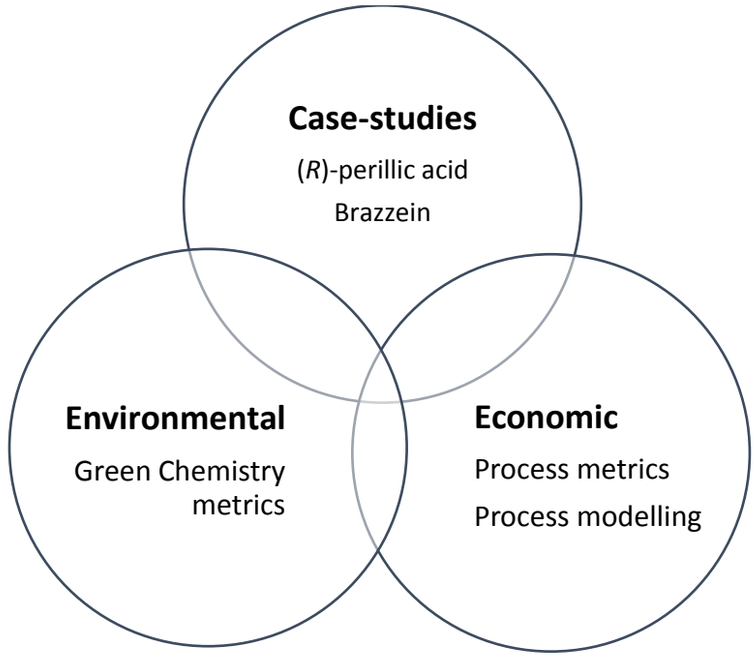


Figure 1.2 Schematic representation of the scope of the thesis.

1.5 Thesis outline

The thesis is organized into three main parts, as described below.

Part I (Chapter 2) – Provides an introduction and background to the different tools for economic and environmental assessment of bioprocesses at different stages of development.

Part II (Chapter 3-4) – Focuses on all the work developed for the two case-studies.

- Chapter 3 is dedicated to case-study 1 – the bioconversion of (*R*)-limonene to (*R*)-perillic acid by a whole-cell biocatalyst.
  It includes the route selection for perillic acid production and an evaluation of the process both from an economic and an environmental perspective. Additionally, experimental work for characterization of the reaction system is also presented as well as a discussion of the main bottlenecks of the process and strategies to improve it.
- Chapter 4 is dedicated to case-study 2 – the fermentation of *Pichia pastoris* for brazzein production.

This chapter is focused on the comparison of different processes for sweeteners production from an environmental perspective. It aims to provide guidelines for implementation of environmental assessment of bioprocesses in early stages of development as a tool for process development.

Part III (Chapter 5-7) – Includes a general discussion of the methodology for evaluation of bioprocesses for natural products biosynthesis in an early stage of development. A discussion of the applicability of the different evaluation tools is included, as well as conclusions and future perspectives for this work.

Part I

Evaluation tools

2. Sustainability assessment of bioprocesses

Sustainability focuses on a triple bottom line - the integration of environmental integrity, social responsibility and economic viability, represented in Figure 2.1. This triple bottom line is also known as the three Ps: Profit, Planet and People. The three pillars of sustainability complement each other and when talking about sustainability one must take all the three parts into consideration.

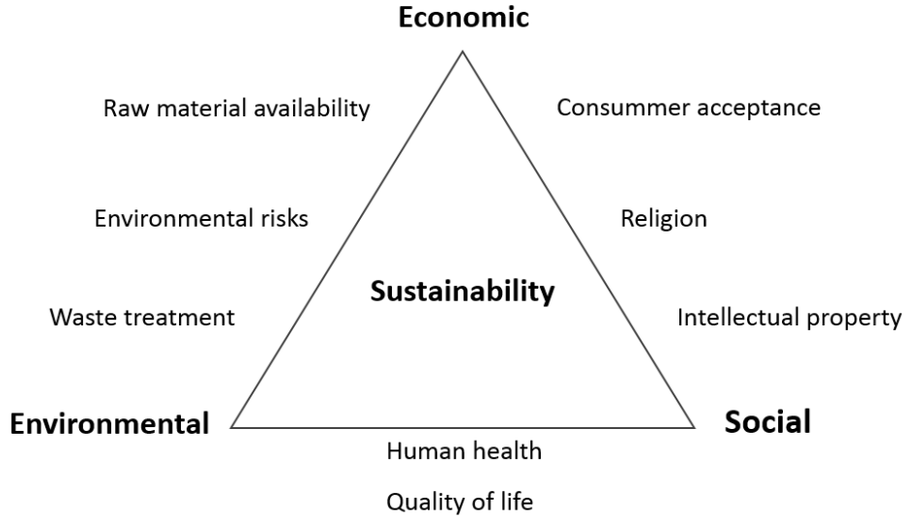


Figure 2.1 Interaction between the three parts of sustainability: economic, environmental and social (adapted from (Heinzle et al., 2008)).

The interactions between the three parts of sustainability are many and complex. For instance, the raw material availability depends on the depletion of natural resources (environmental issue). On the other hand, the availability of a certain raw material affects its price, which in turn affects the economy of the process.

Both economic and environmental assessments, which are further developed in the following sections, were applied to the case-studies considered in this thesis. The social aspects of sustainability have not been included in neither of the two case-studies.

2.1 Economic assessment

2.1.1 Process metrics

The economic feasibility of a biocatalytic process is of vital importance and it determines if a process can be scaled-up to industrial manufacturing or not. The performance of a bioprocess, either a biocatalytic or a fermentation process, can be assessed using a set of metrics that can guide biocatalyst and process development in early stages of development (Lima-Ramos et al., 2014b) (Van Dien, 2013).

In the case of biocatalytic processes these metrics are reaction yield, biocatalyst yield, space-time yield or volumetric productivity and product concentration. The target values for these metrics are influenced by the market value and annual production of the product of interest, which determine the scale of the production process. The minimum values for each process metric are case specific. They depend on the type of catalyst used, whether it is an isolated enzyme or a whole-cell biocatalyst, the biocatalyst formulation (liquid or immobilized) and the type of product and it is therefore difficult to define general targets for biocatalytic processes.

Reaction yield measures the amount of product formed per amount of substrate consumed and it should be expressed on a molar basis (% mol product/mol substrate). Reaction yield has been inaccurately defined as a ratio between the mass of product and the mass of substrate in previous work from Lima-Ramos and co-workers and Lundemo and co-workers (Lima-Ramos et al., 2014b) (Lundemo and Woodley, 2015). However, reaction yield should always be defined as a molar ratio and in the particular case of natural products, it does not make sense to calculate a mass ratio since most of the times the product has a higher molecular weight than the substrates and therefore using a mass ratio could lead incorrectly to a reaction yield higher than 100%. Reaction yield represents a measurement of raw materials cost contribution and therefore this metric is of greater importance for bulk chemicals, which have a low selling price and therefore the gap between the cost of raw materials and a profitable product cost is low. The production costs are often dominated by the cost of raw materials which puts pressure on reaction yield. The other situation where reaction yield can be of major importance is if the substrate is a high-value chemical and the price difference between substrate and product is low. The reaction yield is determined by the substrate concentration in the reaction media and by the enzyme specificity. High reaction yields might be difficult to achieve in many biocatalytic reactions due to catalyst inhibition or instability (Lima-Ramos et al., 2014b) (Ramesh et al., 2016).

Product concentration measures the downstream processing costs, given that for lower product concentrations, a high volume of reaction mixture is sent for product recovery and purification. Therefore, there will be a high cost for removing a high amount of solvent from the reaction mixture for product recovery (Lima-Ramos et al., 2014b).

Demands on reaction yield and final product concentration are relevant in processes with isolated enzymes or whole-cells. Space-time yield (STY) is however more important for processes with growing whole-cells in the same way as it is for fermentation processes. Space-time yield or volumetric productivity corresponds to the mass of product formed per reaction volume per reaction time and is typically expressed in g/L/h. It accounts for capital costs, namely for the reactor cost, and energy and labor related costs during the reaction (Lundemo and Woodley, 2015).

For resting cell processes or processes with purified and isolated enzymes, biocatalyst yield is applied instead. This metric represents the total amount of product that can be produced per mass of biocatalyst, similarly to the total turnover number. It reflects the cost of biocatalyst production (fermentation and biocatalyst purification and formulation) on the biocatalytic process. Hence, its values are influenced by the final cell density and protein expression of the fermentation process for enzyme production. Catalyst recycle is one of the possible strategies to improve the biocatalyst yield (Lima-Ramos et al., 2014b) (Ramesh et al., 2016) (Lundemo and Woodley, 2015).

Several successfully implemented industrial biocatalytic processes show product concentrations higher than 50 g/L or space-time yields higher than 1 g/L/h. Straathof and co-workers reviewed 134 biotransformation processes for production of fine chemicals, both with isolated enzymes or whole-cells as catalysts, and reported space-time yields well over 1 g/L/h, reaction yields typically well over 80% and mostly over 90% (exceptions are kinetic resolutions which give yields close to 50%) and final product concentrations well above 50 g/L. Product concentrations varied from 50 g/L to above 200 g/L for carbohydrates and amides. The differences in product concentrations correlated with product solubility, toxicity and/or stability (reaction systems where product inhibition was seen showed a lower product concentration) (Straathof et al., 2002).

The biocatalyst yield is dependent on the enzyme formulation. For immobilized enzymes or immobilized resting cells, yields of 10^2-10^4 kg per kg of catalyst have been reported (Thomas et al., 2002).

In order to achieve process metrics that allow an economically feasible implementation of the process, it is clear that in most cases a strategy for biocatalyst improvement (via targeted protein engineering) should be complemented by the implementation of process intensification strategies into the reaction and process of interest (Ramesh et al., 2016). The development of a biocatalytic process should then be done in two main

steps - first by process intensification followed by process scale-up and increase of the production volume. In the process intensification stage, the process is kept at a small scale but the process metrics are improved by enzyme modification and reaction and process engineering tools. Once the process metrics have achieved the desired values, there is confidence that the process is indeed scalable and the volume of production can be scaled-up (Lima-Ramos et al., 2014a).

Regarding fermentation processes, product concentration and space-time yield are also critical metrics to evaluate them and are in the same range of values as for biocatalytic processes with growing cells (Oudshoorn et al., 2010). The third metric that is used to assess fermentation processes is the yield of product on the carbon source, measured in %(C-mol/C-mol) or %(g/g). In this case, the rule of thumb is that the yield needs to be higher than 80% of the maximum theoretical yield (calculated from the metabolic pathway) for bulk chemicals (Van Dien, 2013).

Several examples of microbial cells whose metabolism is engineered to produce chemicals and/or fuels, as well as novel enzyme-based reaction systems are described in scientific literature. However, it is still a relatively small number of them that reaches commercialization. At early stages of research, cost calculations and understanding the influence of process metrics on the production cost are critical to estimate targets to obtain an economically feasible process with a given production organism (van't Riet, 1986). It is difficult to estimate the cost and research intensity necessary to modify the production microorganism or enzyme. But having that, one can weight it against the targets for the key process metrics and estimate the cost and development time necessaries to achieve industrial implementation.

2.1.2 Full cost estimation

Economic evaluation is a tool that is often used in chemical engineering to quantitatively estimate the costs and the expected profitability of a given chemical process. It includes cost estimation, revenue estimation and profitability analysis.

Typically cost estimation is made throughout the development process of a chemical process and the accuracy of the estimations increase in later development stages. Cost estimation in a preliminary design stage involves the application of rules-of-thumb and rough estimations (e.g. for equipment cost), which means that the accuracy of estimations at this stage of development is somehow dependent on the experience of the engineer working on this task. Typically in early-stages of development the accuracy is about ±20-30%. As the project design is refined, the estimates become more detailed and accurate. But even

with a certain degree of uncertainty it is extremely useful to estimate the costs of a chemical process during its development stages since it facilitates process debottlenecking and can guide research (Lima-Ramos et al., 2014b).

Cost estimation is divided into two categories: estimation of capital expenditure or capital investment (CAPEX) and operating expenditure or operations cost (OPEX).

CAPEX corresponds to the costs of getting the industrial plant into operation. It includes the fixed-capital investment (FCI), which refers to the capital necessary for all the equipment and accessories required in the process operation and start-up; and working capital, which corresponds to the sum required for day-to-day operation of the plant, including the cost of inventories and supplies.

Operation costs (OPEX) consist of variable production costs; fixed charges and plant overhead costs. Variable production costs include raw materials costs; labor costs; utilities; patents and royalties; waste treatment and disposal; laboratory, quality control and quality assurance. Fixed charges or fixed costs include depreciation of equipment and buildings, local taxes, property insurances and rent and interests. Finally, plant overhead costs include general expenses, such as administrative costs, distribution and marketing, and research and development (R&D) costs (Peters et al., 2004). Figure 2.2 includes a scheme of all categories and sub-categories of cost estimation.

Since in early stages of development the level of uncertainty is high, it is also necessary to evaluate the effect of certain modifications to the original process on the total process environmental and economic profile by performing a sensitivity and/or uncertainty analysis (Lima-Ramos et al., 2014b). Developing a process model even in early stages of development can be extremely useful, since it can be a tool to evaluate the influence of certain process parameters on the final production cost (OPEX).

There are several different softwares for process design and simulation that facilitate cost estimation, since they incorporate an estimator of equipment prices. Examples of these softwares are Aspen Plus®, from Aspen Technology, Inc.; SuperPro Designer®, from Intelligen Inc.; or SimSci PRO/II from Schneider Electric. In this thesis SuperPro Designer® (version 9.5) was selected as the software for process design, mass and energy balances and cost estimation.

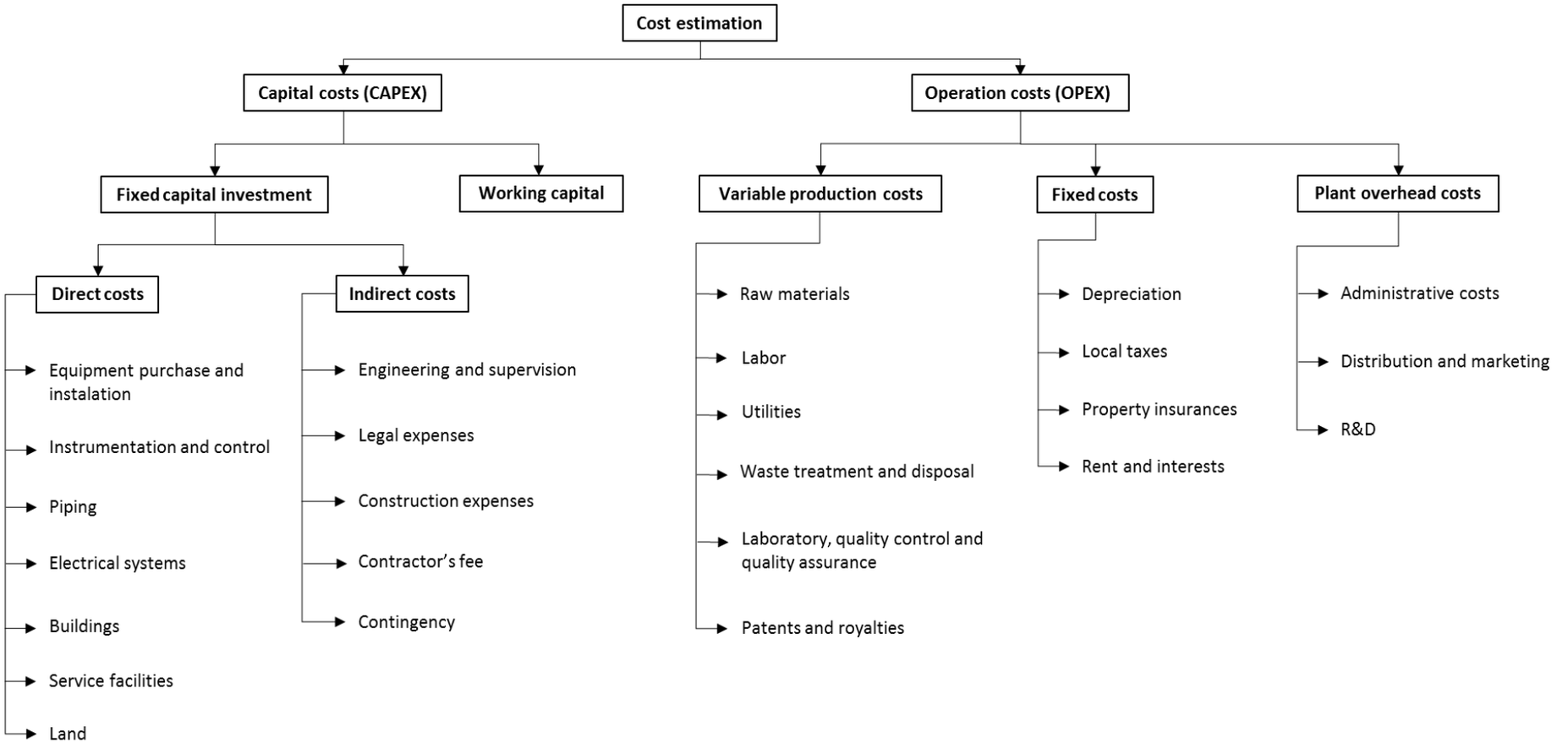


Figure 2.2 Cost estimation different categories and sub-categories.

2.2 Environmental assessment

Over the past decades there has been a rise in public awareness of health and environmental issues of consumer goods. Most companies recognize this trend and feel continued pressure to find greener alternatives. Policy makers are also increasingly concerned with sustainability, which is translated in one hand on tighter regulations on the use of certain raw materials and on the emissions resulting from chemical production processes, and on the other hand in an increase of interest for research projects aiming to develop sustainable and “green” chemicals. This trend has led many companies to evaluate the sustainability of their products and production processes and to include environmental concerns as a driver for product and process development.

Both companies and academia are today faced with the challenge of having to evaluate the environmental impact of a certain chemical synthetic route and/or production process. Hence, there is the need for developing methods for assessing the environmental profile of chemical production processes in different development stages, which is a rather complex task. Different tools for the environmental assessment of chemicals processes, with varying degrees of detail and complexity have been developed and are nowadays applied by many chemical companies, in particular pharmaceutical companies, to assess their processes. On one hand there are reaction- and process-related metrics, which try to comparatively assess processes from a green chemistry and green engineering perspectives. On the other end of complexity we have the Life Cycle Assessment (LCA) methodology – see Figure 2.3.

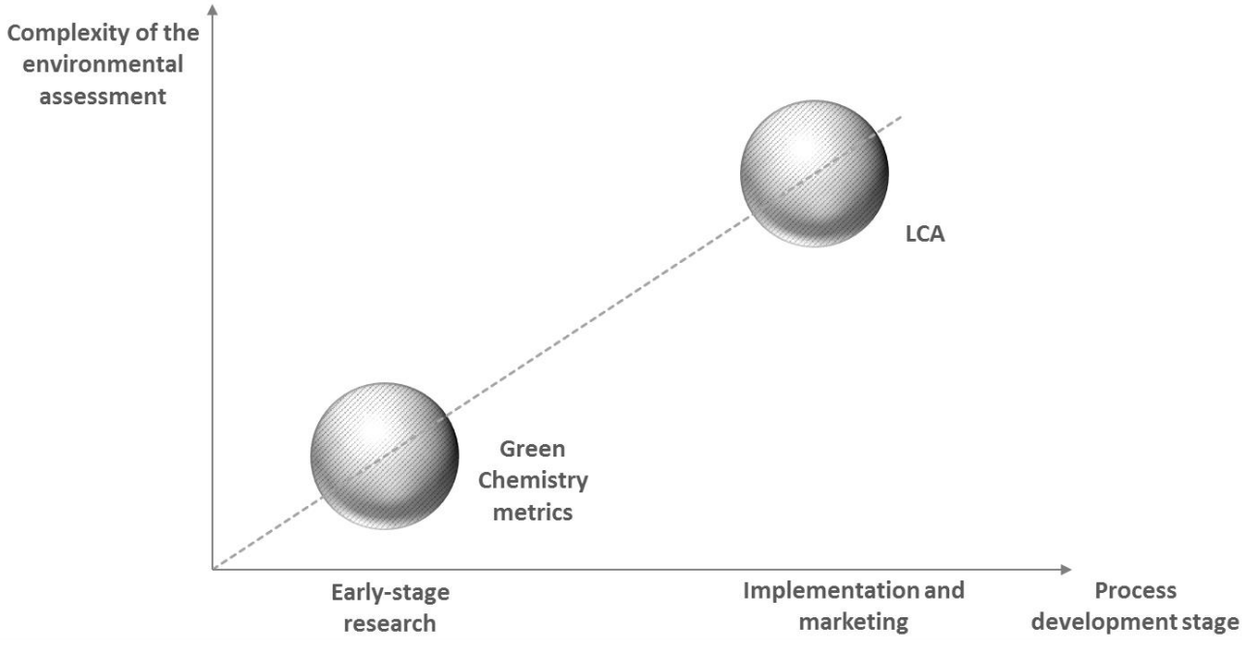


Figure 2.3 Correlation between process development stage and the complexity of the environmental assessment.

Given the complexity of these different tools and the amount and type of data required for each of them, they should be applied in different stages of development of the chemical process.

Green Chemistry metrics try to quantify and measure the "greenness" of a given process or reaction by translating it in terms of simple metrics which should be easy to understand and apply. The amount of data required is relatively low, which makes it an appropriate tool for synthetic route selection and early stages of research and process development. These simple metrics lack however a holistic view of the environmental impacts of the product in its whole life cycle and do not distinguish the different types of waste and emissions generated.

LCA on the other hand is a very detailed tool for evaluation of the product environmental burden in its entire life cycle (including raw materials manufacturing, manufacturing process of the desired product, use, transportation and final disposal). It is a very work intensive, complex and time-consuming tool and is therefore adequate for later stages of development, such as the process implementation and marketing.

Some methodologies aiming to guide the environmental assessment of chemical processes in its different development stages have been published. Examples of these are the metrics toolkit resulting from the CHEM21 project for evaluating the "green credentials" of a certain reaction, developed by McElroy and co-workers (McElroy et al., 2015), or the methodology to guide the development of biocatalytic processes

developed by Lima-Ramos and co-workers (Lima-Ramos et al., 2014b). There are slight differences in the methodologies but it is widely accepted that the level of detail of the environmental assessment should increase with the level of development of the chemical process.

The different tools for environmental assessment are described in more detail in the following sections.

2.2.1 Green Chemistry

Green chemistry is a concept and guiding principle which aims to encourage the development of manufacturing processes and products with the lowest possible environmental impact. The twelve principles of green chemistry were introduced in 1998 by Anastas and Warner and are summarized in Table 2.1 (Anastas and Eghbali, 2010).

Table 2.1 The twelve principles of green chemistry (adapted from (Anastas and Eghbali, 2010)).

Prevention	It is preferable to prevent waste than to treat or clean up waste after it is formed.
Atom economy	Synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product.
Less hazardous chemical synthesis	Whenever possible, synthetic methods should be designed to use and generate substances that pose little or no toxicity to human health and the environment.
Designing safer chemicals	Chemical products should be designed to preserve efficacy of the function while reducing toxicity.
Safer solvents and auxiliaries	The use of auxiliary substances (e.g. solvents) should be avoided whenever possible and, when used, innocuous.
Design for energy efficiency	Energy requirements of chemical processes should be recognized for their environmental and economic impacts and should be minimized. If possible, synthetic methods should be conducted at ambient temperature and pressure.
Use of renewable feedstocks	A raw material or feedstock should be renewable rather than depleting whenever technically and economically feasible.
Reduce derivatives	Unnecessary derivatization (use of blocking groups, protection/deprotection, temporary modification of physical/chemical processes) should be minimized or avoided if possible, since such steps require additional reagents and can generate waste.
Catalysis	Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.
Design for degradation	Chemical products should be designed so that at the end of their function they break down into innocuous degradation products and do not persist in the environment.
Real-time analysis for pollution prevention	Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances.
Inherently Safer Chemistry for accident prevention	Substances and the form of a substance used in a chemical process should be chosen to minimize the potential for chemical accidents, including releases, explosions and fires.

These principles go hand in hand with the economy of a process, since the more efficient a process is, the lower waste production is. Figure 2.4 relates waste production, expressed as the difference between the

total mass of materials used in the production process (M) and the mass of final product (P), with process efficiency, which is expressed as the ratio P/M. An ideal process (which is thermodynamically impossible) would have "zero waste production", *i.e.* all the material used in the process would be converted into the final product (P/M=1 and M-P=0). A real process can improve its efficiency and therefore reduce waste production by means of technological improvements to the process and/or change of the raw material used (Lapkin and Constable, 2009).

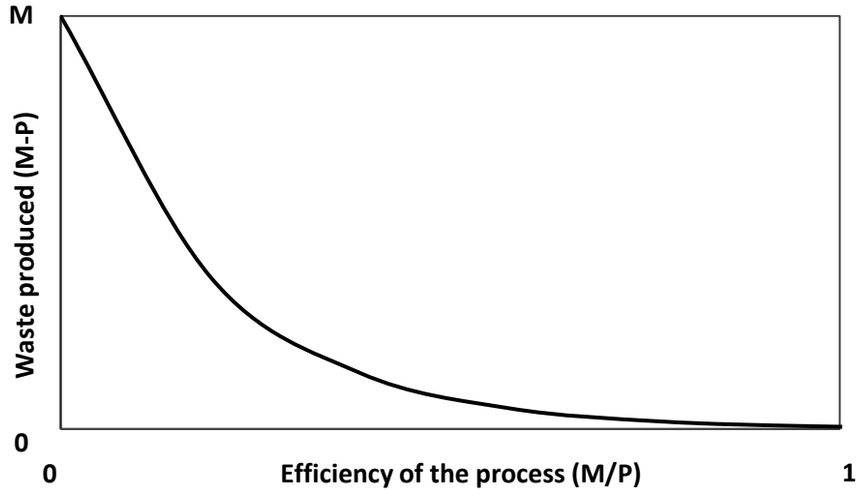


Figure 2.4 Relation between waste produced and process efficiency (adapted from (Lapkin and Constable, 2009)). M represents the total mass of materials used in the production and P the mass of final product.

2.2.1.1 The natural fit of biocatalysis with green chemistry

Biocatalysis fits most of the goals and principles of green chemistry. The high selectivity of enzymes and their ability to carry out difficult chemical reactions eliminate synthesis steps, the associated waste in multistep reactions and the need for protective groups and for resolutions involving chemical derivatives. It also allows most of the starting material atoms to be converted to the product, contributing in this way to a maximization of atom economy.

The preferential solvent used in biocatalytic reactions is water, which eliminates organic solvent waste. The reactions typically run at neutral pH and room temperature, which reduces energy consumption. Mild reaction conditions also minimize the potential for explosions, fires and accidental spills. Furthermore,

enzymes are biodegradable catalysts and are manufactured using renewable feedstocks (Tao and Kazlauskas, 2011).

An evidence that biocatalysis can contribute to green chemistry is the fact that many of the recent winners of the US Environmental Protection Agency's Presidential Award in Green Chemistry have used biocatalysis as the key improvement technique. For instance, in 2012 Codexis, Inc. and Professor Yi Tang from the University of California, Los Angeles received the award for developing a biocatalytic process to manufacture Simvastatin, a drug for treating high cholesterol, (US Environmental Protection Agency) (Xie et al., 2006).

The "green label" given to biocatalysis is often used as a "selling argument". However as appropriately argued by Ni and co-workers, biocatalysis is not green *per se* and there is a big gap between the statement of such green claims and the environmental profiles of reaction systems reported in scientific literature (Ni et al., 2014). In many cases, reactions that are reported in scientific literature take place in very diluted solutions, conditions which are not consistent with an environmentally friendly process. In these conditions large amounts of wastewater are generated, whose treatment consumes additional reagents and energy and can have a negative contribution to the environment (de María and Hollmann, 2015). Moreover, they often face problems of low productivities or space-time yield, which are caused by problems of substrate and/or product inhibition, reactant availability, biocatalyst activity and stability, etc. (Wenda et al., 2011). Claiming that such reactions are "green" just because they fulfill one of the twelve principles of green chemistry (in most cases atom economy) is not truthful since the 12 principles of green chemistry are intended to be used as a cohesive system (Ni et al., 2014).

These reactions that are reported in scientific literature are still under early stages of development and consequently in most of the times they show very high E-factors. In fact, a lot of times these biocatalytic reactions show an E-factor higher than their conventional chemical alternative, as opposed to what would be expected by many researchers. For instance, Kuhn and co-workers compared the biocatalytic production of (*S*)-styrene with three different options of chemical catalysis and it was shown that the biocatalytic option showed an E-factor of 3.42 kg/kg which is lower than two of the chemical options (i) ferric phenanthroline as catalyst with 6.58 kg/kg and ii) manganese sulfate as catalyst with 5.72 kg/kg), but higher than the chemical option using titanium silicate as the catalyst (E-factor of 1.71 kg/kg). This option was also the one with the lowest PMI (3.04 kg/kg) as opposed to the biocatalytic option with a PMI of 21.07 kg/kg (Kuhn et al., 2010).

But in reality, enzymes did not evolve to be an environmentally friendly catalyst. Enzymes have evolved to catalyze a vast number of reactions that contribute to the survival and reproduction of the organisms that produce them. The fact that they can be used for our benefit in industrial production of several types of chemicals is remarkable, but that is not enzymes primary purpose. In fact, many of the characteristics they

have developed during their evolution are not desirable from an industrial perspective. For instance, the fact that many enzymes face product inhibition at certain concentrations of product is what allows them to regulate the flow of metabolites inside the cells, which is fundamental for the cells metabolism. However, for industrial applications this feature is unwelcome since the industrial goal is to produce high concentrations of product in a short period of time (Arnold, 2001). Researchers force enzymes to work in conditions that are completely different from the conditions they have evolved to work on. In nature enzymes have evolved to work in dilute solutions, with low substrate and product concentrations, so it is not surprising that so many enzyme-based reactions show high E-factors.

It is extraordinary to see so many novel enzyme-based reactions being reported and even though most of them do not show an environmentally friendly profile, experience has shown that if the process is successfully intensified by means of biocatalyst modification combined with reaction and process engineering, it is possible to attain enzymes full potential and develop an industrial chemical process with a "green" profile. Several examples have been reported in literature of such processes, and are shown in Table 2.2.

The biocatalytic synthesis of (*S*)-ethyl-4-chloro-3-hydroxybutyrate, an intermediate for atorvastatin production, which was previously mentioned in Chapter 1, shows a total E-factor of 5.8 kg waste/kg product. If water is included E-factor increases to 18 kg/kg, which is still a remarkably low E-factor (Ma et al., 2010).

The biocatalytic synthesis of pregabalin also shows a very low E-factor and PMI. Pregabalin is a lipophilic γ-aminobutyric acid analogue that was developed for the treatment of several nervous system disorders, such as epilepsy or anxiety, and is commercialized under the name Lyrica® by Pfizer. Pregabalin was first synthesized with chemical catalysts, which produced a racemic mixture of pregabalin. The right enantiomer was obtained by classical resolution with a very low efficiency of 30%. The E-factor of this process was therefore relatively high – 86 kg/kg. The chemical synthetic route was then substituted by a biocatalytic route with two lipases. The biocatalytic route reduced the E-factor to 17 kg/kg and the process was still further improved by recycling the wrong enantiomer, (*R*)-cyanodiester, which further reduced E-factor to 8 kg/kg (Dunn et al., 2010) (Dunn, 2012).

Table 2.2 E-factors for some examples of biocatalytic reactions at industrial scale. The E-factors shown only refer to the reaction (DSP is not included) and do not include water.

Product	E-factor ($kg_{waste}/kg_{product}$)	Reference
(*S*)-ethyl-4-chloro-3-hydroxybutyrate	5.8	(Ma et al., 2010)
(*S*)-cyanodiester (Pregabalin)	8	(Dunn, 2012)
(*R*)-tetrahydrothiophene-3-ol	2.5	(Liang et al., 2010)

A quantitative evaluation of these green chemistry metrics at early stages of research and comparison with similar processes at industrial scale can give some valuable insight to the reaction development and provide guidance to researchers working on these reactions in the laboratory. The scientific community working on biocatalysis can greatly benefit from knowledge sharing and reporting of more quantitative studies of the environmental profile of biocatalytic reactions in early stages of research.

2.2.1.2 Green Chemistry metrics

The first green chemistry metrics to be introduced were atom economy by Trost in 1991 (Trost, 1991) and E-factor by Sheldon in 1992 (Sheldon, 1992). Ever since the 90s green chemistry metrics have proliferated in number, several new metrics were introduced (Curzons et al., 2001) (Hudlicky et al., 1999) and a considerable amount of reviews of various metric systems and methodologies on how to assess chemical processes from a green chemistry perspective have been published (Constable et al., 2002), (McElroy et al., 2015), (Jimenez-Gonzalez et al., 2011), (Shonnard et al., 2003), (Roschangar et al., 2015), (Lima-Ramos et al., 2014b).

However, the green chemistry community has not benefited from the introduction of so many new metrics. In contract, there is now an excess of metrics without standardized definitions or boundaries, which inhibits its wide integration in industrial process research and development. There is a lack of unified metrics that researchers both from academia and industry can easily apply in early stages of research, which is a barrier for the fulfillment of green chemistry's full potential (Roschangar et al., 2015).

It is however, a very complex task to select a set of key metrics that have the greatest relevance to evaluate the "greenness" of a given process. It is difficult to find just a few metrics that are able to describe the complexity of a given chemical/biochemical process.

Environmental metrics must be adapted for their context and continuously evaluated as to their utility, applicability and relevance/appropriateness to the process under study. They should be easily understood and accepted by key stakeholders (scientists, engineers, managers, etc.). Overall they should promote strategic analysis and continuous improvement of a process. They should be routinely questioned, assessed, evaluated and evolved to help make strategic decisions and make them useful to a business (Lapkin and Constable, 2009).

Green metrics can be divided into two main groups: i) metrics that are based on the stoichiometric equation of the chemical reaction under study and evaluate its potential environmental impact and ii) metrics that address the amount of waste generated in the entire process (upstream and downstream processing included).

Reaction-related metrics

Atom Economy

The first group of metrics includes atom economy (AE), which was introduced by Trost in 1991 (Trost, 1991). Atom economy (Equation 2.1) is based on a theoretical maximum yield of 100% and it assumes that reactants are used in stoichiometric amounts, disregarding also the use of solvents, acids or bases. It measures how much of the starting material ends in the desired product, assessing in that way the stoichiometry of the reaction. The driver behind this metric is to ensure that chemists design and select a production route where as many as possible of the atoms of the substrate are included in the final product, *i.e.* a synthetic route with a high mass yield and low waste (Lima-Ramos et al., 2014b).

Equation 2.1

$$AE\ (\%) = \frac{Molecular\ weight\ of\ product}{\sum molecular\ weight\ of\ reactants} \times 100$$

Carbon Efficiency

Carbon efficiency (CE) (Equation 2.2) was first developed by GlaxoSmithKline (GSK). It is merely a refinement of AE that takes into account the yield and the amount of carbon in the reactants that is incorporated into the final product. It is defined as the percentage of carbon in the reactants that remain in the final product (Constable et al., 2002). A high carbon efficiency is desired, given that in a reaction system with a low CE there is a greater amount of carbon that will eventually end in waste streams or as emissions (Lima-Ramos et al., 2014b).

Equation 2.2

$$CE\ (\%) = \frac{Mass\ of\ carbon\ in\ the\ product}{\sum mass\ carbon\ present\ in\ reactants} \times 100$$

Reaction Mass Efficiency

Reaction mass efficiency (RME) is a metric that was also developed by GSK. RME (Equation 2.3) takes into account both the concepts of atom efficiency, reaction yields and the molar quantities of reactants. It corresponds to the percentage of the mass of reactants that remain in the product. RME combines key elements of chemistry and process and represents a simple, objective, easily derived and understood metric for use by chemists, process chemists or chemical engineers (Constable et al., 2002).

Equation 2.3

$$RME(\%) = \frac{Mass\ of\ final\ product}{\sum mass\ of\ reactants} \times 100$$

Both atom economy and reaction mass efficiency can be very useful for comparison of different routes for production of a given chemical and complement each other well. Atom economy is a metric that is very simple to calculate and one only needs to know the stoichiometric equation to calculate this metric. It tells

the user if the synthetic route is designed in a way that maximizes the incorporation of all the starting materials used in the reaction into the final product. If so, atom economy should be high (ideally 100%) and the synthetic route has a good potential to be a sustainable synthetic route. Since most of the starting material is incorporated into the final product, the waste and emissions that are generated from this reaction should be low.

However, this metric does not take into account the reaction yield and it disregards the use of auxiliary substance (solvents, acids, bases etc.). Therefore, it can be misleading to look only to atom economy and this is often the case in biocatalytic reactions. Many of these reactions show a very high atom economy and in theory these synthetic routes have a very good potential to be "greener" when compared to conventional chemical synthesis. However, it is also often the case that these reactions have a low reaction yield and run in very diluted aqueous solutions. It is therefore important to complement atom economy with RME, since this metric takes into account the reaction yield and the "real" amounts of reactants and auxiliary chemicals used in the reaction, hence giving a more realistic picture of the environmental profile of the reaction. The biggest disadvantage of RME is the fact that it requires experimental work to be performed prior to its calculation.

Process-related metrics

E-factor

On the group of metrics that assess the environmental impact of the overall process, E-factor is a widely accepted and useful metric, which was first proposed by Roger Sheldon. E-factor (Equation 2.4) is the ratio of waste produced per unit of final product, considering that waste includes all the reactants, solvents, etc., basically all the chemicals used in the process, except the desired product. The higher E-factor is, the higher waste production is and consequently, the environmental impact is greater. Ideally, E-factor should be zero.

E-factor has been widely adopted in chemical industry, particularly in pharmaceutical industry, being widely used by companies such as GlaxoSmithKline, Pfizer or AstraZeneca.

Equation 2.4

$$E\ factor = \frac{total\ mass\ of\ waste}{mass\ of\ final\ product}$$

According to Sheldon definition of E-factor, water is excluded from its calculations since in many cases its inclusion can lead to exceptionally high E-factors that can hinder a meaningful comparison of chemical processes. In many instances, water by itself does not constitute a significant environmental impact. However, in certain industries, such as pharmaceutical industry, the use of highly purified water and the use of high amounts of water in fermentation and biocatalytic processes, can result in a considerable environmental impact. Therefore, there is a trend in the pharmaceutical industry towards the inclusion of water in E-factor. Also because water usage becomes increasingly problematic due to shortage of potable water in certain parts of the world (Lapkin and Constable, 2009).

In Table 2.3 typical values for E-factor for the different sectors of chemical industry are shown. Waste production is typically greater in pharmaceutical industry, since the products are complex molecules whose production involves multistep synthesis (Roschangar et al., 2015).

Table 2.3 E-factor (excluding water) for different sectors of chemical industry (adapted from (Tao and Kazlauskas, 2011)).

Industry sector	Annual production (ton/year)	E factor (kg waste/kg product)
Bulk chemicals	10^4-10^6	< 1-5
Fine chemicals	10^2-10^4	5 to >50
Pharmaceutical chemicals	10-10^3	25 to >100

E-factor has demonstrated a strong positive correlation to process economics. A lower E-factor has been shown to be indicative of reduced manufacturing costs, reduced costs from hazardous and toxic waste disposal, improved manufacturing capacity utilization and reduced energy demand in the pharmaceutical industry, which shows how industry has strong economic incentives to integrate green chemistry into process research, development and manufacturing (Leahy et al., 2013) (Roschangar et al., 2015).

Process Mass Intensity (PMI)

Process mass intensity (PMI) (Equation 2.5) takes into account the reaction yield, stoichiometry, solvents, reagents, etc., and expresses this on a weight/weight basis. It includes all the materials used in the process or in a specific process step. Water may or may not be included in the calculation of PMI. Similarly as to E-factor, the trend in the pharmaceutical industry is to include it (Constable et al., 2002).

Equation 2.5

$$PMI = \frac{\sum mass\ input\ materials}{mass\ of\ final\ product}$$

The American Chemical Society Green Chemistry Institute's Pharmaceutical Roundtable (ACS GCIPR) has adopted PMI as the key metric for evaluating and benchmarking processes and uses it to drive more efficiency and innovation in the pharmaceutical and fine chemical industries (Jimenez-Gonzalez et al., 2011).

PMI and E-factor are similar metrics and one can argue that they give the same type of information. However, while in a broad sense one can argue that it does not matter which of these metrics is used, Jimenez-Gonzalez and co-workers argues that increasing efficiency, translated in PMI, is more likely to capture management attention than waste reduction, translated by E-factor and therefore, PMI should be preferred over E-factor. In the business context, efficiency metrics, e.g. PMI, have an advantage over waste metrics, such as E-factor, since they communicate and frame sustainability in terms of adding value, by adding productivity, instead of managing costs, by waste reduction. Metrics such as PMI allow chemists and engineers to focus on how to make a process more efficient instead of solving the problem of waste management. Focusing on waste reduction can help chemical companies to reduce costs, whereas focusing on efficiency enables innovation to create additional value. If the focus is on maximization of value and efficiency, waste reduction will also consequently be one of the benefits. Hence, PMI can promote a philosophy of efficiency and innovation instead of an end-of-pipe focus on waste (Jimenez-Gonzalez et al., 2011).

When calculating PMI one can also breakdown total PMI in its different contributors and analyze which one of the different materials categories (solvents, reactants, water, etc.) contributes the most to total PMI and this gives an indication of which process unit of operation requires further development and optimization.

It has been shown by the ACS GCIPR that PMI is improved over the course of a pharmaceutical product development. At the transition to an advancing development phase, a decrease in PMI is observed. From the pre-clinical studies until commercial production of an API, PMI is decreased from values in the range of 1405 kg materials/kg API to 168 kg materials/kg API (Roschangar et al., 2015).

Solvent Intensity (SI)

From the assessment of several synthetic routes of different chemicals, solvents have been found to commonly be one of the biggest contributors to mass intensity. This is particularly relevant in the pharmaceutical sector, where Constable and co-workers reported that solvents constitute 56% of total materials mass used to manufacture an API and 80-90% of the total non-aqueous mass of materials (Constable et al., 2007).

Therefore, solvent intensity (SI) (Equation 2.6) was defined as a metric to analyze and quantify the amount of solvents used in a given process.

Equation 2.6

$$SI = \frac{\sum mass\ of\ solvents}{mass\ of\ final\ product}$$

Since solvents are one of the biggest mass contributors to a chemical process, limiting the amount of solvents used and selecting the "greenest" ones can result in a significant reduction of the environmental impact of a pharmaceutical product. The selection of the greenest solvent is a compromise between different constraints. On one hand, safety, health and environmental criteria should be considered, but also industrial constraints (e.g. boiling point, freezing temperature, density, quality) need to be met (Prat et al., 2014).

Solvents are not directly responsible for the composition of a reaction product, nor are the active component of a formulation. Therefore, the use of toxic, flammable and environmentally damaging solvents would seem unnecessary because these characteristics have no impact on the function or progress of the system in which the solvent is applied. However, these non-benign characteristics are often linked to the beneficial attributes of the solvent needed for the application. For instance, the volatility of the solvents permits simple recovery

and purification of the solvent by distillation, but it also creates unwanted air emissions and the risk of worker exposure. Amide solvents have the high polarity required to dissolve a broad range of substrates and accelerate reactions, but this attribute often implies reproductive toxicity. Hydrocarbon solvents provide the ability to dissolve oils in extractions and perform separations, but at the same time they are highly combustible and their low water solubility is related to bioaccumulation and water toxicity. In attempts to eliminate undesirable solvents, replacement strategies often seek structurally related compounds not yet covered by the legislative and regulatory measures that are usually required to force solvents substitution. But even though, solvents that are similar structurally can be an easy and obvious first choice for replacement, they are likely to present many of the same environmental, health and safety (EHS) problems. One example of these substitutions is benzene and toluene. Benzene was recognized as a carcinogen compound in the mid-twentieth century and has generally been replaced by toluene. However, toluene is now suspected of damaging the unborn child and of organ damage through prolonged exposure and is included in the list of "substances of very high concern" by European Union and is likely to be banned by REACH regulation in the future (Byrne et al., 2016).

In an attempt to categorize solvents with respect to their EHS profile, several solvent selection guides have been published - (Tobiszewski et al., 2015), (Byrne et al., 2016), (Prat et al., 2016), (Prat et al., 2014), (Henderson et al., 2011), including the solvent selection guides from several pharmaceutical companies, such as GSK's (Henderson et al., 2011), Pfizer (Alfonsi et al., 2008) and Sanofi (Prat et al., 2013). These intend to promote the substitution of conventional organic solvents with greener solvents by academics and researchers giving more information on solvent selection than the regulatory assessments do.

Water Intensity (WI)

A particular case of solvent intensity is water intensity (WI) (Equation 2.7), which aims to analyze the amount of water used in the process. WI is particularly relevant in biocatalytic and fermentation processes, since it is one of the biggest mass contributors to these processes. Even though water is an environmentally benign solvent, it is becoming a scarce and overexploited natural resource. Furthermore, bioprocesses generate a large amount of wastewater that needs to be treated, which is usually very energy intensive. Therefore, water usage should also be optimized and WI appears as a metric that intends to promote a critical thinking on water usage (Lima-Ramos et al., 2014b).

Equation 2.7

$$WI = \frac{\sum mass\ of\ water}{mass\ of\ final\ product}$$

There are several other metrics that have been proposed to evaluate the environmental profile of a chemical process, such as Effective Mass Yield (EMY).

Effective Mass Yield (EMY) is a metric proposed by Hudlicky and co-workers that tries to highlight the environmental effect of the reagents. It is defined as the percentage of the mass of product relative to the mass of all non-benign materials used in its synthesis (Equation 2.8). Hudlicky defines benign as "those by-products, reagents or solvents that have no environmental risk associated with them" (e.g. water, dilute ethanol, etc.) (Hudlicky et al., 1999).

Equation 2.8

$$EMY(\%) = \frac{mass\ of\ final\ product}{\sum mass\ of\ non-benign\ reagents} \times 100$$

However, this definition lacks clarity and objectivity. The applicability of this metric is also limited since there is no information on human toxicity and ecotoxicity for all the chemicals. Besides, depending on the situation even water, ethanol and other "benign chemicals" can have a significant environmental impact (Constable et al., 2002).

Table 2.4 shows a summary of the most commonly used green chemistry metrics for assessment of chemical and/or biocatalytic processes.

Table 2.4 Summary of green chemistry metrics (adapted from (Roschangar et al., 2015)).

Green chemistry metric	Abbreviation	Equation	Units	Consideration for				Optimum value	First introduced by
				Reaction yield	Stoichiometry	Solvents	Water		
Reaction-related metrics									
Atom economy	AE	$\frac{MW\ of\ product}{\sum MW\ of\ reactants} \times 100$	%	X	X	X	X	100%	(Trost, 1991)
Carbon efficiency	CE	$\frac{m(carbon\ in\ the\ product)}{\sum m\ (carbon\ in\ reactants)} \times 100$	%	✓	✓	X	X	100%	(Curzons et al., 2001)
Reaction mass efficiency	RME	$\frac{mass\ of\ final\ product}{\sum mass\ of\ reactants} \times 100$	%	✓	✓	X	X	100%	(Curzons et al., 2001)
Process-related metrics									
Environmental impact factor	E-factor	$\frac{total\ mass\ of\ waste}{mass\ of\ final\ product}$	kg/kg	✓	✓	✓	X	0	(Sheldon, 1992)
Process mass intensity	PMI	$\frac{\sum mass\ input\ materials}{mass\ of\ final\ product}$	kg/kg	✓	✓	✓	✓	1	ACS GCIPR (2007)
Solvent Intensity	SI	$\frac{\sum mass\ of\ solvents}{mass\ of\ final\ product}$	kg/kg	X	X	✓	X	0	(Curzons et al., 2001)
Effective mass yield	EMY	$\frac{mass\ of\ final\ product}{\sum mass\ of\ non-benign\ reagents} \times 100$	%	✓	✓	✓	X	100%	(Hudlicky et al., 1999)
Water intensity	WI	$\frac{\sum mass\ of\ water}{mass\ of\ final\ product}$	kg/kg	X	X	X	✓	0	(Curzons et al., 2001)

Most of these reaction-related and process-related metrics have the big advantage of being simple to use and can give instant feedback of a change made in the process or in the synthetic reaction, and therefore can easily be used for modelling and comparison of different scenarios. Thus there is a great interest in using environmental metrics in the process development stage. However, it obviously also presents some drawbacks and limitations. One evident limitation is the fact that the same weight is given to all different types of waste generated. For instance, when calculating E-factor the same weight is given to 1 kilogram of sodium chloride (NaCl) and to 1 kilogram of hexane, when clearly the health and safety hazards of both chemicals is completely different resulting in different impacts on the environment (Tufvesson et al., 2012). This is the reason why the calculation of these green chemistry metrics should be combined with an analysis of the health and safety hazards of the chemicals used in the process.

In this thesis atom economy and carbon efficiency in combination with reaction mass efficiency were selected for the comparison of the environmental profile of the different synthetic routes for (*R*)-perillic acid production (section 3.3).

For the environmental assessment of an entire process a combination of several process-related metrics were used in (*R*)-perillic acid and brazzein case-studies, but PMI was selected as the preferred metric to guide process development.

2.2.2 Environmental, Health and Safety hazards

Assessing health and safety of the chemical inventories is an important part of an environmental assessment. Besides human toxicity it is also important to take into account the hazards to the environment.

EHS aspects of the chemicals should be assessed based on the globally harmonized system of classification and labelling of chemicals (GHS) and certain chemical hazard categories should be avoided (e.g. carcinogens, mutagens, endocrine disrupting substances, flammable, explosive, etc.).

In addition to the health and safety of the chemicals, acute and chronic toxicity data should also be gathered in response to legislation such as REACH. “Registration, Evaluation, Authorization and Restriction of Chemicals” (REACH) is a regulation of the European Union, adopted to improve the protection of human health and the environment from the risks that can be posed by chemicals, while enhancing the competitiveness of the EU chemicals industry. It affects the import and usage of a wide range of chemicals in Europe, and any products found to not comply with the conditions established in REACH are removed from

the market through the “Rapid Alert System for Dangerous Non-food Products” (RAPEX) information scheme (Byrne et al., 2016). As a result of REACH, in 2015 several products containing toluene, chloroform or benzene have been banned from EU, and several solvents are currently under scrutiny, such as N,N-dimethylformamide (DMF), N,N-dimethylacetamide (DMAc), N-methylpyrrolidinone (NMP) as well as certain hydroxyethers and chlorinated solvents (Byrne et al., 2016).

There are highly respected and trustworthy databases which compile information on the hazards of chemical substances and help with the often complicated task of substituting a certain chemical. Examples of these databases are the SIN LIST and the SUBSPORT, the Substitution Support Portal. The SIN (Substitute it Now!) List is a globally used database of chemicals likely to be banned or restricted in a near future. The chemicals on the SIN List have been identified by ChemSec (International Chemical Secretariat) as Substances of Very High Concern based on the criteria established by the EU chemicals regulation REACH (http://sinlist.org, accessed on 22.09.2016). SUBSPORT, the Substitution Support Portal, is a database/search engine on hazardous substances that are legally or voluntarily restricted or subject to public debate (www.subsport.eu, accessed on 22.09.2016).

These portals aim to support companies in fulfilling substitution requirements within EU legislation, as well as being a useful resource for scientific institutions interested in substituting hazardous chemicals. It is imperative that researchers look into the health, safety and environmental hazards of the chemicals used in a certain process in an early stage of development, since substituting chemicals in later stages of research and development is much more costly and complicated.

2.2.3 Life Cycle Assessment

In comparison with green chemistry metrics, Life Cycle Assessment (LCA) is at the other end of the spectrum in terms of complexity and comprehensiveness (Lima-Ramos et al., 2014b). LCA is defined as “the compilation and evaluation of the inputs, outputs and potential environmental impacts of a product system throughout its life cycle” (European Comission for Standardization, 2006a). Life cycle thinking is an approach covering all life cycle stages of a process, product or service system, from extracting and processing of raw materials, manufacturing, transportation and distribution, use/reuse, recycling and disposal. This is often called a “cradle to grave” approach, represented in Figure 2.5.

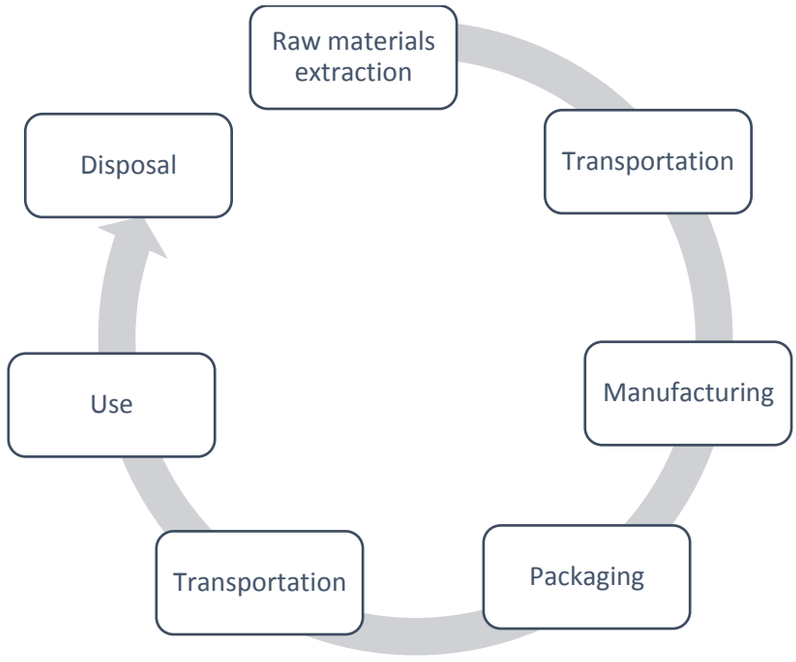


Figure 2.5 Life cycle thinking - a cradle to grave approach.

Unlike green chemistry metrics, LCA is a methodology that is not specific for chemical and/or biochemical processes. It was developed to be suitable for the environmental assessment of all types of products, processes or services (Lima-Ramos et al., 2014b).

LCA is a methodology that has been standardized by the International Standard Organization (ISO) – ISO14040:2006 and ISO14044:2006 (European Comission for Standardization, 2006a) (European Comission for Standardization, 2006b). The level of detail and the boundaries of an LCA study depend on the subject and goal of the study, but the ISO standards need to be followed. The LCA methodology comprises of four phases. The first phase is the goal and scope definition, where the system boundaries and goal of the study are defined. This step is followed by the Life Cycle Inventory (LCI). This is the most time-consuming step and consists of performing mass and energy balances to all the different steps in the product life cycle, in order to quantify the material and energy inputs into the system as well as wastes and emissions from the system. Then comes the Life Cycle Impact Assessment (LCIA) where all the inventory data is processed and aggregated into a limited set of recognized environmental effects. The inputs and outputs quantified during the LCI step are related to different impact categories, which include Global Warming Potential (GWP), Stratospheric Ozone Depletion Potential (SOP), Photochemical Ozone Creation Potential (POCP), Acidification Potential (AP), Nutrient Enrichment Potential (NEP), Eco-toxicity Potential (ETP), Human Toxicity Potential (HTP) and Hazardous Waste Potential (HWP) (Lima-Ramos et al., 2014b). The last step consists of the interpretation of

the results obtained within the Life Cycle Inventory and/or Life Cycle Impact Assessment in the light of the goal and scope defined. This last step typically includes a sensitivity and/or uncertainty analysis of the results obtained in order to draw conclusions and make recommendations (Lapkin and Constable, 2009).

There are multiple commercially available software tools for the implementation of the LCA methodology, e.g. SimaPro or GaBi. The biggest asset of these software tools is the inclusion of comprehensive inventory databases, which facilitate the mass and energy balances of up- and downstream stages of the process under investigation. One of the most comprehensive databases is the ecoinvent database (latest version is ecoinvent 3.3) (www.ecoinvent.org accessed on 20.09.2016).

LCA provides a framework for reporting the environmental impacts reflecting the whole life cycle of a given product and is therefore a valuable tool to support the implementation and marketing of a given product. However, on the research and development stage of a process or product, a full LCA is too time-consuming, laborious and complicated. The methodology is too comprehensive and there are difficulties in finding all the necessary inventory data, which makes its applicability reserved to late stages of development when all this information and data is certain and available. For these reasons, LCA does not enjoy widespread practice for environmental assessment of bioprocesses, with the exception of biofuels (Tufvesson et al., 2012).

Given the complexity of the LCA methodology, several chemical companies have developed simplified in-house LCA methods, based on the guidelines in the ISO standards for evaluating the sustainability of its products. Examples of these methods are the FLASC-tool developed by GlaxoSmithKline to systematically evaluate synthetic organic reactions and processes (Curzons et al., 2007), or the Eco-Efficiency Analysis method developed by BASF (Shonnard et al., 2003).

2.3 Concluding remarks

There is a large number of available tools to assess the economic and environmental profile of a given bioprocess and the selection of the most appropriate ones is not a straightforward task.

Regarding the assessment of the economic feasibility of a given process, process metrics such as product concentration, space-time yield and reaction yield can provide meaningful information while being simple and easy to calculate.

Similar metrics also exist for the environmental assessment of processes in early stages of development. These metrics, commonly designated as Green Chemistry metrics, can be grouped into reaction-related or

process-related metrics and provide a quick assessment of the environmental profile of a chemical or biochemical process.

Given the inherent differences in the processes, different tools should be used to evaluate biocatalytic and fermentation processes, which are further explored throughout the thesis.

Part II

Case-studies

3. Case-study I: Bioconversion of (*R*)-limonene to (*R*)-perillic acid by a whole-cell biocatalyst

3.1 Introduction

In this first chapter the work done for the first case-study is presented, including benchmarking of the different routes for perillic acid production, economic and environmental assessment and development of different improvement strategies for the process.

This case-study corresponds to the bioconversion of (*R*)-limonene to (*R*)-perillic acid by *Pseudomonas putida* GS1. Perillic acid is a monoterpenoic acid that is naturally present in some plants of the genus *Perilla*, namely in *Perilla frutescens*. It has a strong growth-inhibitory effect on bacteria and fungus, which makes it an attractive candidate to be used for preservation purposes, e.g. in cosmetic industry (Mirata et al., 2009) (Rieks et al., 2004). It has also been shown that perillic acid has anti-cancer properties, namely as an inhibitor of metastic progression of melanoma cells, and could potentially be of interest to the pharmaceutical industry (von Kitzing et al., 1994) (Yeruva et al., 2007).

The bioprocess for *(R*)-perillic acid production is presented in section 3.2. Section 3.3 corresponds to the benchmarking of the bioprocess with other routes for perillic acid production. Section 3.4 corresponds to an economic and environmental assessment of the bioprocess from both an economic and an environmental perspective. Section 3.5 includes an experimental characterization of the process and section 3.6 a discussion of the possible process technologies that can be used to improve this bioprocess.

3.2 Bioprocess description

(*R*)-Limonene is converted to (*R*)-perillic acid in a three-steps reaction represented in Figure 3.1. In the first step (*R*)-limonene is converted to (*R*)-perillyl alcohol by a monooxygenase, which is then converted to (*R*)-perillyl aldehyde by an alcohol dehydrogenase, and finally the aldehyde is converted to (*R*)-perillic acid by an aldehyde dehydrogenase. This reaction is part of the degradation pathway for *p*-cymene that is naturally expressed in *Pseudomonas putida* GS1 metabolism. This degradation pathway is an adaptation mechanism that is induced in the presence of toxic solvents. Limonene is the monocyclic monoterpenoic analogue to the

aromatic *p*-cymene and it also activates the *p*-cymene degradation pathway. In this degradation pathway, *p*-cymene is oxidized to *p*-cumate in three steps, which are catalyzed by a monooxygenase, an alcohol dehydrogenase and an aldehyde dehydrogenase, which are also active towards limonene and its derivatives, perillyl alcohol and perillyl aldehyde. But in contrast to *p*-cumate, perillic acid is not further catabolized and it is secreted into the medium as a result of the narrow substrate specificity of the cumate dioxygenase that further degrades *p*-cumate (Speelmans et al., 1998).

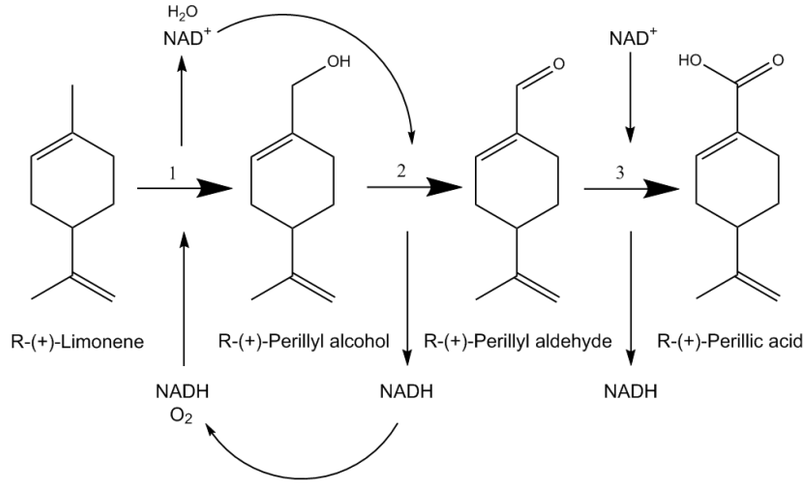


Figure 3.1 Bioconversion of (*R*)-limonene to (*R*)-perillic acid by *P. putida* GS1: (A) monooxygenase; (B) alcohol dehydrogenase; (C) aldehyde dehydrogenase.

As most organic solvents (*R*)-limonene is toxic to most bacterial strains, but *Pseudomonas putida* GS1 is able to grow with concentrations of (*R*)-limonene as high as 500 mM since solvent tolerance mechanisms are activated. On the other hand, (*R*)-perillic acid inhibits cell growth and the biocatalytic reaction and feedback inhibition was claimed to determine the maximum product concentration achievable in batch cultures (Speelmans et al., 1998). Previous work by Mirata and co-workers determined the critical inhibitory concentration of (*R*)-perillic acid to be 165±7 mM. Product inhibition was confirmed experimentally and total inhibition of growth of *P. putida* GS1 was observed with a concentration of 167 mM of (*R*)-perillic acid (cultivations in TB media at pH 10). (Mirata et al., 2009).

A process for biotechnological production of (*R*)-perillic acid with a wild-type strain of *Pseudomonas putida* GS1 was developed and established at BRAIN AG. Since the reaction system faces product inhibition, there is the need of having an *in situ* product removal (ISPR) step to alleviate product inhibition.

3.3 Benchmarking of different routes for perillic acid production

In the first place, an evaluation of the environmental profile of different routes for perillic acid production was made, with the purpose of identifying the most promising route for industrial production of perillic acid.

In the case of (*R*)-perillic acid all the three possible production routes represented in Figure 1.1 – extraction from natural sources, chemical synthesis and biosynthesis – are theoretically possible. The routes for (*R*)-perillic acid production that were identified in literature are: i) extraction from the plant *Perilla frutescens*; ii) chemical synthesis starting from β-pinene, and iii) biocatalytic conversion of (*R*)-limonene to (*R*)-perillic acid by *Pseudomonas putida* GS1.

Perillic acid has been identified as a monoterpene glucoside in the plant *Perilla frutescens* (Fujita and Nakayama, 1993). However, it is only found in nature in very low concentrations as most terpenoids. There are several oxyfunctionalised monoterpenes and sesquiterpenes which are industrially interesting to be used as flavor and fragrances. But they are present in the essential oils of plants, which constitute around 0.1 to 5% of the plant. In the specific case of *Perilla frutescens* leaves, they are constituted by 1.04 %(w/w) of essential oil (Yu et al., 1997). The extraction of minor compounds, such as perillic acid, from the essential oils is only in very rare cases economically feasible (Berger, 2007). For this reason and since it was not found in the literature the exact amount of perillic acid in the leaves of *Perilla frutescens*, this route for perillic acid production was not included and it was not further analyzed.

On the other hand, terpene hydrocarbons such as (*R*)-limonene and pinenes are abundantly available in nature as the main component of citrus and turpentine oils, respectively. Orange oil contains more than 90% of (*R*)-limonene and turpentine oil up to 75-90% pinenes, which makes these compounds as an interesting starting material for oxyfunctionalizations that lead to industrially relevant terpenoids, such as perillic acid (Berger, 2007).

The biocatalytic conversion of (*R*)-limonene to (*R*)-perillic acid by *Pseudomonas putida* GS1 (Mars et al., 2001) (Speelmans et al., 1998) (Mirata et al., 2009) described in section 3.2 and the chemical synthesis of perillic acid starting from β-pinene (Wang et al., 1993) were therefore compared from an environmental point of view.

The biocatalytic conversion of (*R*)-limonene to (*R*)-perillic acid by *Pseudomonas putida* GS1 is shown in Figure 3.1.

The chemical synthesis of perillic acid starting from β-pinene is shown in Figure 3.2.

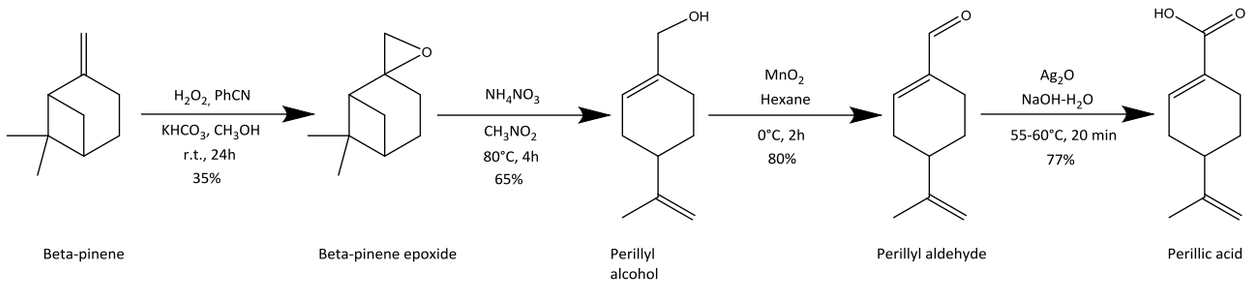


Figure 3.2 Chemical synthesis of perillic acid starting from β-pinene (Wang et al., 1993).

Based on the mass balances of both routes, the key reaction-related green chemistry metrics were calculated for both synthetic routes and are shown in Table 3.1. For easier interpretation, Figure 3.3 shows a graphic representation of these green chemistry metrics for both synthetic routes.

Table 3.1 Comparison of reaction-related green chemistry metrics for the biocatalytic and chemical synthetic routes for perillic acid production.

	Biocatalytic route	**Chemical route**
Atom Economy (%)	99	25
Carbon Efficiency (%)	100	59
Reaction Mass Efficiency (%)	20	3

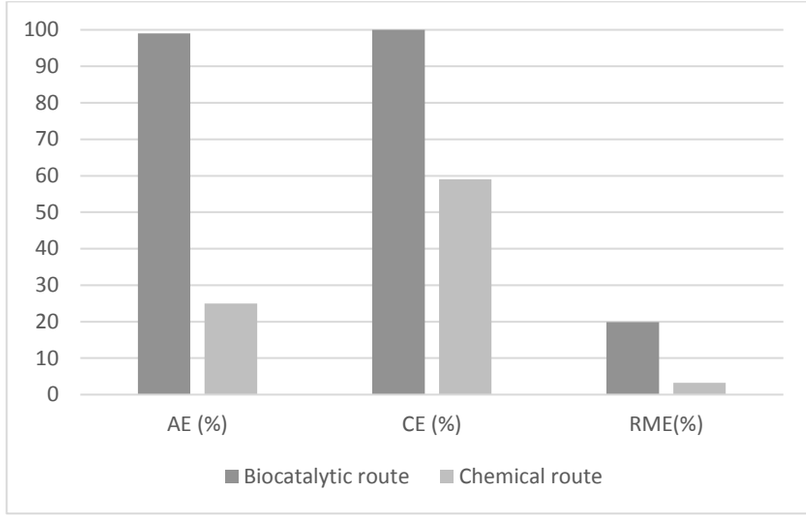


Figure 3.3 Reaction-related green chemistry metrics for the biocatalytic and chemical synthetic routes for perillic acid production.

From Table 3.1 and Figure 3.3 it is clear that the biocatalytic route has the potential to be a "greener" process, since all the three metrics are considerably superior to the chemical route. Carbon efficiency is 100%, which corresponds to the ideal value, and atom economy is also extraordinarily close to the ideal. The starting material for the biocatalytic route, (*R*)-limonene is chemically very similar to the final product, (*R*)-perillic acid and only oxygen is consumed as a reactant in this oxyfunctionalization. Therefore, this is a very good example of a synthetic route that obeys to the principles of atom economy – the synthetic route maximizes the incorporation of the starting materials into the final product.

However, the situation is a little different when it comes to RME. This metric also has a higher value for the biocatalytic route, but in both routes its value is very low which indicates that both synthetic routes require further development and optimization.

Another important issue to take into account when selecting a synthetic route is the qualitative analysis of EHS aspects of the chemicals and solvents used in the reaction. McElroy and co-workers grouped several solvents that are typically used in the pharmaceutical industry and attributed green, yellow or red flags to the use of hazardous and highly hazardous solvents, problematic and recommended solvents, respectively. The same procedure was adopted for the safety and health of the reactants used, based on the H-statements defined by the globally harmonized system of classification and labeling of chemicals (GHS) (McElroy et al., 2015). This flags system is very simple, illustrative and easy to interpret and has therefore been followed to compare the chemical properties of the chemicals used in both routes.

Table 3. 2 Analysis of the environmental, health and safety hazards of the reactants and solvents used in the biocatalytic and chemical synthetic routes.

		Green flag	Yellow flag	Red flag
Biocatalytic route	**Solvents**			
	No solvents used.	X		
	Reactants			
	(*R*)-Limonene			
	H410: Very toxic to aquatic life with long lasting effects			X
Chemical route	**Solvents**			
	Nitromethane – highly hazardous			X
	Hexane - hazardous			X
	Methanol	X		
	Water	X		
	Reactants			
	Hydrogen peroxide (H_2O_2)			X
	H330: Fatal if inhaled			
	Manganese dioxide (MnO_2)			
	H373: May cause damage to organs through prolonged or repeated exposure		X	

Analyzing Table 3. 2 one can see that the chemical synthesis shows more EHS concerns (translated by the number of red and yellow flags) than the biocatalytic route. The chemical synthesis consists of four different steps, each one with different reactants, solvents and catalysts. So in total, the number of chemicals used in the chemical route is considerably higher than the biocatalytic route. Two of the solvents used in the chemical route are hazardous. Nitromethane is highly hazardous, since it is a flammable liquid and vapour (H226) and highly explosive, and hexane is also a hazardous solvent which shows several health, safety and environmental issues – it is a highly flammable liquid and vapour (H225), it is suspected of damaging fertility (H361), may cause damage to organs through prolonged or repeated exposure (H373) and is toxic to aquatic life with long lasting effects (H411). Hexane has also been considered a chemical of very high concern by ChemSec and it is part of the SIN List because it is classified as a possible reprotoxic chemical (R3) and has been reported to have endocrine and neurological effects (sinlist.org accessed on 18.09.2016). The reactants and catalysts also show EHS hazards, for instance manganese dioxide (MnO_2) may cause damage to organs through prolonged or repeated exposure (H373). Another environmental issue of the chemical route is the fact that each synthetic step takes place at a different temperature in a range from 0 °C to 80 °C, which is undesirable from an energetic point of view.

On the other hand, the biocatalytic route also shows some EHS concerns. (*R*)-limonene is very toxic to aquatic life with long lasting effects (H410) and it is therefore not the “greenest” starting material. Moreover, when

making the chemical inventory of the biocatalytic route, $CoSO_4$ has been identified as a chemical of very high concern, present in the SIN List since it has been classified as carcinogenic, mutagenic, reprotoxic (CMR) according to Annex 1 of Directive 67/548/EE (sinlist.org accessed on 20.08.2016). However, cobalt sulphate is only used in the trace metals solution and it is present in the reaction media in a concentration in the range of nanograms per liter. Since, the concentration of cobalt sulphate used is so low, this should not be a major health and safety concern. When assessing the EHS hazards of the different chemicals used in a chemical reaction it is also important to analyze the EHS hazards of the final product. However that was not possible since data on the safety and toxicology of (*R*)-perillic acid could not be found in scientific literature.

The use of a biocatalyst is preferable from an environmental point of view, as opposed to the use of excess of reagents in the chemical route. And the fact that the reaction takes place at a constant temperature close to room temperature (30 °C) is also a positive point from an environmental and an economic perspective.

In conclusion both the reaction-related green chemistry metrics and the EHS hazards of the chemical inventory point towards the selection of the biocatalytic route as the most promising to be investigated for industrial application. The economic and environmental profile of the biocatalytic reaction were further investigated in the following sections of the thesis.

3.4 Bioprocess evaluation

3.4.1 Economic assessment

3.4.1.1 Process metrics

In order to make an initial assessment of the economy of the bioprocess for (*R*)-perillic acid production, the process metrics that are relevant in this case, *i.e.* product concentration, space-time yield and reaction yield, were calculated based on the mass balances of the process. The process metrics for are shown in Table 3.3.

Table 3.3 Process metrics for the bioprocess for (*R*)-perillic acid production. The reaction yield is calculated on a molar basis.

Process metrics	(*R*)-perillic acid bioprocess
Product concentration (g/L)	4.81
Space-time yield (g/(L.h))	0.17
Reaction yield (%)	20.6

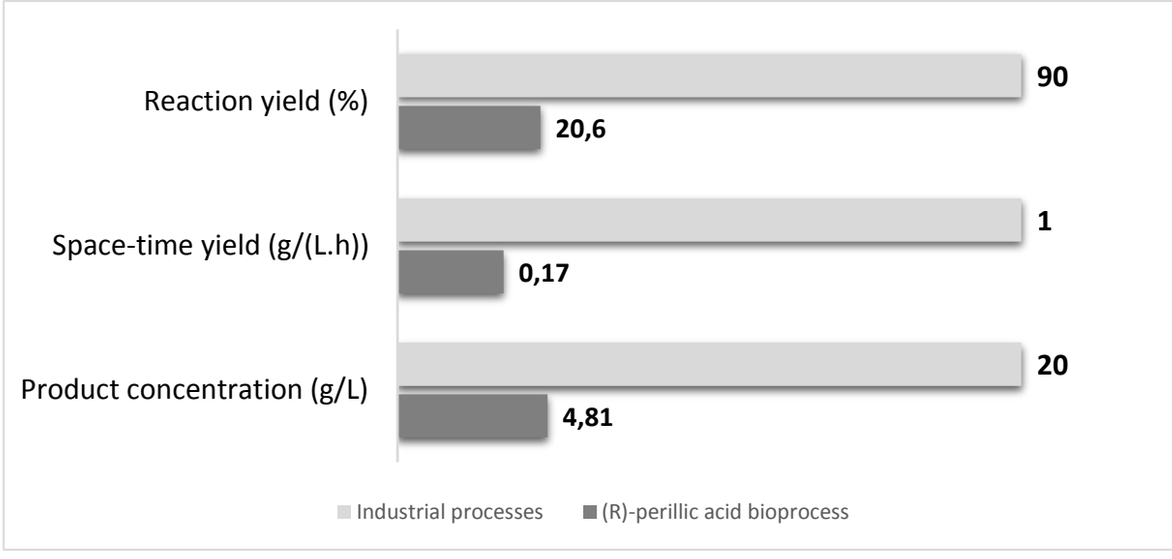


Figure 3.4 Process metrics for the (*R*)-perillic acid bioprocess presented along with typical values for an economically feasible process.

Characterization and identification of the limitations of a bioprocess is the first step towards development of an economically feasible process. Translating the performance of the bioprocess into process metrics and benchmarking against quantitative targets is a very valuable tool. In Figure 3.4 typical values for a biocatalytic pharmaceutical process implemented at industrial scale are represented together with the process metrics for the bioprocess for (*R*)-perillic acid production. Pharmaceutical chemicals can be used as an appropriate benchmark given the low annual production that is expected for (*R*)-perillic acid. Since it is a novel chemical product that is not commercially available in the cosmetic, food, pharmaceutical or any other market, it is assumed a very low initial annual production of (*R*)-perillic acid in the range of 1 ton/year.

As shown in Figure 3.4 all the three process metrics for the bioprocess for (*R*)-perillic acid production are far from reasonable values for an economically feasible process. The process undoubtedly needs further reaction and process development for its intensification and it is still far from economic feasibility.

Furthermore, all of the three process metrics are approximately equally far from the targets, *i.e.* none of them is significantly better than the others. And all the process metrics are equally important for the economy of a biocatalytic process. So the question that is raised from this simple economic assessment is – which metric should be improved in first place? Which metric has a greater influence on the overall economy of the process and should therefore be tackled first?

In order to further explore this question a process model and a full cost estimation were developed which are shown in section 3.4.1.2.

3.4.1.2 Process model

Economic feasibility was assessed by means of a method designed for utilization at early development stages of chemical processes (Heinzle E.; Weirich and K., 1998) (Heinzle et al., 1998). A process model for the chromatography-based process was developed in the commercial process simulation software SuperPro Designer®, version 9.5 (Intelligen Inc., Boston, USA).

The model is based on experimental data collected at 10L scale and scalability to 5 m^3 was assumed.

The bioconversion reaction was described as in equation Equation 3.1 and a reaction yield of 20.6% was assumed. It was assumed that the remaining (*R*)-limonene that is fed to the fermenter is lost in the exhaust gas, a phenomenon that is observed at laboratory scale.

Equation 3.1

$$C_{10}H_{16} + 1.5\ O_2 \rightarrow\ C_{10}H_{14}O_2 + H_2O$$

The annual production was assumed to be 1 ton (*R*)-perillic acid per year and the annual production time is only 3 months/year. It is assumed that the final product would be stored in storage facilities for the remaining time of the year.

The scheduling of the process was done in order to maximize equipment usage. Total batch time is approximately 139 hours (6 days) and 17 batches run in one production year.

SuperPro Designer® software comprises comprehensive databases for the calculation of numerous economic parameters. These values were used for the economic assessment unless otherwise stated. Raw material costs were complemented with industrial market prices from the ICIS webpage (www.icis.com accessed on 15.07.2016) or, if necessary, by division of the lab-scale prices from Sigma-Aldrich (www.sigmaaldrich.com accessed on 20.10.2016) by a factor of 10.

Basic labor costs are estimated from the labor requirements of each operation step in the factory and assuming a standard wage of 30 $/h (approximately 28 €/h) for an operator (excluding benefits) (Kuhn et al., 2010). It was also assumed that the industrial facility would be a multi-purpose plant with shared utilities and services, which would be rented for (*R*)-perillic acid production. The facility-dependent costs, which represent a rental fee for the use of the equipment, are calculated based on equipment usage. Wastewater treatment costs were not included in the cost assessment.

Based on this process model, which includes mass as well as energy balances, an economic assessment of the process including a full cost estimation was made.

The economic assessment that was performed focused solely on the production cost (€/kg product). Capital cost (CAPEX) and depreciation of the equipment were not considered in the economic assessment.

The results of the economic assessment revealed that the production costs are higher than what would be desired, which confirmed the previous conclusions from the assessment of the process metrics that the process needs to be further intensified.

Figure 3.5 shows the contribution of different elements to the total annual operation cost. These different elements are raw materials, labor, facility-dependent costs, utilities and consumables, which include resins, filters, etc.

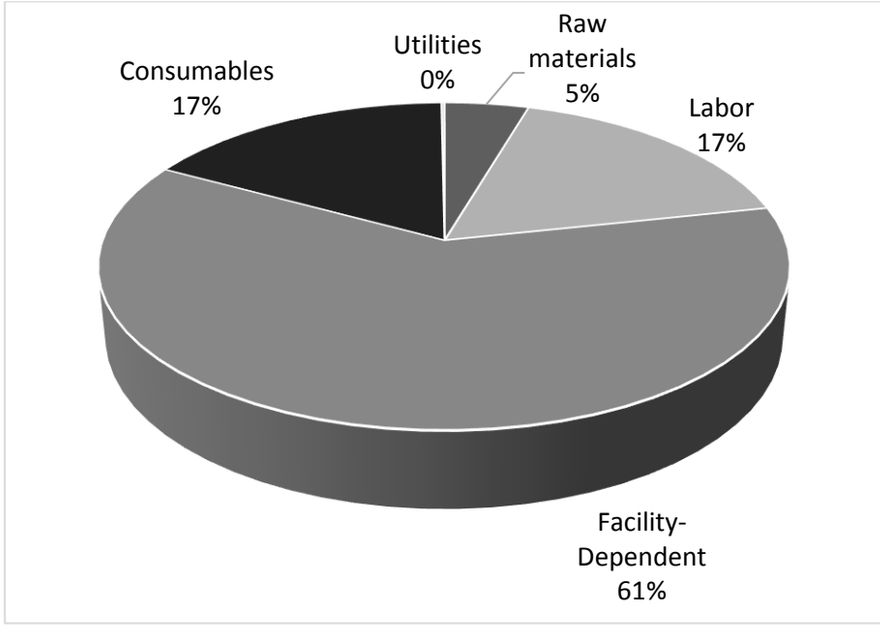


Figure 3.5 Annual operation cost breakdown for the chromatography-based production process.

Facility-dependent costs are the biggest contributor to the total operations costs, which is not uncommon for bioprocesses. Bioprocesses typically produce high-value products in small quantities in relatively expensive facilities, with equipment that is more sophisticated, e.g. fermenters and chromatographic columns, than equipment that is used in conventional chemical processes (Harrison et al., 2015). That is precisely the case here where (*R*)-perillic acid is produced in a very small scale (1 ton/year), therefore it is expectable that facility-dependent costs contribution to total operation cost is substantial.

Biocatalytic processes are typically capital intensive because large and complex fermenters are required. The main reasons for this are usually dilute reaction systems and complex requirements for process control to guarantee optimal growth of microorganisms (Straathof and Adlercreutz, 2000). Sterilization and biocontainment are also extra features of fermenters (when compared with chemical reactors) that result in an additional cost. The comparably dilute product concentrations in the fermenter are the reason why the equipment has to be designed larger as compared to chemical plants to achieve similar product amounts. This results in a higher cost of the equipment, which can be alleviated if the equipment is rented, as it happens in this case. The considerably high facility-dependent costs stress the importance of process intensification by different process technologies and/or biocatalyst modification. (Woodley, 2008) (Chotani et al., 2000).

(*R*)-perillic acid production at a 1 ton/year scale was clearly dominated by fixed costs (facility-dependent and labor costs), which account for 78% of the total operation costs. That is a similar scenario to other published economic assessments of biocatalytic processes, e.g. (*S*)-styrene biocatalytic production where fixed costs account with 63% of total operations costs (Kuhn et al., 2010) (Kuhn et al., 2012).

The high fixed costs and the low variable production costs (raw materials and utilities) of the biocatalytic process would clearly favor larger annual production volumes, as the contribution of the fixed costs with respect to total costs decreases with increasing scale (economy of scale). This was confirmed by running a sensitivity analysis on the volume of annual production, whose results are shown in Figure 3.6.

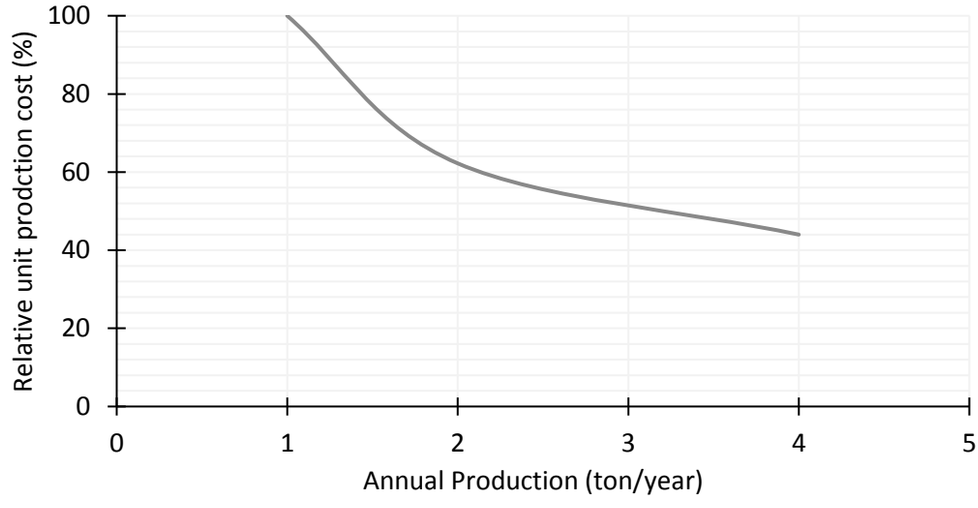


Figure 3.6 Relative unit production cost (€/kg) as a function of annual production (ton/year).

But more interesting than merely calculating the production cost and the weight of its different contributors is to analyze what influences it. Therefore a sensitivity analysis on the different process metrics was made and the variation of production cost was analyzed.

The sensitivity analysis was performed by analyzing the influence of reaction yield and reaction time (expressed by the space-time-yield) on unit production costs (€/kg).

For the sensitivity analysis on reaction yield, the annual production was kept constant at 1 ton/year and the results are shown in Figure 3.7.

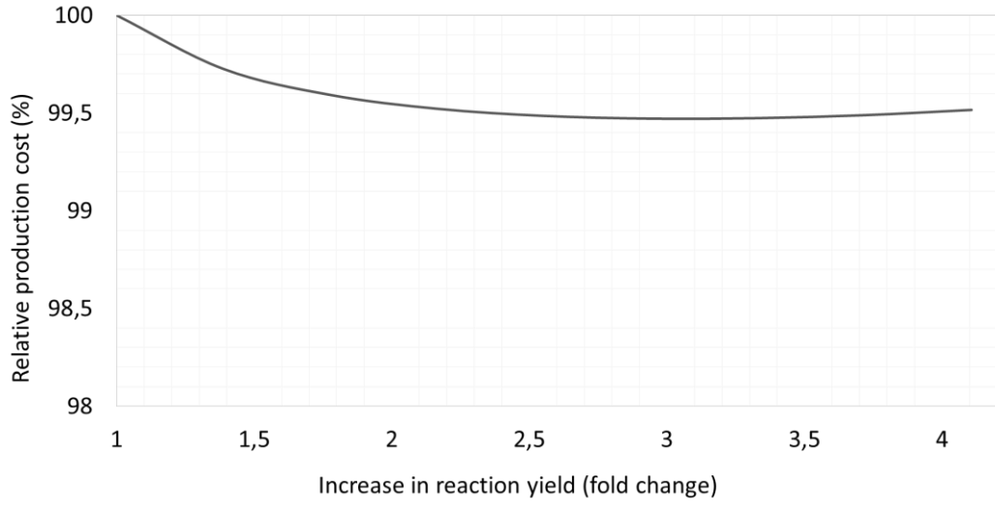


Figure 3.7 Relative production cost as a function of increase in reaction yield.

Increasing the reaction yield up to 4 folds, only decreased the production cost by 0.5%, which is an insignificant difference from an economic perspective. This minor decrease can be explained by the substrate low selling price. By increasing the reaction yield, the raw materials cost contribution to total operation costs is reduced. But since the substrate is a low-value chemical, the cost contribution of the raw materials is anyway low (around 5% of total operation costs as shown in Figure 3.5). So increasing reaction yield does not have a significant impact on total operation costs.

On the other hand, the sensitivity analysis to the space-time yield revealed a different picture. Figure 3.8 shows the results of this sensitivity analysis, where space-time yield was varied by only changing the reaction time (reaction yield was kept constant). The results show that the production cost is very sensitive to space-time yield. For instance, when reducing transformation time by 80%, total operation costs can be reduced up to 40%, which is a very significant reduction.

In this sensitivity analysis, annual production was also kept constant as 1 ton/year. The reduction in production cost can be explained by the fact that if the transformation time is reduced, the total batch time is also reduced. Hence, to produce 1 ton of (*R)*-perillic acid a lower annual production time is necessary, which reduces the cost of equipment usage and labor. Since the facility-dependent costs are the biggest contributor to total production costs, reducing the reaction time can have a very significant effect on the economy of the process.

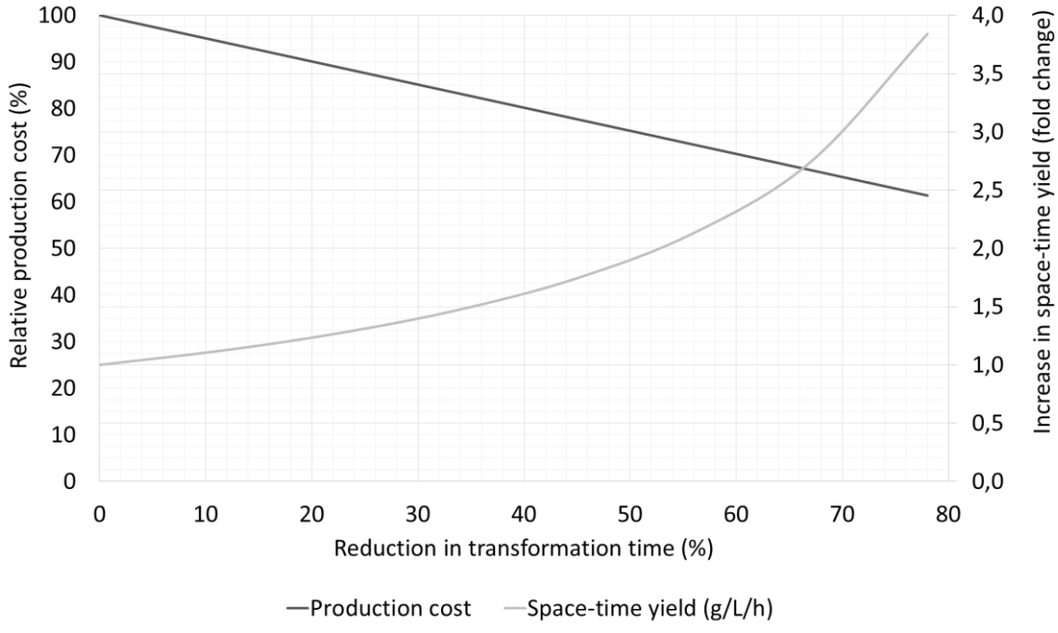


Figure 3.8 Relative production cost and increase in space-time yield as a function of reduction in transformation time.

In conclusion, the sensitivity analysis on different process metrics allowed the identification of the space-time yield, or more specifically the reaction time, as one of the parameters that mostly affect the economic feasibility of this process. Therefore, the focus of process development should be on increasing space-time yield and accelerating the reaction.

The process model and subsequent full cost estimation proved to be a very useful tool in ranking the importance of the different process metrics and identifying the space-time yield as the process metric that mostly affects the economy of this bioprocess.

3.4.2 Environmental assessment

The environmental profile of the biotechnological production of (*R*)-perillic acid was also assessed in terms of process-related green chemistry metrics. The green chemistry metrics (PMI, E-factor and SI) for the bioprocess for (*R*)-perillic acid production were calculated based on experimental data at 10L scale and are shown in Table 3.4.

E-factor of the bioprocess for (*R*)-perillic acid production is relatively high, but within the typical range of values for pharmaceutical chemicals (25 to >100). PMI is also very similar in both cases and relatively high, which again demonstrates that the process needs further intensification.

Table 3.4 Process-related green chemistry metrics for the bioprocess for (*R*)-perillic acid production.

Process-related metrics		**(*R*)-perillic acid bioprocess**
SI ($kg_{solvent}/kg_{product}$)		109.8
E-factor ($kg_{waste}/kg_{product}$)		226.9
PMI ($kg_{materials}/kg_{product}$)	USP	83.1
	DSP	219.8
	Total	302.8

Figure 3. 9 shows the contribution of different materials to total PMI of the bioprocess for (*R*)-perillic acid production.

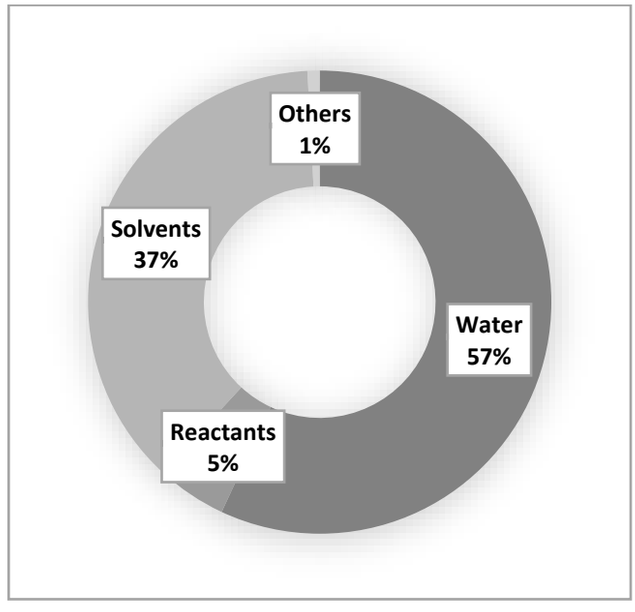


Figure 3. 9 Composition by mass of the type of materials used in the bioprocesses for (*R*)-perillic acid production.

The biggest contributor to PMI is water, which is typical of bioprocesses. Solvents contribute with 37% to PMI due to ethanol consumption in the downstream processing section of the bioprocess. Even though ethanol is not a hazardous solvent, solvents use should always be minimized.

3.5 Experimental characterization of the bioprocess

In the previous sections an economic and environmental assessment of the bioconversion of (*R*)-limonene to (*R*)-perillic acid was done. One of the conclusions from the economic assessment was that space-time yield or volumetric productivity (g/L/h) greatly affects the economy of the process.

Here, as an addition to the *in silico* analysis of the bioprocess, some experiments to characterize it were done in order to investigate the reasons for the low space-time yield and better understand the limitations of this reaction system.

3.5.1 Materials and methods

3.5.1.1 Determination of the reaction rate

To determine the reaction rate of (*R*)-limonene conversion to (*R*)-perillic acid, a fermentation following the same process conditions as the ones followed by BRAIN AG was run.

Chemicals, strain and media

(*R*)-limonene (99% purity) was purchased from Erich Ziegler (Aufsess, Germany), (*S*)-perillic acid (95% purity) and all other chemicals have been purchased from Sigma-Aldrich (Seelze, Germany).

P. putida GS1 was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). Minimal media with glycerol as the main carbon source was used both for pre-cultivation and cultivation in the fermenter.

Fermentation

The bioconversion of (*R*)-limonene to (*R*)-perillic acid was performed according to standard procedures (Mirata et al., 2009).

Analytical methods

The concentration of (*R*)-perillic acid was measured by HPLC analysis. Samples from the fermentations were centrifuged at 13,000 rpm for 10 minutes and the supernatant was diluted in an equal volume of acetonitrile. The sample was again centrifuged at 13,000 rpm for 10 minutes for protein removal and further diluted with acetonitrile until a concentration that fits the calibration curve was reached (calibration curve shown in Appendices). Commercially available (*S*)-perillic acid (Sigma-Aldrich, Germany) was used as an external standard, since commercially available (*R*)-perillic acid could not be acquired.

The HPLC analysis was performed on a Dionex UltiMate 3000 HPLC equipped with a Photodiode Array Detector (Dionex, Thermo Scientific). The column used in the HPLC was a Luna 3µm C18(2) 100 Å, 150x4.6 mm. The mobile phase was 70:30 (v/v) acetonitrile/water containing 0.1% formic acid, at 0.8 mL/min and 40 °C. The UV detector wavelength was selected at 217 nm.

Measurement of cell concentration

To measure growth kinetics, cell concentrations were analyzed photometrically at 600 nm (OD_{600nm}). Before measuring cell concentrations in limonene conversions, 1 mL samples were centrifuged at 13,000 rpm for 10 minutes and cell pellets were resuspended in a sterile saline solution (0.9 %(w/v) NaCl) after removing the supernatant and limonene phase.

For determination of cell dry weight, 1 mL of cell culture was transferred into a pre-weighed and pre-dried Eppendorf tube. The sample was then centrifuged at 13,000 rpm for 10 min in an Eppendorf centrifuge. The supernatant was carefully extracted and the cell pellet was washed with a sterile saline solution 0.9 %(w/v) and centrifuged again (13,000 rpm; 10 min). The cell pellet was then dried at 80°C for more than 24 hours and placed in a desiccator to cool to room temperature until a constant weight at four decimal places was measured.

3.5.1.2 Measurement of substrate loss under fermentation conditions

Limonene loss under fermentation conditions was measured in two different sets of experiments. In the first set, aeration rate was fixed and limonene loss in the exhaust gas was measured over time. In the second set of experiments, the aeration rate was varied using a flow control valve and limonene loss was measured for a fixed period of time.

These measurements were made in an experimental setup simulating the fermentation conditions but without cells. The temperature was kept constant at 30 °C, agitation was constant at 600 rpm and 2 %(v/v) of limonene was added to the fermentation media at the beginning of the experiment. The experimental setup, represented in Figure 3.10, consisted of a reactor vessel with an incorporated sparger at the bottom (a ring sparger, the same type of spargers used in the fermenter). The off-gas was collected in a Viton tube, which is impermeable to limonene and condensed using dry-ice as the cooling agent. For the experiments with variable aeration rate, the air flow was controlled and measured with a flow control valve. Aeration rates between 200 mL/min and 1000 mL/min were tested.

To measure the remaining limonene in the fermenter after a certain period of time, limonene was extracted from the fermentation media with methyl *tert*-butyl ether (MTBE) and measured in a UV-VIS spectrophotometer (Shimadzu, Japan). It was not possible to take reproducible samples from the reactor vessel since limonene is poorly-soluble in water (solubility in water is 0.1 mM) and droplets of limonene are dispersed in the aqueous media. Therefore, the entire volume of media was used in a liquid-liquid extraction with MTBE to collect the remaining limonene in the fermenter after a certain time of operation. UV-VIS measurements were possible because limonene is slightly yellow. Different wavelengths were tested and it was seen that the UV-VIS absorption spectrum of MTBE and glycerol do not overlap the limonene absorption peak. A calibration curve at 254 nm was made for concentrations of limonene in the range of 0.1-2 %(v/v) limonene.

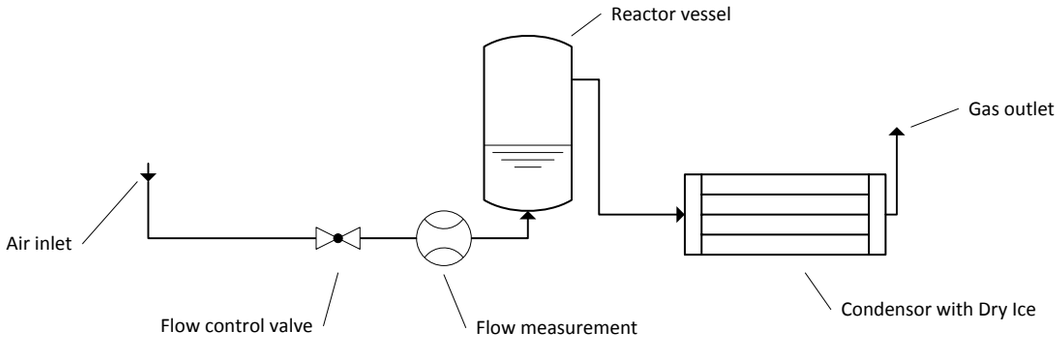


Figure 3.10 Experimental setup for experiments measuring limonene stripping from the fermenter.

3.5.2 Results

3.5.2.1 Determination of the reaction rate

To determine the rate of the conversion of (*R*)-limonene to (*R*)-perillic acid the reaction was followed for 48 hours. The progress curve for the reaction is shown in Figure 3.11. and Figure 3.12 shows the progression of biomass growth and (*R*)-perillic acid formation for the entire time frame of the experiment.

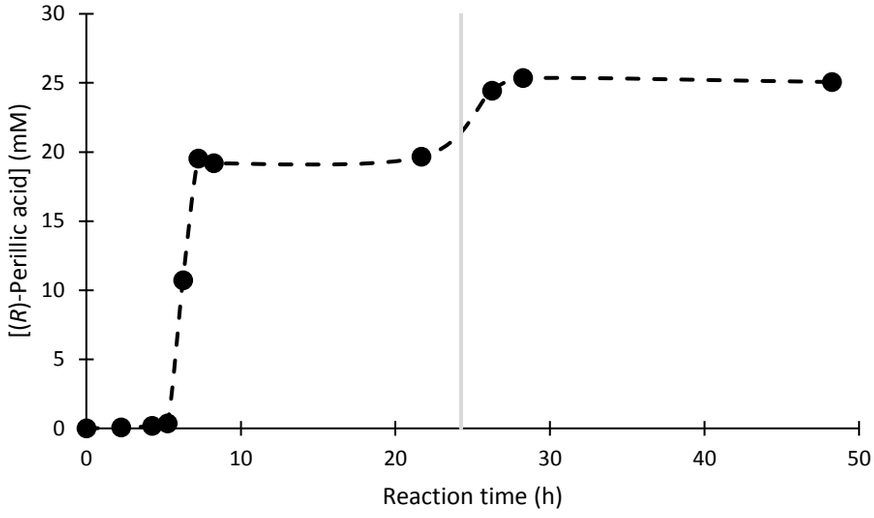


Figure 3.11 Progress curve of the conversion of (*R*)-limonene to (*R*)-perillic acid in 1 L fermenter. A pulse of (*R*)-limonene was added to the fermenter at time 0 h and a second pulse was added after 24 hours of reaction (represented by the grey line).

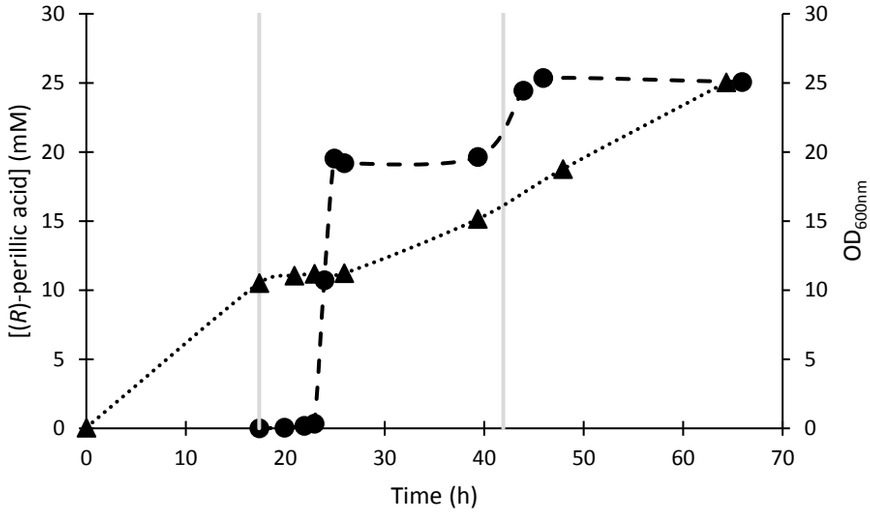


Figure 3.12 Biomass growth (▲) and product formation (●) kinetics in 1 L fermenter. The grey lines represent the time of (*R*)-limonene addition (first addition 17h after inoculation and the second addition is 24h after the first addition).

In Figure 3.11 one can observe that after (*R*)-limonene addition to the fermenter there is a lag phase of approximately 3 hours where the production of (*R*)-perillic acid is negligible. This suggests that some time is

required to activate the induction of cellular adaptation mechanisms in response to the toxic effect of (*R*)-limonene. After this adaptation phase, the conversion of (*R*)-limonene to (*R*)-perillic acid takes place at a given rate for the following 5 hours until a concentration of approximately 20 mM is reached. The reaction reaches then a plateau and the concentration of (*R*)-perillic acid remains constant until the addition of a second pulse of (*R*)-limonene. Then the concentration of (*R*)-perillic acid increases again but at a lower rate than before and again reaches a plateau at a concentration of 25 mM.

Taking into account only the first 8 hours of reaction (after which the concentration of (*R*)-perillic acid remains approximately constant) the conversion takes place at an average rate of 2.3 mM/h, which corresponds to 0.38 g/L/h. This value is slightly higher than the ones reported by Mirata and co-workers that report a conversion rate of 1.8 mM/h (0.30 g/L/h) in the first 4 days of operation of the bioprocess developed by them (Mirata et al., 2009).

The plateau of (*R*)-perillic acid concentration could be explained by either i) issues on substrate availability, ii) product inhibition, or a combination of both problems.

Regarding substrate availability, it was easily detected that a significant part of (*R*)-limonene that is fed to the fermenter is lost in the exhaust gas, since (*R*)-limonene has a characteristic smell to orange. Limonene vapor pressure at 30 °C is approximately 3.4 mmHg (valor estimated from (Perry et al., 1997)), which is lower than water vapor pressure at 30 °C (31.8 mmHg from (Perry et al., 1997)). Therefore, its volatility is lower than water volatility under fermentation conditions. However, since limonene is poorly-soluble in water and there is an inlet air stream, stripping of (*R*)-limonene occurs. (*R*)-Limonene is stripped by the gas stream and lost in the exhaust gas. Hence, when the conversion of (*R*)-limonene to (*R*)-perillic acid reaches a stationary phase, it could be because there is no more limonene available in the fermenter and most of it was lost in the exhaust gas.

Regarding product inhibition, Mirata and co-workers have investigated product inhibition in this reaction system and they observed that maximum specific activities (µmol perillic acid/min/g cdw) decreased exponentially with increasing product concentrations. Already at (*R*)-perillic acid concentrations as low as 2 mM, the maximum specific activity of the biocatalyst decreases (Mirata et al., 2009). Therefore, at a concentration of 20 mM of (*R*)-perillic acid the specific activity of the whole-cell biocatalyst is significantly impaired, which can contribute to the reduction in conversion rate and the plateau on (*R*)-perillic acid formation. After the second addition of (*R*)-limonene the conversion of (*R*)-limonene to (*R*)-perillic acid takes place, but at a much lower rate which might indicate that the whole-cell biocatalyst's activity has been reduced due to a prolonged contact with the product.

But since the biocatalyst is a whole-cell there are several different factors influencing the conversion rate besides the activity of the enzymes involved in the biosynthetic reaction. During the fermentation the cells are under constant stress and its metabolism is overloaded. A high amount of energy is spent in activating the solvent tolerance mechanisms to cope with (*R*)-limonene presence in the cultivation media, in the conversion of (*R*)-limonene to (*R*)-perillic acid and finally in pumping (*R*)-perillic acid out of the cells. Therefore, it is possible that with time the cells are no longer viable and able to properly function under these conditions. The cell's membrane might be compromised and the cells unable to pump (*R*)-perillic acid out of their membrane, which would be translated in a reduction of the experimentally measured reaction rate. The cells might also be unable to properly regenerate the cofactor necessary for the bioconversion, or it is also possible that being unable to cope with all these stress factors, a large number of cells die which means that in the end of the fermentation only a small number of cells is able to perform the bioconversion. Finally, given the fact that the strain that is used is a wild-type strain it is also possible that a large number of cells mutate and lose the p-cymene pathway, losing their ability to convert (*R*)-limonene to (*R*)-perillic acid.

The experimental determination of the reaction rate proved to be very useful for the development of the process. In the bioprocess for (*R*)-perillic acid production, the ISPR operation takes place only 24 hours after the addition of limonene. But according to the progression curve obtained in this experimental work, the ISPR should take place much earlier and be more frequent. If a plateau is reached after 8 hours of reaction, the remaining 16 hours until ISPR are merely wasted since there is not a significant improvement on product formation. By keeping the reaction always at a steady rate and avoiding that the conversion reaches a stationary phase, the space-time yield will be higher. This results in a more efficient use of the equipment, namely the fermenter, and can further reduce facility-dependent costs that have been shown to be one of the biggest cost contributors in this process.

3.5.2.2 Measurement of substrate loss under fermentation conditions

To further investigate the causes of a reduced reaction rate, (*R*)-limonene loss under fermentation conditions has been investigated. (*R*)-Limonene loss in the exhaust gas stream affects negatively the reaction yield and the conversion rate. Besides, (*R*)-limonene is very toxic to aquatic life with long lasting effects (H410), so it should not t be released to the environment. Hence, experiments were run in order to quantitatively measure how much (*R*)-limonene is lost during the conversion time.

In the first set of experiments the aeration time was fixed to 4 hours but the aeration rate was varied. Whereas in the second set of experiments, the aeration rate was kept constant at 1 vvm for different periods of time (4 hours and 12 hours). The results for both experiments are shown in Figure 3.13.

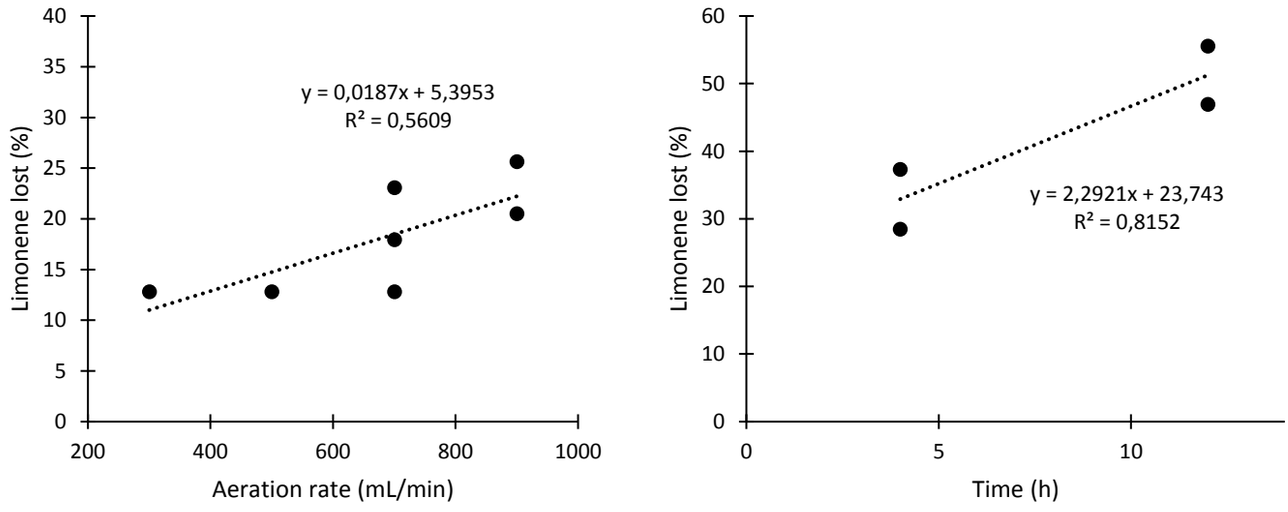


Figure 3.13 Limonene loss under fermentation conditions. **Left:** Limonene loss at different aeration rates for a fixed period of aeration of 4 hours. **Right:** Limonene loss at different periods of time for a fixed aeration rate of 1 vvm.

The experimental method showed some practical difficulties and limitations and some variability in the measurements was observed (Figure 3.13). Given the variability of the experimental measurements it was not possible to quantitatively measure the amount of (*R*)-limonene that is lost during the bioconversion.

But nonetheless, it was possible to observe and experimentally show that (*R*)-limonene stripping under fermentation conditions takes place. As expected, the loss of limonene is more pronounced for higher aeration rates and when the aeration runs for longer periods of time, *i.e.* as reaction time goes by more (*R*)-limonene is lost in the exhaust gas. However, it was not possible to draw any conclusions on the extent that aeration rate or aeration time influence limonene loss under fermentation conditions. Moreover, these experiments were run in the absence of cells, but their presence could also influence limonene stripping.

Even though the accuracy of the experimental method does not allow an accurate quantification of limonene loss under fermentation conditions, it clearly shows that stripping of the substrate does occur which confirms that substrate availability is a major issue in this reaction system. Since (*R*)-limonene is stripped from the fermentation media over time, it should be fed to the fermenter more frequently than only every 24 hours, or possibly in a continuous mode. Given that (*R*)-limonene stripping is reduced at lower aeration rates, the

possibility of running the reaction at lower aeration rates should also be considered. The current bioprocess runs with growing cells, which means that oxygen is required both for cell growth and for the bioconversion of (*R*)-limonene to (*R*)-perillic acid. Therefore, there is a limit of how much the aeration rate can be reduced and alternative methods for oxygen supply, such as supplying enriched air or producing oxygen *in situ* by the decomposition of hydrogen peroxide by catalase, should be considered. Another option to reduce (*R*)-limonene stripping would be to run the reaction in a two-phase system. An organic solvent layer could act as a (*R*)-limonene reservoir and in this way stripping could be reduced. This system has also the benefit of potentially reducing substrate and product inhibition, but would require some modifications in the process operation.

3.6 Strategies for improvement of the bioprocess

In previous sections was shown that space-time yield is the metric that affects the most the production cost. After an experimental characterization of the process, it was observed a reduction of the conversion rate approximately 8 hours after the start of the reaction. The production of (*R*)-perillic acid reaches a stationary phase where there is no significant increase on its concentration. Investigation on substrate loss under fermentation conditions showed that there are problems with substrate availability in the reaction. And previous work from Mirata and co-workers showed that the reaction faces product inhibition from very low concentrations of (*R*)-perillic acid (Mirata et al., 2009). All these issues affect negatively space-time yield.

Space-time yield is a very complex metric which is affected by several different parameters, summarized in Table 3.5. Different strategies for improvement of these parameters, including tools for both biocatalyst modification and process development, are shown in Table 3.6. These different parameters are reviewed and discussed for whole-cell P450 processes by Lundemo and co-workers (Lundemo and Woodley, 2015).

Table 3.5 Typical parameters affecting space-time yield.

Biocatalyst-related parameters	**Specific activity (U/g cdw)**	Fermentation cell density (g cdw/L) Enzyme activity Cofactor regeneration Electron transport from the cofactor to the active site
Substrate and product related parameters	Transport of substrate and product across the cell membrane Product and/or substrate inhibition	
Process related parameters	Oxygen requirements Transport limitations between phases in a two-phase system	

Table 3.6 Different strategies to improve space-time yield.

Biocatalyst specific activity (U/g cdw)	**Fermentation cell density (g cdw/L)**	High cell-density fermentation Fermentation optimization (media, feeding, etc.)
	Enzyme activity	Protein engineering
	Cofactor regeneration	Change operating mode Metabolic engineering Protein engineering Heterologous enzymatic regeneration of the cofactor
	Electron transport from the cofactor to the active site	Engineering of redox partners
Substrate and product related parameters	**Transport of substrate and product across the cell membrane**	Permeabilization of the cell membrane (physical or chemical methods)
	Product and/or substrate inhibition	*In situ* product removal (ISPR) *In situ* substrate supply (ISSS)
	Substrate solubility	Addition of co-solvents
Process-related parameters	**Oxygen requirements**	Alternative methods for O_2 supply
	Transport limitations between phases in a two-phase system	Addition of surfactants or co-solvents

A whole-cell biocatalyst is a very complex system and therefore there are several different parameters affecting space-time yield. Regarding the parameters related to the biocatalyst, it is the biocatalyst specific activity (U/g cdw) that mostly affects the space-time yield. The biocatalyst specific activity is influenced by the cell density in the fermentation, the enzyme activity, in terms of k_{cat} (s^{-1}, turnover per active site and second), cofactor regeneration and electron transport from the cofactor to the active site. The bioconversion of (*R*)-limonene to (*R*)-perillic acid is a multi-enzyme system, with three different enzymes involved in the reaction (or four enzymes if the reductase shuttling NADH to the first reaction step is also included). Therefore, for improvement of any of the biocatalyst-related parameters affecting space-time yield, such as enzyme activity, cofactor regeneration or electron transport from the cofactor to the active site, it is

necessary to determine the rate limiting step and improve the enzyme(s) involved in that step. From a process engineering point of view it is possible to improve the fermentation cell density by optimizing the fermentation conditions, e.g. the fermentation media or the feeding of the carbon source. In order to increase the fermentation cell density, a high-cell-density fermentation of *Pseudomonas putida* GS1 was developed and is shown in the following sections.

Regarding the substrate and product related parameters, this process faces product inhibition and low solubility of the substrate in water. One of the strategies to improve problems of product inhibition in the reaction is to have ISPR. The ISPR operation of this process was also further investigated in the following sections.

High-cell density fermentation and ISPR were further investigated and tested experimentally and are presented as examples of process technologies that can be applied to intensify this bioprocess.

3.6.1 Materials and methods

3.6.1.1 High-cell-density fermentation

The seed culture and the fermentation ran in the same conditions as the ones applied in the base-case fermentation. The only difference in this experiment was the feeding profile of the carbon source used in the biomass growth phase. The feeding of 40 %(w/v) glycerol was done exponentially, according to Equation 3.2, in order to keep a constant specific growth rate of 0.1 h^{-1}. In Equation 3.2 μ_0 corresponds to the specific growth rate (h^{-1}); Y_{SX} is the yield of biomass on carbon source; S_f is the concentration of carbon source in the feed stream (g/L); S_0 is the concentration of carbon source in the culture vessel at the end of the batch phase (g/L); X_0 is the biomass concentration in the culture vessel at the end of the batch phase (g/L) and V_0 is the initial culture volume, before the fed-batch has started.

Equation 3.2

$$F(t) = \frac{\mu_0}{Y_{S/X}\,(S_f - S_0)}\,X_0 V_0\,\exp(\mu_0 t)$$

In these experiments, Y_{SX} was assumed to be 0.26 g cdw/g glycerol based on shake flask fermentations, the initial culture volume (V_0) was 700 mL, S_f is equal to 0.4 g glycerol/mL; it is assumed that the carbon source is entirely consumed by the end of the batch phase (S_0 is equal to zero) and X_0 was measured after the batch phase of 5 hours.

The exponential feed lasted 24 hours after which the feeding strategy was changed to a constant feeding. (*R*)-Limonene was fed to the fermenter after the exponential feed in order to start the bioconversion of (*R*)-limonene to (*R*)-perillic acid and the reaction was followed for 48 hours.

3.6.2 Results

3.6.2.1 High-cell-density fermentation

As previously mentioned, the cell density in a fermentation affects the biocatalyst specific activity in a whole-cell process. The cell density can be improved by optimization of the fermentation conditions, e.g. media optimization, or by applying different feeding strategies of the carbon source.

Here a high-cell density fermentation of *Pseudomonas putida* GS1 was developed by applying an exponential feed of the carbon source (Equation 3.2) in the phase for biomass growth.

Mirata and co-workers have determined the maximum specific growth rate (μ_{max}) of *P. putida* GS1 to be 1.43 h^{-1} in the absence of perillic acid (Mirata et al., 2009). In the first batches of fermentations, μ was set to 0.25 h^{-1} based on previous work from Sun and co-workers with a similar *Pseudomonas putida* strain (Sun et al., 2006). However, at this growth rate oxygen deprivation occurred approximately 10 hours after the start of exponential feed and it was not possible to keep dissolved oxygen concentration above 30% air saturation. The growth rate was then lowered to 0.1 h^{-1} and the progress curve for the fermentation is shown in Figure 3.14.

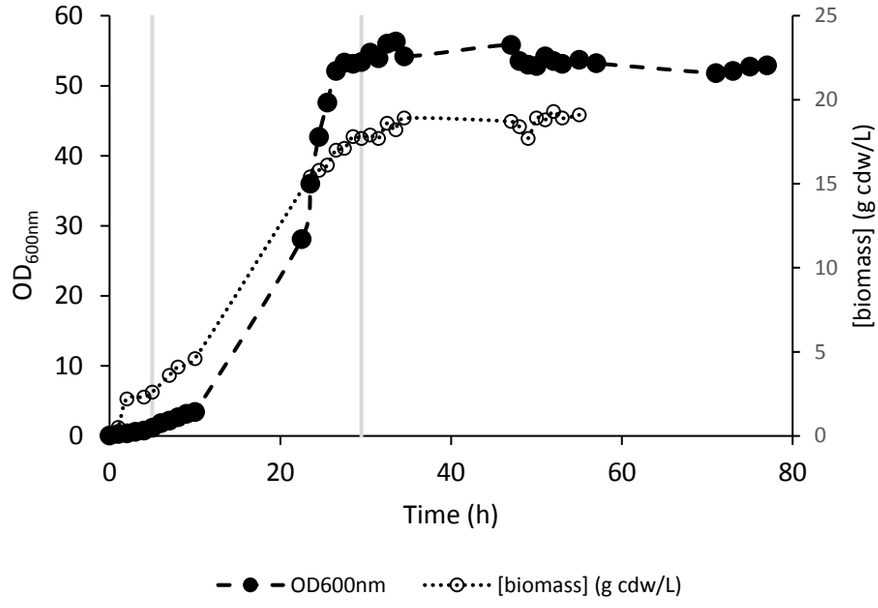


Figure 3.14 Progress curve for a fed-batch fermentation with exponential feed keeping a constant specific growth rate of 0.1 h^{-1}. The vertical lines represent the time points at which the exponential feed started and finished.

By having an exponential feed of the carbon source that kept the growth rate constant at 0.1 h^{-1}, it was possible to reach an OD_{600nm} of approximately 55, which corresponds to a biomass concentration of approximately 19 g cdw/L. According to Equation 3.2, after 24 hours of exponential feeding 18.48 g cdw should theoretically be produced in the fermenter. Therefore, it was possible to achieve the expected biomass concentration.

It was possible to successfully increase biomass concentration from an OD_{600nm} of approximately 25 in the end of the base-case fermentation, which corresponds to approximately 10 g cdw/L, to an OD_{600nm} of approximately 55 at the end of the high-cell density fermentation, which corresponds to approximately 19 g cdw/L.

But even though biomass concentration was considerably increased, it was not possible to verify if this leads to a higher space-time yield of the bioprocess since (*R*)-perillic acid formation was not observed. Even though (*R*)-limonene was added to the fermentation media in the end of the exponential feed, (*R*)-perillic could not be detected in any of the HPLC chromatograms.

3.7 Concluding remarks

In this chapter the biocatalytic conversion of (*R*)-limonene to (*R*)-perillic acid by a wild type strain of *Pseudomonas putida* was benchmarked with other possible routes for perillic acid production, namely with the chemical synthesis starting from β-pinene developed by Wang and co-workers (Wang et al., 1993). Reaction-related green chemistry metrics and EHS hazards of the different chemicals used in the different routes proved to be useful tools for benchmarking of the different synthetic routes and showed that the biocatalytic route has a greater potential to be a sustainable route for perillic acid production. However, even though atom economy and carbon efficiency show that the biocatalytic conversion of (*R*)-limonene to (*R*)-perillic acid by *P. putida* is a very promising route where the principles of maximizing the incorporation of starting materials into the final product are obeyed, reaction mass efficiency revealed how it is still far from reaching its full potential. It is not surprising that the reaction yield and volumetric productivity of the reaction are still far from the values necessary for industrial implementation given the fact that the strain that is used is a wild-type strain, which did not evolve to work in industrially relevant conditions.

The bioconversion of (*R*)-limonene to (*R*)-perillic acid by a whole-cell biocatalyst was further explored by means of an economic and environmental assessment. A process model was developed in SuperPro Designer® software and a sensitivity analysis on different process metrics and its influence on production costs allowed the identification of space-time yield as the most critical process metric.

Following the identification of low space-time yield as the main bottleneck of this bioprocess, different parameters affecting space-time yield and strategies to improve it were reviewed.

In order to understand the reasons for a low space-time yield some additional experimental work was developed. The reaction rate was measured over time and it was seen a considerable decrease in reaction rate approximately 8 hours after the start of the reaction. The reduction in reaction rate could be explained by product inhibition and substrate loss by stripping of (*R*)-limonene in the exhaust gas, which was demonstrated experimentally.

The strategies for improvement of space-time yield include both techniques to modify and improve the biocatalyst and process technologies. One process technology the development of a high-cell density fermentation, was tested experimentally in order to exemplify some of the strategies that can be applied in the development of this bioprocess and the improvements that can be obtained with this type of tools. Even though the cell density was successfully improved in the fermenter, the improvements in space-time yield could not be verified. The fermentation process showed a very high instability and variability on the

production levels of (*R*)-perillic acid, which is a major concern for industrial implementation of this bioprocess.

Several other strategies for improvement of space-time yield should be tested experimentally, including protein and metabolic engineering for biocatalyst modification. The bioprocess analyzed in this chapter used a wild-type strain of *Pseudomonas putida*. However, as reviewed in section 1.3 most processes for production of natural products that have reached commercial scale use genetically improved microorganisms. The genetic modification of the strain could also potentially improve the robustness of the strain, which is an important factor for industrial implementation. On the other hand, the natural label of this product is important for its application in cosmetic or food industry and the use of genetically modified organisms is not well accepted by many consumers, especially in the European market. But could this bioprocess ever reach the necessary yields and productivities for industrial implementation without the use of an improved biocatalyst? Further experimental work on the process would be necessary to answer this question.

4. Case-study II: Fermentation of recombinant *Pichia pastoris* for brazzein production

4.1 Introduction

In this chapter the second case-study that was explored in this thesis is introduced. It corresponds to the production of brazzein by fermentation of the yeast *Pichia pastoris*. Brazzein is a natural sweetener present in the wild African plant *Pentadiplandra brazzena* Baillon that is much sweeter than sucrose by several orders of magnitude. It is a protein comprising of 53 amino acid residues, and therefore this case-study differs from the first one since the product is a protein associated to cells' growth. This bioprocess is classified as a fermentation process and not a biocatalytic process.

This chapter is organized in six different sections. First a brief introduction to different sweeteners and its productions processes is made in section 4.2. Following that, the fermentation process for brazzein production is described in section 4.3. A very brief evaluation of the economy of the process is shown in section 4.4 where the key process metrics of this bioprocess are analyzed. But the main focus of this case-study is on the tools for environmental assessment and on the challenges of comparing the environmental profile of different types of processes in different stages of development. In section 4.5 the environmental profile of brazzein fermentation process is first benchmarked with different routes for brazzein production (extraction from the African fruits and extraction from genetically modified maize), and secondly with production processes of other sweeteners, namely stevia and aspartame. Finally, section 4.5.4 presents some concluding remarks on this case-study.

4.2 Sweeteners

Sweet-tasting substances, generally designated as sweeteners, have gained increasingly importance in food industry over the last decades. This industry has been for long in a demand to substitute sugar by a sweetener that has the same taste and texture as sugar but with less calories. The increase in obesity and diabetes in industrialized countries has resulted in governmental actions to promote health and wellness, e.g. Mexico has introduced a one-peso-per-liter tax on sugar-sweetened soft drinks in 2014. In addition, consumers

became more concerned with their health and are increasingly demanding. Nowadays, consumers seek for healthier products but with the same tasting and texture characteristics as sugar-sweetened products.

One of the first sweeteners to be widely applied in several food products was aspartame and it is still today the most common intense sweetener with a worldwide consumption of 10,000 tons/year. Aspartame is a chemically synthesized sweetener that was discovered in 1965, it is 160 to 220 times sweeter than sucrose and its taste is described to be very similar to sucrose, showing no bitter or metallic aftertaste. It is a dipeptide composed of two amino acids, L-aspartic acid and the methyl ester of L-phenylalanine. The synthetic route of aspartame is represented in Figure 4.1 (Zorn and Czermark, 2014). However, aspartame safety has been subject of several controversies and critics have linked aspartame to several health problems, including cancer. Even though several scientific publications dismiss the potential health risks of aspartame and it is considered as a safe food additive by worldwide health and food organizations, aspartame became a very unpopular sweetener and consumers seek new sweeteners preferably from natural sources.

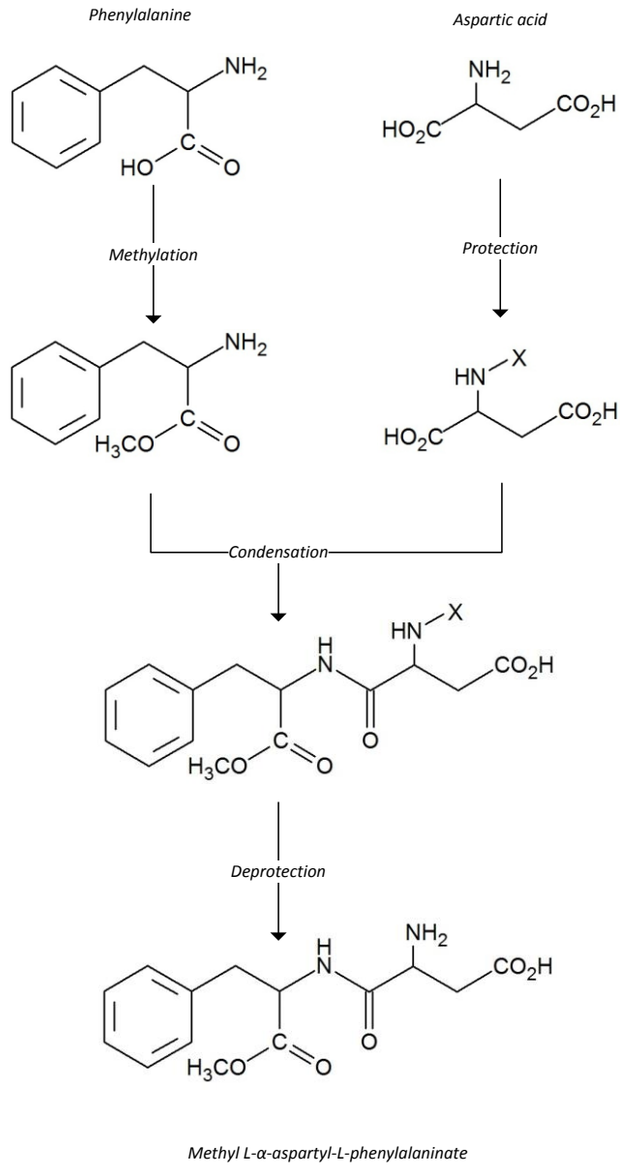


Figure 4.1 Chemical synthesis of aspartame.

So as a result of the current global demand for healthy and natural products, there is a clear market for novel sweeteners to be applied in beverages and food products.

Stevia, a natural sweetener present in high concentrations on the South American plant *Stevia rebaudiana* Bertoni, has become a popular alternative to sugar and is already being used by some of the world's biggest beverage brands, e.g. Coca-Cola Life®. Stevia is the common designation given to steviol glycosides (shown in Figure 4.2) that naturally occur in *Stevia rebaudiana* and has been consumed for centuries in Paraguay and Brazil.

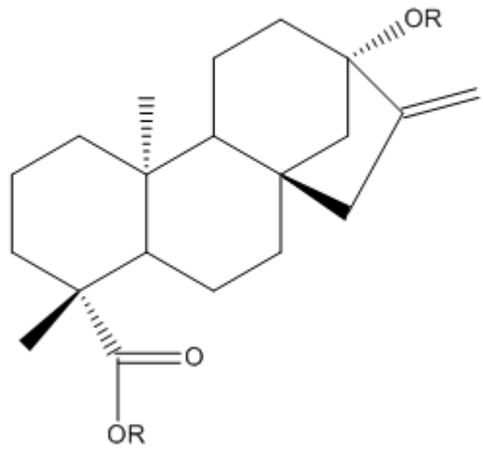


Figure 4.2 Structure of steviol glycosides. R: mono- or disaccharide residues.

The most abundant glycosides in the plant are stevioside and rebaudioside A, with a content of 4-8.5 % (w/w) of stevioside and 1.5-5% (w/w) for rebaudioside A. The ratio of the different components varies depending on the plant, and it can be manipulated by breeding aiming at an increase in Rebaudioside A, the product with the best sensory properties. Depending on the composition, steviol glycosides are 200-300 times sweeter than sugar. However, they have a bitter and licorice aftertaste, which is an undesirable property in the food industry. Steviol glycosides have been approved as food additives in 2011 by the European Food Safety Authority (European Food Safety Authority (EFSA), 2010). The manufacturing process of stevia, which is described in more detail in section 4.5.2.3, consists of three basic steps – i) water extraction from the plant leaves, ii) preliminary purification with ion exchange chromatography, and iii) recrystallization from methanol or aqueous ethanol. There are several variations of this basic process and several different approaches to extract and purify the molecules have been investigated (Puri et al., 2011). A novel process for stevia production by fermentation of recombinant yeast has also been developed by Evolva (Mikkelsen et al., 2014) and will start commercial manufacturing in a collaboration with Cargill®.

In this quest for the perfect sweetener with great taste and no calories, brazzein appears to be a very promising alternative. It is much sweeter than conventional sucrose and stevia and it does not have the undesired licorice aftertaste. Brazzein is a sweet protein from a wild African plant *Pentadiplandra brazzena* Baillon. It is a single chain polypeptide, which is comprised of 53 amino acids residues. It has a molecular weight of 6,5 kDa. The protein was discovered and named by Ding Ming and Göran Hellekant in 1994 (Hellekant and Ming, 1998). The content of brazzein in the fruit is about 0.2 - 0.05 % by weight and mainly

placed in the pulp. *Pentadiplandra brazzeana* grows in central Africa, more precisely from Nigeria east to the Central African Republic and south to Democratic Republic of the Congo and Angola. It grows as a shrub or a liana. The sweet fruit, and other parts of the plant, have been used for centuries and are still in use by local people. But there is not an agricultural cultivation of the plant. The fruits are harvested from wild plants.

Brazzein is 500 times sweeter than a 10 % (w/v) sucrose solution. In comparison with a 2 % (w/v) solution, brazzein is 2000 times sweeter. The taste of brazzein is described as purely sweet, with no sourness, saltiness or bitterness, but the mouth feeling differs from the sweetness of sucrose. The sweetness grows slower, is easily washed from the tongue and does not last long. Brazzein is a thermo stable protein. The sweetness lasts even after incubation at 80 °C for 4 h.

An obvious possibility for the production of brazzein is the extraction from fruits of *Pentadiplandra brazzeana*. However the large scale production of brazzein from natural sources is described as uneconomical. The reasons indicated are limited availability of the fruit and complications associated with large-scale production of the native plant (Lamphear et al., 2005). Therefore, the production of brazzein in a recombinant host appears to be an interesting approach. The genetic code of brazzein has been sequenced in 1994 by Hellekant and Ming (Hellekant and Ming, 1998) and since then there has been several attempts to produce brazzein in different hosts. One of the options, which is further analyzed in this chapter, is the expression of the protein in maize which has been demonstrated by Lamphear and co-workers (Lamphear et al., 2005). One of the advantages of this option would be that the final product is sweet maize flour which simplifies the purification. Assadi-Porter and co-workers have reported in 2000 the production of brazzein in *Escherichia coli* (Assadi-Porter et al., 2000) and the company Natur Research Ingredients Inc. is currently working on the commercialization of brazzein produced by fermentation of *E. coli* under the name Cweet™. BRAIN AG has focused on the expression of brazzein in yeast cells from the species *Pichia pastoris*, and this process is further analyzed throughout this chapter.

4.2.1 Intensity of sweetness

As mentioned before sweeteners have different intensities of sweetness. A high intensity sweetener, such as brazzein, can be up to thousands times sweeter than sucrose. So the sweetness of a substance is represented in terms of sweet potency, also called relative sweetness, which expresses how much sweeter a substance is in comparison to sucrose. Sweet potency is correlated on a mass basis, which means that solutions with different mass concentrations of the sweetener are compared with sucrose solutions with different

concentrations in a dose-response diagram. For example, brazzein has a sweetness potency of 2000 when compared to a 2% sucrose solution, which means that brazzein is 2000 times sweeter than a 2% solution of sucrose.

Since sweeteners have different sweetness intensities, it is important to take this into account when comparing the environmental impacts of the different sweeteners. Therefore, a comparison in terms of sweetness potency was made as shown in section 4.5.

4.3 Bioprocess description

The process flowsheet for the fermentation of *Pichia pastoris* for brazzein production is shown in Figure 4.3. The data used for the process flowsheet and for the mass balances is experimental data from 10 L scale experiments run at BRAIN AG facilities. The mass balances for the whole process are shown in detail in the Appendices.

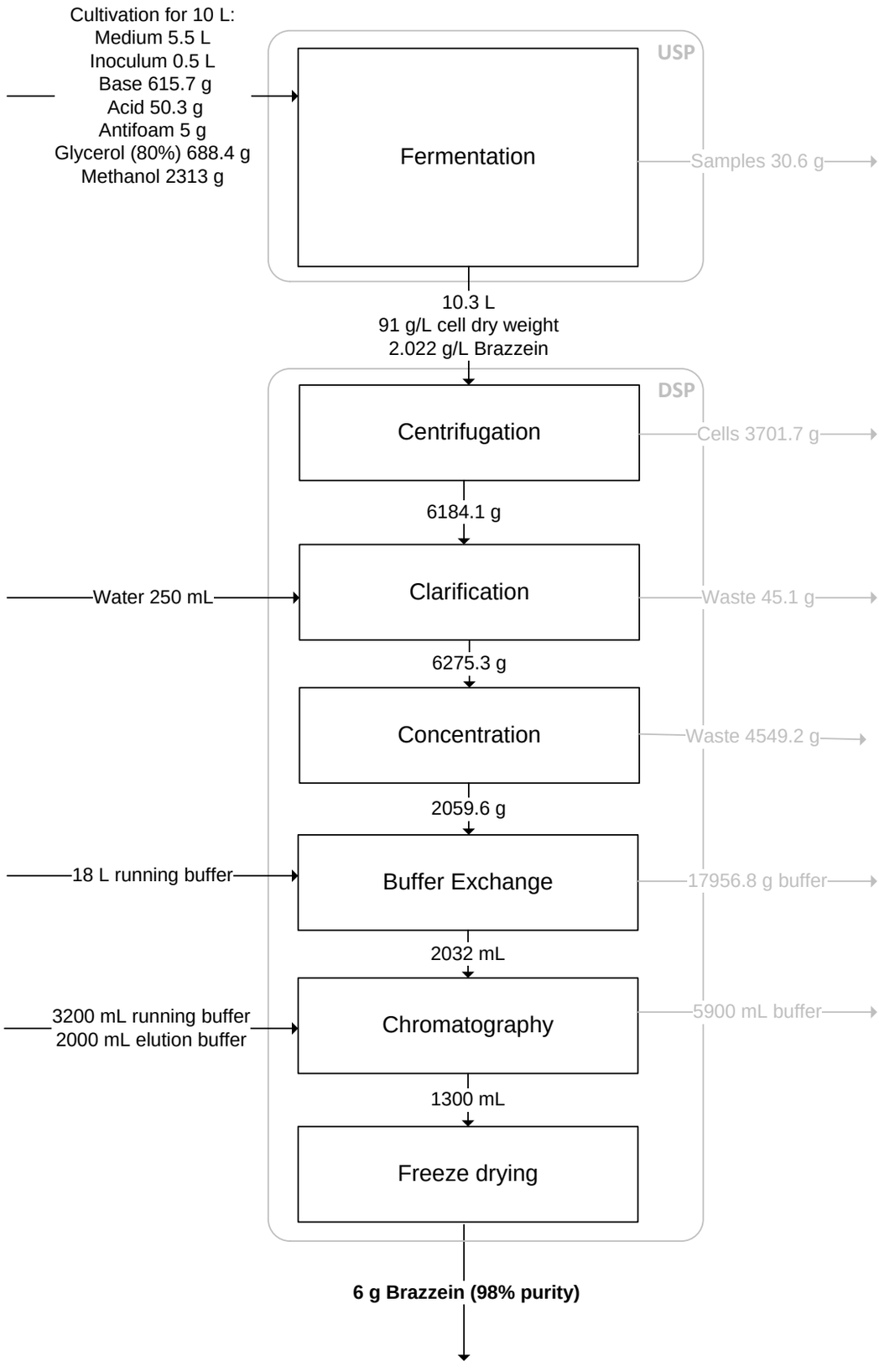


Figure 4.3 Process flowsheet for brazzein production by fermentation of recombinant *Pichia pastoris*.

The established bioprocess for brazzein production corresponds to a fed-batch fermentation of *Pichia pastoris*, where glycerol and methanol are used as carbon sources. The media composition is shown in Table 4.1. 1 mL/L of trace metals solution, whose composition is shown in Table 4.2 is added to the fermentation broth in the beginning of the fermentation. The fermentation is aerobic and takes place at a constant temperature of 30 °C and at a constant pH (balanced with phosphoric acid (17% (v/v) and/or ammonia (25 % (v/v)), and lasts for approximately 50 hours. Foam production is controlled through addition of antifoam of polypropylene glycol. Brazzein is produced by the cells with a product yield of 0.012 g/g of methanol and excreted to the media.

Table 4.1 Composition of the fermentation media for fermentation of *Pichia pastoris*.

Component	Concentration (g/L)
Glycerol	40
KH_2PO_4	42.9
$(NH_4)_2SO_4$	5
$CaCl_2$	1
K_2SO_4	14.3
$MgSO_4$	11.7

Table 4.2 Composition of the trace metals solution used in the fermentation of *Pichia pastoris*.

Component	Concentration (mg/L)
$CuSO_4$	2
NaI	0.08
$MnSO_4$	3.0
Na_2MoO_4	0.2
H_3BO_4	0.02
$CaSO_4$	0.5
$CoCl_2$	0.5
$ZnCl_2$	7
$FeSO_4$	22
Biotin	0.2
H_2SO_4	1 µL/L

Since the product is extracellular, cell lysis is not necessary. After fermentation, there is a centrifugation for separation of the cells from the fermentation broth. Following that there is a clarification step through filtration at 4 °C and a concentration step at 10 °C by ultrafiltration where proteins and particles bigger than 50 kDa are removed from solution. Finally there is a diafiltration step for exchanging the product to a different buffer of sodium acetate (10 mM) and sodium chloride (50 mM) and an ion exchange chromatography where brazzein is selectively bound to the resin. The product is then eluted from the chromatography column using a buffer of sodium acetate (10 mM) and sodium chloride (1 M). The last step is drying the product in a freeze-drying step until a final concentration of 98% is achieved. However, the downstream processing of this process is highly inefficient with a yield of 28.8%. Approximately 71.2% of brazzein that is produced in the fermentation is lost in the purification steps.

4.4 Bioprocess evaluation

In the same way as for the first case-study the key process metrics described in section 2.1.1 have been calculated and are shown in Table 4.3. In this case-study the bioprocess in analysis is a fermentation and not a biocatalytic reaction, which means that there is not a reaction yield but a yield of product on carbon source. This yield on carbon source should be compared with the maximum theoretical yield calculated from the metabolic pathway. However, this comparison was not done since the pathway for brazzein production in *Pichia pastoris* could not be found in literature.

Table 4.3 Process metrics of brazzein production process.

Product concentration (g/L)	2.02
Space-time yield (g/L/h)	0.075
Yield of brazzein on glycerol (g/g)	0.035
Yield of brazzein on methanol (g/g)	0.012

This process refers to a fermentation for production of a protein and not a chemical. In this case, the requirements for the process metrics are lower since normally the selling price of proteins, which are typically used for therapeutic purposes, is higher than the price of chemicals. However, brazzein is not a therapeutic protein. It is aimed to be applied in the food industry, so its price should be lower than therapeutic proteins.

The minimum values for the process metrics that guarantee economic feasibility of the process depend on the final selling price of brazzein.

4.5 Environmental assessment

There are only two possible routes for brazzein production, the extraction from natural sources or the biosynthesis. The chemical synthesis is not an option since brazzein is a protein.

In this section of the thesis, an environmental comparison of different production processes was made and is graphically summarized in Figure 4.4. In section 4.5.3 the biosynthetic route for brazzein production using *Pichia pastoris* as a production host is benchmarked with two possible extraction routes – extraction from the African fruits of *Pentadiplandra brazzena* and extraction from genetically modified maize. The biosynthetic route for brazzein production is then also benchmarked with the production processes for other two sweeteners. The two different sweeteners used as a benchmark are stevia and aspartame. The method selected for stevia production was stevia extraction from the leaves of *Stevia rebaudiana*, which is the process that is currently used for commercial production of stevia. It would also be interesting to include in the assessment a comparison with stevia production by yeast fermentation, since this process should be similar to *Pichia pastoris* fermentation for brazzein production. However, it was not possible to include it due to lack of published data. The method selected for aspartame production was the enzymatic reaction, with the enzyme thermolysin.

It is important to note that all these processes are in different stages of development. Stevia and aspartame are commercially available and their production processes have gone through an extensive development and optimization until commercial production was economically feasible. Brazzein production by fermentation of *Pichia pastoris* or extraction from modified maize are still in research and development and have only been applied at laboratory scale. Finally, the production of brazzein by extraction of the African fruits is only a conceptual process based on the patent from Hellekant and co-workers (Hellekant and Ming, 1998).

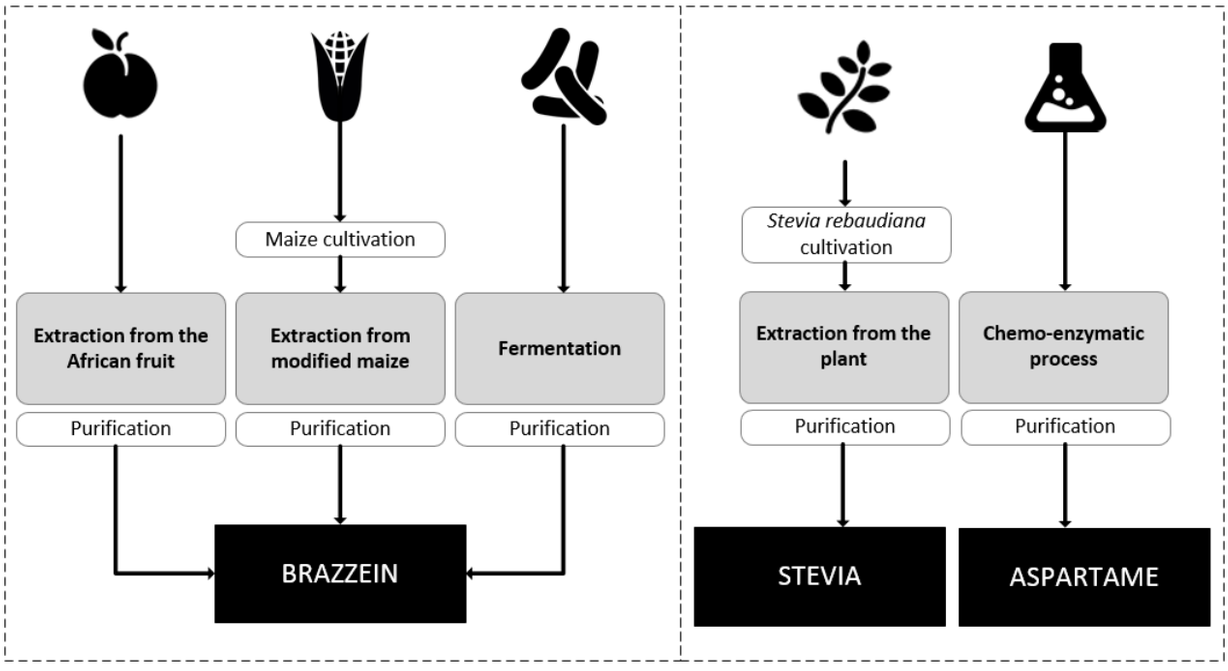


Figure 4.4 Graphical summary of the different processes benchmarked in the environmental assessment.

4.5.1 Scope of the environmental assessments

In environmental assessments the first step is to define the scope of the assessment. In general, a more holistic approach will give more meaningful results. However, the amount of data required and the complexity of the analysis will also increase. Therefore, a compromise between the complexity and the significance of the analysis needs to be reached.

The processes analyzed in this chapter are different types of processes – three of the five processes are extraction from natural sources, one process is a fermentation and the last is a chemical synthesis (aspartame production). Therefore it is not straightforward to define a common scope for the five processes. Apart from the production of aspartame, all the other processes have in common a phase for growing of the biomass, where the product is included. This phase corresponds to the growth of yeast cells in the fermentation process and to the growth and cultivation of plants in the extraction processes. This phase is the upstream processing where the product is formed. The resulting biomass containing the product is then sent to downstream processing where the product is separated from the biomass and further purified. A scheme representing these different steps that constitute the scope of the assessment is shown in Figure 4.5.

In the case of aspartame production, only the chemical synthesis of aspartame is considered in the upstream processing. The starting point for the synthesis are the two amino acids, L-aspartic acid and D,L-phenylalanine. The synthesis of the amino acids as well as all the other reactants is not included in the assessment.

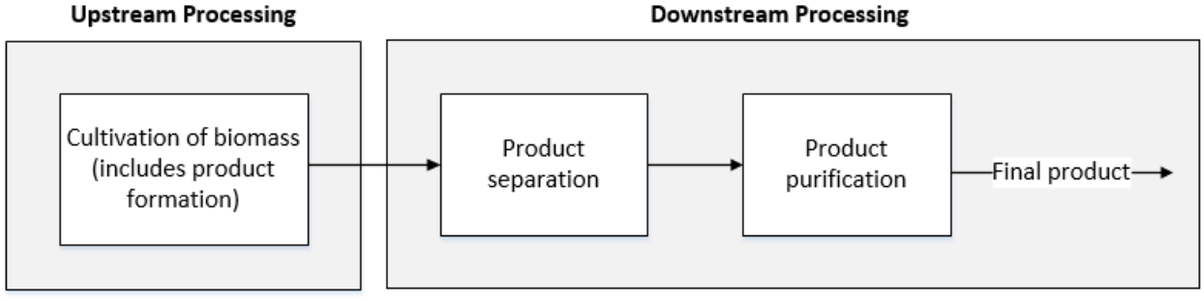


Figure 4.5 Scope of the environmental assessment.

The system boundaries end at the last step of the manufacturing process, the so called cradle to gate approach. Packaging and shipping of the product are not included, neither the use nor disposal of the product.

4.5.2 Modeling and inventory of the processes

An extensive literature was made to collect data on all the different processes. In this section all the processes are described in detail as well as all the assumptions made, apart from brazzein fermentation which is described in section 4.3. The mass balances of all the processes are shown in the Appendices.

4.5.2.1 Brazzein extraction from genetically modified maize

A conceptual process for brazzein extraction from genetically modified maize has been developed based on the work from Lamphear and co-workers (Lamphear et al., 2005). One advantage of this process option would be that after dry-milling of the seeds, the sweet maize flour could be used directly in food products. However,

for a meaningful comparison with the other processes for brazzein production, a process for brazzein purification is assumed. Furthermore, the sweet maize flour could not be used in baking processes at temperatures higher than 98 °C, which would limit the options for product formulation. In their work, Lamphear and co-workers have proposed a process for purification of brazzein. It was developed for analytical purposes but it is very similar to other processes for purification of recombinant proteins recovered from plants (Menkhaus et al., 2004) (Ma and Wang, 2012). Therefore, the same conditions were assumed in the conceptual process developed for the environmental assessment, whose flowsheet is shown in Figure 4.6.

The process starts with the cultivation of the genetically modified maize. It was assumed that the cultivation of genetically modified maize would be done in the same conditions as regular maize, with the same yield of maize per hectare, fertilizer and water consumption, etc. The amount of fertilizers, pesticides, manure, etc. consumed was taken from the Agri-footprint® LCA Food Database available in the LCA software SimaPro. The values used are for corn cob mix (CCM) production (CCM consists of the grains and cobs of maize) but it is assumed that they are the same as for maize cultivation (Vellinga and Boer, 2012). The yield of maize per hectare was taken from the Federal Statistical Office of Germany. The average yield was 10 ton/ha for the years 2012-2014. The maize that is harvested needs to be matured and dried. The harvested maize is composed of 35% (w/w) of dry mass (Federal Statistical Office of Germany, 2016). Hence 3.5 tons of dry weight of maize is obtained per hectare.

Lamphear and co-workers measured an average brazzein content of 50 µg per gram dry weight of maize. Single seeds reach a concentration of brazzein of 400 µg per gram of seed. This is still a process in early stages of research, therefore the authors of the work expect an increase in brazzein content through backcrossing of lines. Thus, a yield of 400 µg per gram of dry weight of maize was assumed (Lamphear et al., 2005).

The process for brazzein extraction starts with standard dry milling of the maize. The milling process leads to a concentration of brazzein, since the germ of maize which is the part of maize with the highest concentration of brazzein is separated from the hulls. The germ-rich fraction of maize is then sent to downstream processing, which starts with an extraction step with a buffer of 20 mM of sodium acetate and 30 mM of sodium chloride. In the extraction step the ratio solids:buffer is 5:1. After extraction, there is a centrifugation and filtration with a 0.2 µm filter to separate the particulate matter. Then a cation exchange chromatography separates brazzein from other proteins. Finally, there is an ultrafiltration at 3 kDa for concentration of the solution and desalting. The final step is freeze-drying (Lamphear et al., 2005).

The method described by Lamphear and co-workers does not include the yield of each individual step. Since the purification process is very similar to the one from the fermentation of *Pichia pastoris*, the same yield of

28.8 % was assumed for the downstream processing. The conditions in each individual step, e.g. amount of buffer used, were also considered to be the same as in the fermentation process unless otherwise stated. It was also assumed that the product has the same purity of 98% in the end of the purification process.

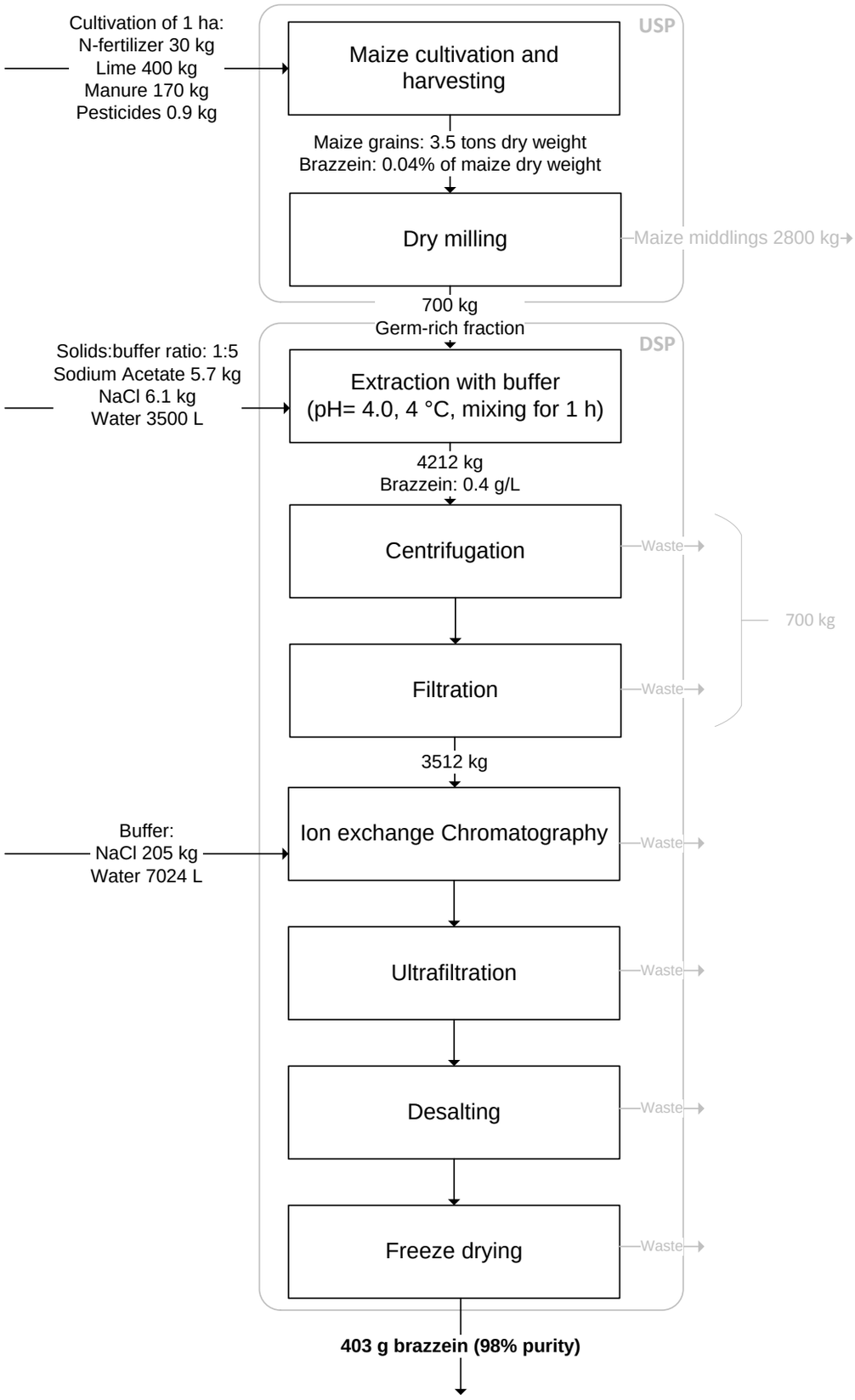


Figure 4.6 Process flowsheet for brazzein extraction from genetically modified maize.

4.5.2.2 Brazzein extraction from the African fruits of *Pentadiplandra brazzeana* Baillon

A theoretical possibility for the production of brazzein is the extraction from the African fruits of *Pentadiplandra brazzeana* Baillon. The patent from Hellekant and Ming protects among other things the isolation of brazzein from the African fruits and even though the method described is not at industrial scale it is here assumed to be a possible method to produce brazzein (Hellekant and Ming, 1998).

A conceptual process for brazzein production through extraction from the African fruits is proposed and the flowsheet is presented in Figure 4.7.

The production process starts with harvesting the fruits of the plant *Pentadiplandra brazzeana* Baillon. The large scale cultivation of the plant is complicated and it was not possible to find data in literature for the agricultural cultivation of this plant. Therefore, it was not included any water for irrigation of the plants, pesticides or manure. It was just considered that the fruits are harvested from the wild. The content of brazzein in the ripe fruit is described in the range 0.05%-0.2% by weight (Hellekant and Ming, 1998). An average value of 0.125% (w/w) of brazzein was assumed in this environmental assessment.

Brazzein only exists in the soft pulp of the fruit, so the first step is the separation from the seeds which are considerably large. Hence this separation would be easy to perform manually or by mechanic means. Given the large size of the seeds, it was assumed that the mass of pulp is approximately the same as the mass of seeds. The next step is a solid-liquid extraction using an acidic buffer (pH 5) at a ratio of solid:liquid of 1:40 (w/v). Afterwards, there is a filtration and centrifugation to separate the solids from the liquid phase that contains the sweet protein. The proteins are then precipitated with a high salt concentration, which is followed by a gel filtration and a cation exchange chromatography. Afterwards there is an ultrafiltration for concentration and desalting of the solution. Finally, there is another chromatographic step, this time an anion exchange chromatography and the last step is drying. The yield of the purification process is stated by Hellekant and Ming as 36%. And it is assumed that the final purity is 98% as in the previous processes. As in the previous process, process conditions similar to the process for fermentation of *Pichia pastoris* have been assumed unless otherwise stated.

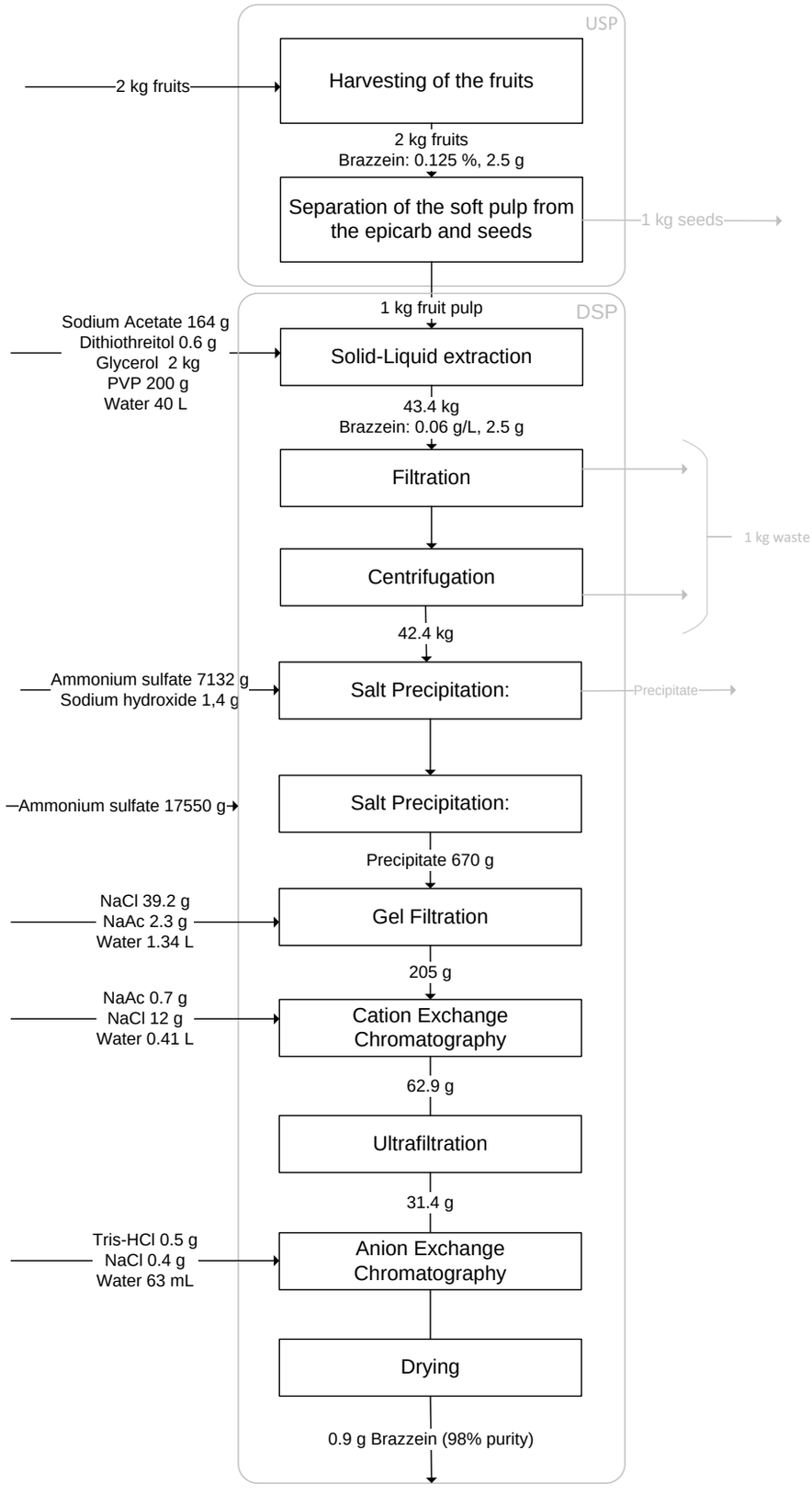


Figure 4.7 Process flowsheet for brazzein extraction from the African fruits of *Pentadiplandra brazzeana.*

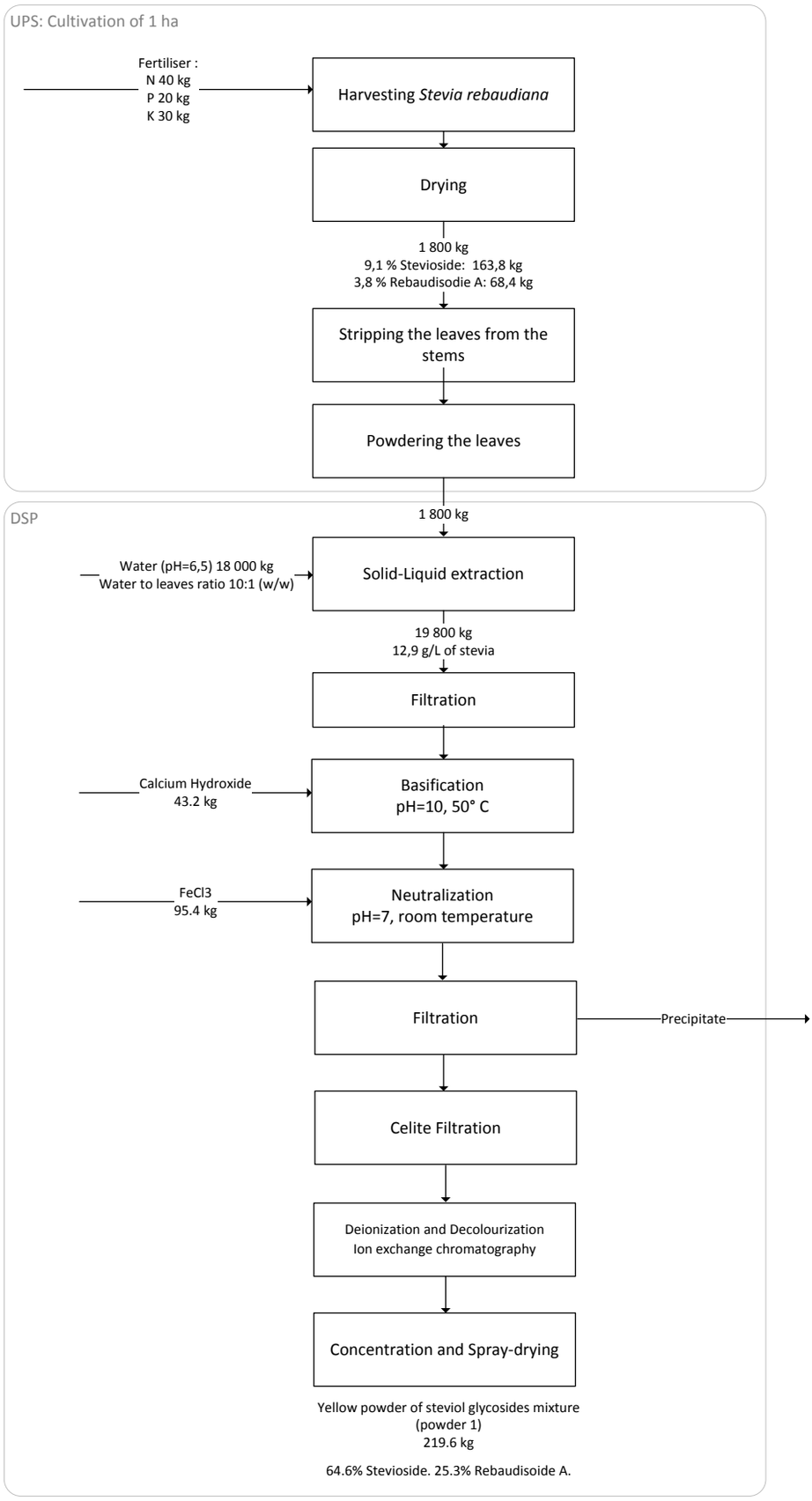
4.5.2.3 Stevia extraction from leaves

Stevia is commercially produced by extraction of the glycosides from the leaves of the plant *Stevia rebaudiana* Bertoni. The process used for the environmental assessment is based on the patent from the company PureCircle that commercializes Stevia (Purkayastha et al., 2012). The process described in the patent fulfills the European requirements to extract stevioside and rebaudioside A with high purity. The process consists of the following main steps – brazzein is extracted with hot water from the dried and powered leaves, then there is a pre-treatment with base and iron chloride, filtration and ion exchange chromatography and the purification consists of several filtration, drying and crystallization steps.

The process flowsheet is sub-divided into two parts which are shown in Figure 4.8.

In the environmental assessment it is also considered the cultivation of the plants. The data for the cultivation of the plants is taken from a report on stevia cultivation from the Rural Industries Research and Development Corporation (RIRDC) and the Australian Government (Midmore and Rank, 2002). The crop yield is in average 1.6-2 tons dried stevia leaves/ha/year. The average value of 1.8 tons/ha was used in this assessment. In the cultivation of one hectare of stevia, 40 kg of nitrogen, 20 kg of phosphorus and 30 kg of potassium are used as fertilizers. Supplementary irrigation is generally assumed to be essential to avoid any water stress on plants unless the growing area has reliable rainfall throughout most of the year. However, it is dependent on the amount of rainfall on the cultivation site. Therefore, water for irrigation as well as herbicides and pesticides were not considered in this analysis, since they are site-dependent.

The plant leaves contain in average 9.1% of stevioside and 3.8% of rebaudioside A (Purkayastha et al., 2012). After harvesting the leaves are dried, separated from the stems and powdered before extraction of the glycosides with hot water. Afterwards, the leaf residues are removed by filtration. The following basification of the solution to a pH of 10 should flocculate unwanted substances, which are removed by filtration. The following step is an ion exchange chromatography that separates the glycosides from remaining substances. Afterwards, a concentration and drying steps originate a yellow sweet powder, which is designated as “powder 1”. This powder is basically a mixture of different steviol glycosides. This powder is then solvated in methanol and filtered. The filtrate contains rebaudioside A and the precipitate contains stevioside. For further purification of the stevioside the precipitate is dried and crystallized with ethanol. A series of filtration, crystallizations and drying steps are taken until stevioside crystals are recovered with a purity of 99.3% and rebaudioside A crystals with a purity of 98.9%. The two glycosides are then mixed in the final product which contains 29% rebaudioside A and 71% stevioside.


UPS: Cultivation of 1 ha
Fertiliser :
N 40 kg
P 20 kg
K 30 kg
Harvesting *Stevia rebaudiana*
Drying
1 800 kg
9,1 % Stevioside: 163,8 kg
3,8 % Rebaudisodie A: 68,4 kg
Stripping the leaves from the stems
Powdering the leaves
1 800 kg
DSP
Water (pH=6,5) 18 000 kg
Water to leaves ratio 10:1 (w/w)
Solid-Liquid extraction
19 800 kg
12,9 g/L of stevia
Filtration
Calcium Hydroxide
43.2 kg
Basification
pH=10, 50° C
FeCl3
95.4 kg
Neutralization
pH=7, room temperature
Filtration
Precipitate
Celite Filtration
Deionization and Decolourization
Ion exchange chromatography
Concentration and Spray-drying
Yellow powder of steviol glycosides mixture
(powder 1)
219.6 kg
64.6% Stevioside. 25.3% Rebaudisoide A.

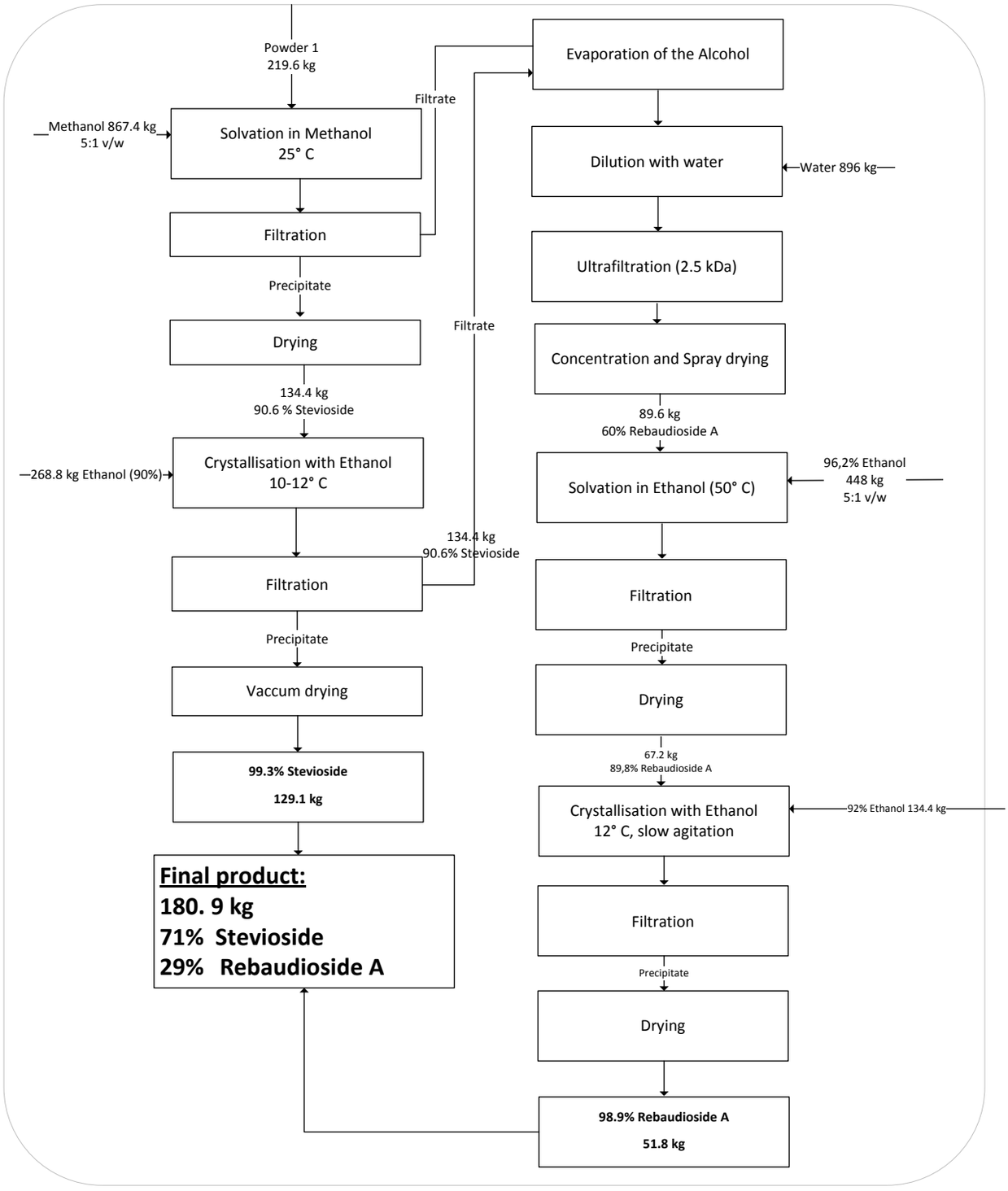


Figure 4.8 Process extraction for stevia extraction from the leaves of *Stevia rebaudiana* Bertoni.

4.5.2.4 Aspartame enzymatic production

There are different methods for production of aspartame. The condensation of the two modified amino acids, phenylalanine and aspartic acid, can be a chemical or an enzymatic reaction (Hanzawa, 2010). In this case an enzymatic production of aspartame was selected.

The data for the mass balances of this process is taken from a patent from the company Ajinomoto Co., Inc., which is one of the major producers of aspartame, and from a synthesis procedure from Lindeberg (Kiyotaka Oyama et al., 1985) (Lindeberg, 1987).

The process flowsheet for the enzymatic production of aspartame is shown in Figure 4.9.

The process starts with the preparation of the two amino acids. L-aspartic acid needs a protection group for the amino group - a Z-protection. So a *N*-benzyloxycarbonyl group is introduced in alkaline media, to form *N*-benzyloxycarbonyl-aspartic acid (ZA). Afterwards, the protected amino acid is washed with an organic solvent, toluene, to remove unreacted compounds. DL-phenylalanine is prepared through esterification in methanol in acid conditions, to form phenylalanine methyl ester (PM). Afterwards, methanol is evaporated through distillation and the amino acid is dissolved in water.

The aqueous solutions of the two protected amino acids are mixed with the enzyme thermolysin and the condensation reaction takes place at 40 °C. The L- form of the methylated phenylalanine, L-phenylalanine methyl ester (L-PheOMe) reacts stereo and regio selectively with the *N*-protected aspartic acid. The reaction product, Z-Aspartame, forms an insoluble addition compound with one molecule of remaining D-PheOMe and precipitates. When the reaction is completed, the Z-Aspartame-D-PheOMe addition compound is collected as a solid product and thermolysin is recovered from the solution. D-PheOMe is then separated from Z-Aspartame by acid washing with hydrochloric acid. The protection group, Z- is removed from Z-aspartame by hydrogenolysis. Finally, aspartame, or α-L-aspartyl-L-phenylalanine methyl ester, is obtained by crystallization and drying. D-PheOMe is recycled after racemization by alkaline treatment and reesterification in methanol (Hanzawa, 2010).

For simplification of the mass balances, the reuse of the enzyme and the Pd/C catalyst used in the hydrogenolysis step was not considered as well as the recovery of D-phenylalanine methyl ester. It was assumed a loss of 10% of product in the purification steps.

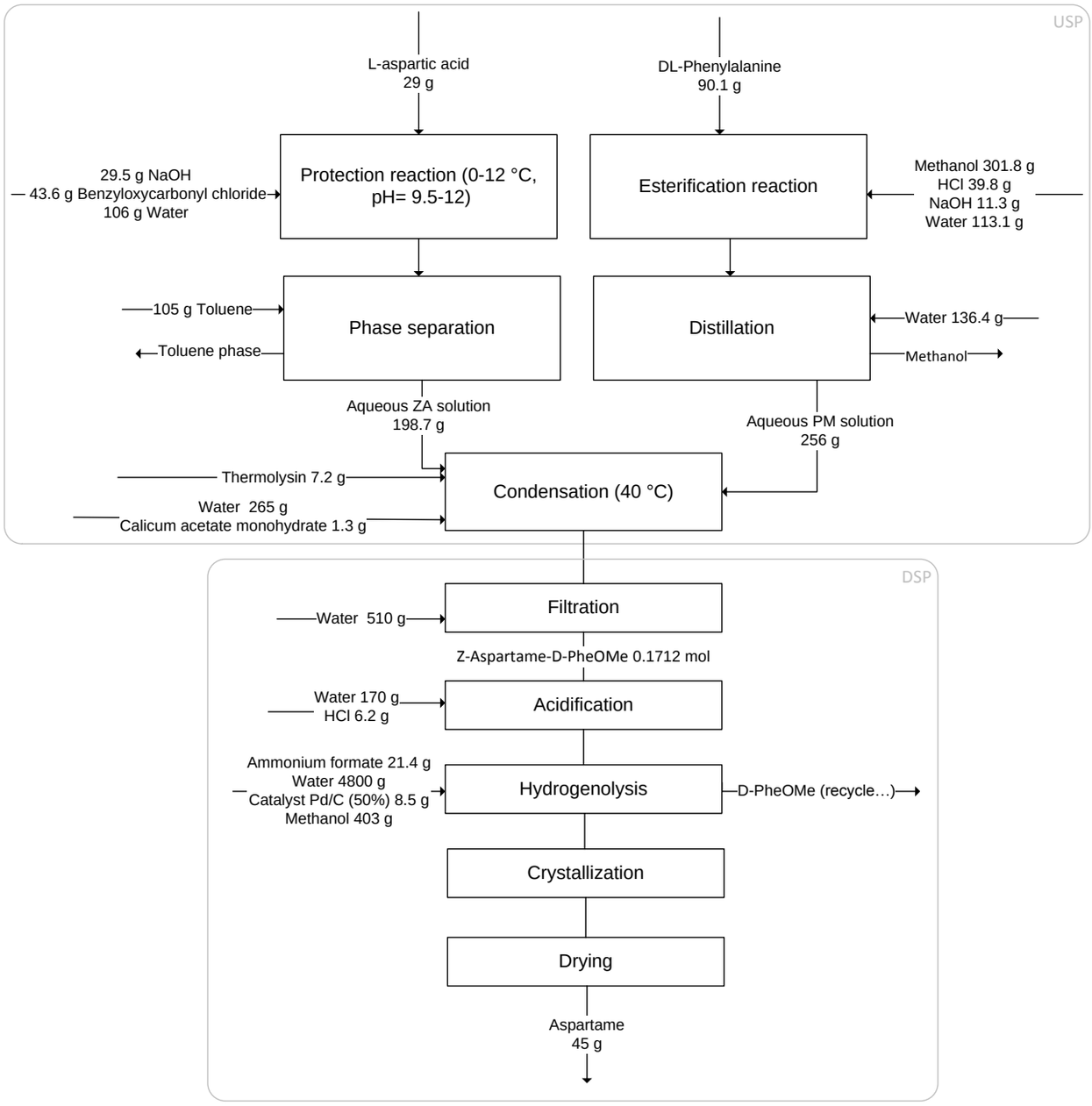


Figure 4.9 Process flowsheet for aspartame production process.

4.5.3 Benchmarking of brazzein fermentation process with similar processes

4.5.3.1 Process Mass Intensity

Since PMI is one of the environmental metrics more widely used, it was calculated for the five production processes previously described and it is shown in Table 4.4.

Table 4.4 Process mass intensity for the five different processes analyzed in the environmental assessment.

	Brazzein fermentation	Brazzein extraction from maize	Brazzein extraction from the African fruits	Stevia production	Aspartame production
Total PMI (kg materials/kg product)	5,647	28,130	76,576	115	155

The PMI values shown in Table 4.4 include water, which is the biggest contributor to total PMI. Water intensity of the different processes will be further discussed in section 4.5.3.3.

From the five different production processes, brazzein extraction from the African fruits of *Pentadiplandra brazzeana* Baillon is the one that shows the highest PMI, approximately 77 kg of materials are required to produce 1 gram of brazzein. This demonstrates the fact that brazzein extraction from the fruits is not an economically or environmentally feasible process, as it was expected. Furthermore, the process for brazzein extraction from the fruits is only a conceptual process, where all the data used in the assessment comes from the patent describing the analytical procedure to isolate brazzein. This analytical procedure did not go through any development or optimization, so it was expected to have a very high PMI for this process.

The second highest PMI is for brazzein extraction from maize, with a total PMI of approximately 28 kg/g. This process is at laboratory scale like brazzein fermentation, so one can say that they are in comparable stages of development. But the fermentation process for brazzein production shows a total PMI 5 times lower than brazzein extraction from maize. The fermentation for brazzein production shows a total PMI of 5.6 kg/g, which is high as it would be expected for a process that is still under development at laboratory scale. At this stage of development, the optimization of materials consumption is not a concern as it is when the process is at production scale. However, it also shows how the process of brazzein fermentation still requires some work on process development and optimization. It is not uncommon that fermentation processes for

chemicals production at commercial scale have a total PMI below 100 kg materials/kg product. Some examples are citric acid production, with a total PMI of 17 kg/kg, pyruvic acid production, with a PMI of 53-35 kg/kg depending on the operations used in the downstream processing, or riboflavin with a total PMI of 39 kg/kg. But fermentation processes for recombinant proteins production show a much higher PMI, since the downstream processing is much more complex and consists of several units of operation. One good example is insulin , which shows a PMI of 55 kg/g (Heinzle et al., 2008).

The relation between total PMI and the stage of development of the process is again verified if one looks at the values for PMI of stevia and aspartame production, which show a PMI of 120 kg/kg and 160 kg/kg respectively. These two values are higher than the above mentioned values for fermentation processes for chemicals production but are in any way 50 times lower than the fermentation process for brazzein production. So there is a clear relationship between the development stage of the process and total PMI, which would be expected since processes at commercial scale have gone through an intensive research and development. Clearly it is not appropriate to compare processes in different development stages, they will obviously show values very different for PMI as well as all the other metrics. But on the other hand, how else can the benchmarking of novel natural products be made? The benchmarking of novel products with products that are already at commercial scale can provide a development target for processes that are still under research and development, like brazzein fermentation.

It is also interesting to put these values into context with sucrose production. For processing of sucrose, total PMI is 10-25 kg/kg if water is included and 0.4 kg/kg excluding water (Gerbens-Leenes and Hoekstra, 2009). These values exclude the cultivation of the biomass that is used as a source of sucrose, e.g. sugarcane or sugar beets.

But perhaps a more interesting analysis is to see how much can PMI of the fermentation process decrease if the process is improved. Therefore, a sensitivity analysis on PMI was performed to analyze how much it is affected by the product concentration and the yield of downstream processing and the results are shown in Figure 4.10.

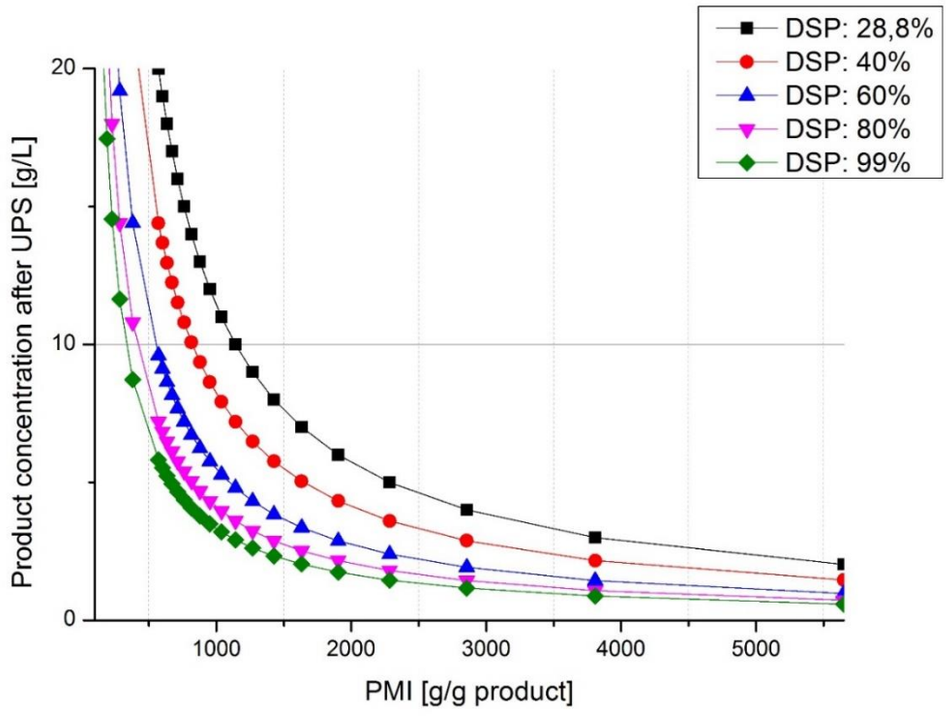


Figure 4.10 Variation of PMI of brazzein fermentation process for different product concentrations and different yields of downstream processing.

It is clear that PMI is very much affected by product concentration in the fermentation broth and by the efficiency of DSP. If during the process development and optimization phase product concentration is increased to 10 g/L and the efficiency of downstream processing is improved to 80%, PMI would be 10 times lower, approximately 400 kg/kg. Even though this is a considerable improvement of the process, it is still not enough to reach PMI values comparable to the commercial processes for stevia or aspartame production, so further optimization would be required. If product concentration could be improved to approximately 35 g/L and downstream processing efficiency kept at 80%, PMI would be 115 kg/kg, which is a comparable value to stevia production.

Hence, one can conclude that both upstream and downstream have a big impact on PMI and it is not enough to improve only one of the parts of the process. Both USP and DSP need to be improved to reach the full potential of the process. The application of a sensitivity analysis proved to be a valuable tool to define targets for process development.

However, the PMI values previously shown do not take into account the relative sweetness of the different sweeteners. Brazzein, stevia and aspartame have different sweetness intensities and are much sweeter than

sucrose. Therefore, to have the same sweetness intensity in the final food products, the necessary amount of for instance brazzein will be different from the required amount of aspartame or sucrose. Hence, a correction of PMI taking into account the relative sweetness of the different sweeteners was done. The sweetness potency of the different sweeteners in comparison to a 2% and a 10% solution of sucrose is shown in Table 4.5.

Table 4.5 Comparison of the sweetness potency of aspartame, brazzein and stevia components (rebaudioside A and stevioside) in relation to sucrose. (SE – Sucrose Equivalent)

Sweetener	Sweetness potency in relation to a 2% solution of sucrose (2% SE)	Sweetness potency in relation to a 10% solution of sucrose (10% SE)	Reference
Aspartame	250	107	(Walters et al., 1991)
Brazzein	2000	500	(Hellekant and Ming, 1998)
Rebaudioside A	320	170	(Mitchell, 2006)
Stevioside	213	190	(Mitchell, 2006)

Process mass intensity of the different processes corrected on the different sweetness intensities is shown in Table 4.6.

Table 4.6 Process mass intensity corrected on sweetness potency for 2% SE and 10% SE (SE – Sucrose Equivalent).

	Brazzein fermentation	Brazzein extraction from maize	Brazzein extraction from the African fruits	Stevia production	Aspartame production
PMI (kg/kg) per sweetness potency in relation to 2% SE	3	14	38	0.5	0.6
PMI (kg/kg) per sweetness potency in relation to 10% SE	11	56	153	0.6	1.4

Taking into account the relative sweetness of the different sweeteners, PMI values are considerably lower. However, PMI for brazzein fermentation is still higher than PMI for stevia and aspartame production. On the other hand, PMI values for brazzein fermentation (3 kg/kg per sweetness potency (for 2%SE) and 11 kg/kg

per sweetness potency (for 10% SE)) are now on the same range as PMI values for sucrose processing (10-25 kg/kg). Taking into account that the process is still under development, once the brazzein fermentation process reaches its full potential PMI values should be even lower. This shows that the fermentation of *Pichia pastoris* for brazzein production can be an environmentally promising option for sweeteners production if it is successfully intensified.

4.5.3.2 Product concentration

The product concentration of the different sweeteners on biomass is shown in Table 4.7, as well as the product concentration before DSP.

Table 4.7 Product concentration on biomass in % (w/w) and in solution before downstream processing in g/L for the different production processes.

	Brazzein fermentation	**Brazzein extraction from maize**	**Brazzein extraction from the African fruits**	**Stevia production**	**Aspartame production**
Product concentration in biomass (% (w/w)	2.2	0.04	0.125	12.9	na
Product concentration before DSP (g/L)	2.02	0.4	0.06	12.9	40

Looking at the product concentration in biomass and in solution before downstream processing it is clear that there is a correlation between product concentration and total PMI. The processes with a lower product concentration in biomass and in the solution before DSP, namely brazzein extraction from maize and from the African fruits, are the ones with a higher PMI. On the other hand, the processes with a higher product concentration show a lower PMI. This again shows the importance of product concentration for both the economy and the environmental profile of a process.

It is also interesting to note that stevia leaves have a quite high concentration of sweetener, which grants a natural advantage to the process for stevia production by extraction from natural sources.

4.5.3.3 Water Intensity

In Table 4.8 is shown the water intensity and the solvent intensity of the five different processes.

Table 4.8 Water intensity and solvent intensity of the different processes.

	Brazzein fermentation	Brazzein extraction from maize	Brazzein extraction from the African fruits	Stevia production	Aspartame production
WI (kg/kg)	4,932	26,101	46,460	105	130
Contribution to total PMI (%)	87	93	61	91	84
SI (kg/kg)	na	na	na	9.2	18.3
Contribution to total PMI (%)	na	na	na	8	11.5

In all the five processes water represents 60-90% (w/w) of all the materials used and is therefore the biggest contributor to total PMI.

Solvent intensity, on the other hand is not significant. The three processes for brazzein production do not involve the use of any solvent besides water. Stevia production has a solvent intensity of 9.2 kg solvents/kg product. The solvents used are ethanol (4.4 kg EtOH/kg product) and methanol (4.8 kg MeOH/kg product). In aspartame production process 18.3 kg solvents/kg product are used. The solvents used are methanol (16 kg MeOH/kg product) and toluene (2.3 kg toluene/kg product). None of the solvents used are highly hazardous. Toluene is classified by McElroy and co-workers as a problematic solvent (McElroy et al., 2015), but it is not used in high amounts (it represents 1.5% of total PMI).

The results clearly show that water contributes in all of these bio-based processes with more than 50% to total PMI. Hence, water consumption should not be underestimated and it should be included in the calculation of PMI. These results highlight the importance of water consumption on bioprocesses. In a world where water scarcity is increasingly problematic, there should be more research focus on water reutilization or reduction/optimization of water consumption in biochemical processes. A bio-based industry can have several environmental benefits in comparison to conventional petrochemical based industry. However, one cannot forget that these bio-based processes have an inherent high consumption of water, in most cases

higher than in conventional chemical processes. If water use is not addressed as a topic of high priority, the bio-based economy could potentially have a negative environmental impact in terms of water consumption.

4.5.3.4 E-factor

E-factor as defined by Sheldon, *i.e.* excluding water, was also calculated and is shown in Table 4.9.

Table 4.9 E-factor for the different processes.

	Brazzein fermentation	Brazzein extraction from maize	Brazzein extraction from the African fruits	Stevia production	Aspartame production
E-factor (kg waste/kg product)	716	2,028	30,115	11	24

All of the processes for brazzein production show a high value for E-factor, as it would be expected given the early stage of development of these processes.

E-factors of stevia and aspartame production processes are in the range of typical E-factors for the fine chemical industry, with an annual production of 10^2-10^4 tons/year (Sheldon, 2011).

It is interesting to compare the E-factor for brazzein fermentation with typical E-factors of fermentation processes. However, very few have been reported. Sheldon reports an E-factor of 1.4 kg/kg for the bulk fermentation of citric acid. E-factors for the fermentation processes for the production of therapeutic proteins (biopharmaceuticals) are much higher. The production of recombinant human insulin has an E-factor of 6000 kg/kg, and this value increases to 50,000 kg/kg if water is included in the calculation (Sheldon, 2011).

4.5.3.5 Origin of the raw materials

It is also interesting to look at the origin of the raw materials used in the processes. The raw materials have been grouped according to their origin and this categorization is shown in Table 4.10.

Table 4.10 Categorization of the origin of the raw materials used in the different processes.

Category	Raw materials
Renewables	Ethanol; glycerol, manure
Mining	P-fertilizer; K-fertilizer; calcium hydroxide ($Ca(OH)_2$); Iron (III) chloride ($FeCl_3$); sodium chloride (NaCl); phosphoric acid (H_3PO_4); monopotassium phosphate (KH_2PO_4); calcium chloride ($CaCl_2$); potassium sulfate (K_2SO_4); magnesium sulfate ($MgSO_4$); lime ($CaCO_3$); sodium hydroxide (NaOH); catalyst Pd/C
Petrochemical	Methanol; N-fertilizer; ethanol; ammonia; sodium acetate; antifoam; polyvinylpyrrolidone; ammonium sulfate; benzyloxycarbonyl chloride; toluene; hydrogen chloride; hydrochloric acid; ammonium formate
Complex substances	Pesticides; L-aspartic acid; DL-phenylalanine; thermolysin

The raw materials have been grouped in four different categories: renewables, which include all the natural resources that can renew themselves naturally over time or all the materials that are produced from renewable resources; mining, which include all the materials that are extracted from ores through mining or derive from products extracted from ores; petrochemical are all the materials that are produced from oil or gas; and finally complex substances are built up from several different materials. Water was not included in this categorization of the raw materials. Since the production of biomass is included in the scope of the assessment, stevia leaves, yeast cell or the African fruits of *Pentadiplandra brazzeana* Baillon are not included as raw materials. Ethanol can be produced by conventional means from petrochemical materials or by fermentation. Therefore, it was included in both categories and in the processes where ethanol is used it was considered that 50% was from renewable sources and 50% from petrochemical sources.

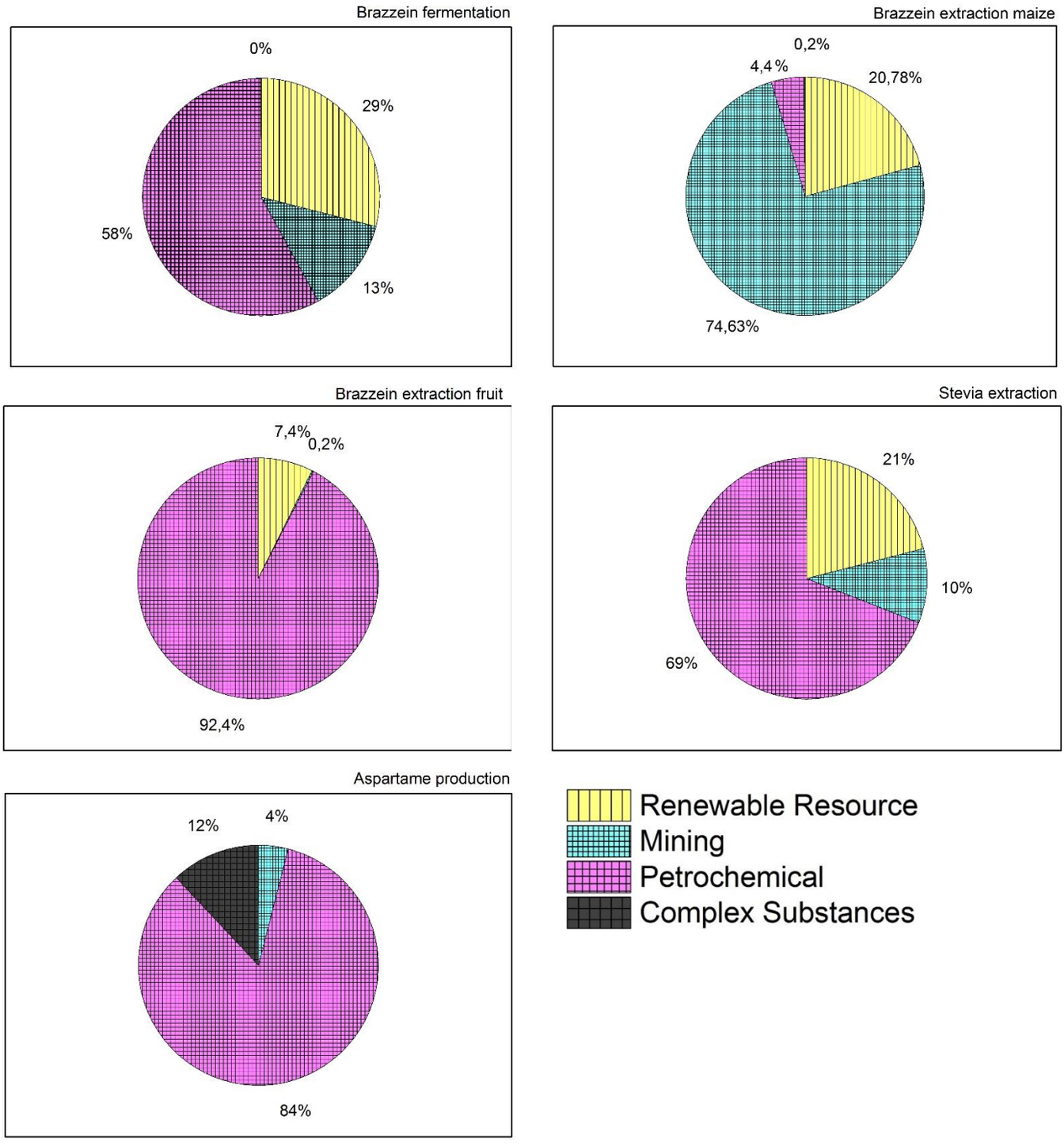


Figure 4.11 Distribution of origin of raw materials of the different processes.

It is interesting to see in Figure 4.11 that even though all these processes are bio-based, the majority of raw materials used are of petrochemical origin. But the fermentation process is the one with a highest percentage of renewable raw materials (29%), mainly due to glycerol that is a by-product of biodiesel production. The high percentage of petrochemicals in the fermentation process comes mainly from the use of methanol as carbon source.

4.5.4 Environmental, health and safety aspects of *P. pastoris* fermentation

In addition to the comparison with other production processes, it was also made an identification of the environmental hotspots of the fermentation process with the aim to draw guidelines for the improvement of the environmental profile. Some recommendations regarding the health, safety and environmental aspects of the process are given in this section.

The procedure that was adopted in (*R*)-perillic acid case-study for assessment of the health, safety and environmental aspects of the reactants and solvents used, was also applied in this second case-study. The flags system adopted by McElroy and co-workers (McElroy et al., 2015) and based on the H-statements of the different chemicals was applied and is shown in Table 4.11.

Table 4.11 Analysis of the health, safety and environmental hazards of the reactants and solvents used in the fermentation of *Pichia pastoris* for brazzein production.

	Green flag	Yellow flag	Red flag
Solvents			
Water	X		
Reactants			
Methanol			
H301: Toxic if swallowed			
H311: Toxic in contact with skin		X	X
H331: Toxic if inhaled			
H370: Causes damage to organs			

The only solvents used in this fermentation process are water-based buffers which gives to this process a "green flag" in terms of solvents use.

From all the chemicals used in the fermentation media, methanol is the only one that shows some concerning H-sentences, namely the H-sentences related to toxicity (H301, H311 and H331) which give a "yellow flag" to

the use of this chemical, and the H-sentence H370 which concerns damage to organs and gives a “red flag” to this chemical. It could be interesting to consider substituting methanol by another carbon source or to use only glycerol as a main carbon source, if cell growth or protein expression are not affected. This substitution would avoid using a toxic chemical and would also considerably decrease the contribution of petrochemical raw materials to the process.

It was also verified if any of the chemicals used in the process are in the SIN List and classified as chemicals of very high concern. Boric acid (BH_3O_3) has been classified as carcinogenic, mutagenic and reprotoxic (CMR) according to Annex VI of Regulation 1272/2008 and is classified as a chemical of very high concern. It is only used in concentrations of nanograms per liter, since it is only added as a trace element to the fermentation. Therefore it should not constitute any danger for the safety of the workers.

4.6 Concluding remarks

The five different processes were compared in terms of PMI, water and solvent intensity, E-factor and origin of the raw materials. Benchmarking the fermentation of *Pichia pastoris* with the other processes for brazzein production (extraction from genetically modified maize and extraction from the African fruits of *Pentadiplandra brazzeana* Baillon) it is clear that the fermentation process is the most promising option.

However when comparing it with the processes for production of stevia or aspartame at a commercial scale, the fermentation process shows a much higher PMI, E-factor and water intensity which would be expected given the early stage of development. In anyway, the benchmarking of the fermentation process with other processes revealed itself as a useful tool since it provided some targets for process development and optimization. Running a sensitivity analysis on the process mass intensity of the fermentation process, it was concluded that product concentration in the fermentation broth needs to be increased to 35 g/L and the efficiency of DSP to 80% in order to have a PMI comparable to commercial processes.

Looking at the origin of the raw materials and the EHS aspects of the chemicals used, some suggestions for improvement of the environmental profile of the process could also be drawn. Methanol is a toxic chemical and contributes largely to the high proportion of petrochemicals used in the process (58%). Therefore, substituting it by another carbon source such as glycerol could be an interesting option. On the other hand, glycerol can be produced from propylene or can be a waste product from biodiesel industry. From an environmental point of view it would be preferable to use a raw material that is a waste from another

industry. The best practice is to make a comparison of different sources of the raw materials and choose the one that is preferable from an environmental point of view. This is however a laborious task.

Some of the environmental metrics that were used in this case-study are different from the (*R*)-perillic acid case-study. Since the process is a fermentation and not a biocatalytic reaction it makes no sense calculating atom economy, reaction mass efficiency or any of the reaction-related metrics. In this case there is not a single reaction for product formation. The product is a protein that is built from several molecules in the cell. Developing a set of metrics specific for the environmental assessment of fermentations would be very interesting and a valuable tool, but it is also quite a challenge given the complexity of all the reactions involved.

It is very important to note that the quality of data is critical for an environmental assessment. In this assessment it was necessary to make some assumptions for the modelling and inventory of the production processes due to lack of data, and the influence of these assumptions on the final results should be analyzed in an uncertainty analysis. Furthermore, the fermentation of *Pichia pastoris* for brazzein production is still at laboratory scale and further development and optimization will still be performed. This final process optimization, through the adjustment of the operation conditions and equipment sizes, has also an influence in the environmental performance (Brunet et al., 2012).

Since assumptions and estimations need to be made in these environmental assessments, the accuracy of the method is limited to a certain degree. These methods for environmental assessment should be seen as a valuable supportive tool for process development and not as a tool to accurately measure waste production or other environmental-related topics.

One could also argue that an economic assessment would be sufficient and there is no need to perform environmental assessments in early stages of development, given the fact that if the economy of the process is improved so is also the environmental profile, e.g. by improvement of PMI. However, the work done in this case-study revealed how an environmental assessment can bring a new perspective and different information relevant for process development, e.g. information on substitution of chemicals.

Likewise, one could argue that if we just apply environmental commonplaces such as reducing water consumption, avoiding the use of toxic chemicals, etc. the environmental profile would anyway be improved without having to do a full environmental assessment involving extensive literature review for collection of data. But the environmental assessment allowed a comparison with other similar processes and allows the research teams working in R&D to see how far their processes are from commercial processes which can help them to define targets for process development and optimization. Obviously, the ideal process would have

an E-factor of zero and a PMI of 1. The goal is always to reduce E-factor and PMI, but how much can they realistically be reduced? Benchmarking processes in early stages of development, such as brazzein fermentation process, puts some realistic targets for E-factor, PMI and other metrics. In this case, for instance, a realistic target for brazzein fermentation process would be a PMI in the range of 100 kg/kg. This is a very valuable tool for scientists, e.g microbiologists or molecular biologists, working in protein and metabolic engineering to improve the expression levels of the proteins in the cells. It puts the values for E-factor and other metrics into context, and it would otherwise be difficult to find a meaning for them.

Part III

Discussion, Conclusion and Perspectives

5. Discussion

This chapter aims to connect the different parts of the thesis, the different tools for bioprocess evaluation, the two different case-studies and the different strategies for bioprocess improvement. General guidelines and recommendations for the successful implementation of processes for production of natural products are presented.

5.1 Methodology for development of processes for the biosynthesis of natural products

Natural products show an incredible potential for use in several industries - pharmaceutical, cosmetics, food, etc. However, its industrial production is hindered by the difficulties in synthesizing these complex molecules. Bioprocesses, both biocatalytic or fermentation processes, appear as a promising possibility for the biosynthesis of these molecules and there are some examples of natural products that have been successfully developed and applied in industrial scale, e.g. artemisin production. Metabolic engineering and protein engineering are areas where great development is being achieved and offer great potential for the biosynthesis of natural products.

However, the development of these processes is rather complex and requires a holistic view of the several limitations and challenges of these processes, from a production host cell and/or biocatalyst, reaction and process perspectives.

In this thesis two fundamentally different case-studies were assessed. The first case-study corresponds to a biocatalytic multi-step reaction for the production of a small molecule, (*R*)-perillic acid which intends to be used in cosmetic industry and could also have potential uses in pharmaceutical or food industry. The second case-study corresponds to a fermentation for recombinant protein production. The recombinant protein, brazzein is intended to be used in food industry. Therefore, this thesis offers a comprehensive tool for the development of natural products by covering different types of bioprocesses, different types of natural products and different sectors of chemical industry. As a result of the work developed for both case-studies, a general methodology for the development of bioprocesses for natural products production was developed and it is shown in Figure 5.1.

This methodology is sub-divided into three parts. The first part corresponds to a route selection/process screening of the different options for production of a given natural product. It is generally assumed that extraction from natural sources is not an economically feasible or sustainable alternative. However, that is not always the case and stevia is a very good example of a process where extraction is feasible. The economic feasibility of extraction is dependent on the amount of natural product existent in the natural source. In this route selection all the possible routes for production of a given natural product must be reviewed and there might be several possibilities for chemical synthesis or biosynthesis using different starting materials. This literature review is followed by the mass balances and an environmental assessment of all the possible options. In the environmental assessment, an analysis of the EHS hazards of all the chemicals used in the chemical inventory of each option is required as well as the calculation of some key green chemistry metrics. The green chemistry metrics are however dependent on the type of molecule being produced. If the product is a small molecule produced in a single or multi-step reaction catalyzed by a chemical catalyst or a biocatalyst, as it is the case for (*R*)-perillic acid, reaction-related metrics (AE, RME and CE) should be used for comparison of the two synthetic routes. But these metrics do not make sense for assessment of large molecules that are built up in several steps of a metabolic pathway, e.g. brazzein. In this case and also when analyzing the extraction from natural sources, process-related metrics such as PMI or E-factor should be preferred.

In case route selection shows that the biosynthetic route is the most promising one, an *in silico* evaluation of this route should be done (part 2 of the methodology) which leads to the identification of the process bottlenecks and strategies for its improvement. These strategies, both from a process/reaction and biocatalyst or host cell perspectives, should then be tested and further explored in the laboratory (part 3 of the methodology). The laboratory work is an essential part of the development of a bioprocess and it should not be underestimated or undervalued. But an *in silico* evaluation of the bioprocess prior to experimental work can guide and focus the experiments on the most critical bottlenecks.

The novelty of this thesis lies on this systematic procedure for route selection and process screening, as well as putting the biosynthesis of natural products into context in relation to demands of an industrially feasible process. There are several novel bioactive natural products with very interesting properties being discovered. But the path to commercialization is rather long and there is not a straightforward procedure for process design and process development of these novel products. Developing novel natural products until commercialization is a challenge faced by many small-medium sized biotechnology enterprises and this thesis tries to understand how these bioprocesses can be developed and offer some guidelines for process development.

Moreover, it reports the environmental profile of bioprocesses for natural products production, which is not often found in scientific literature. The early-stage environmental assessment that was applied in both case-studies can give very meaningful insight into process development.

Besides the environmental assessment, different tools for exploring the economy of a process are described throughout the thesis. Calculating process metrics, such as product concentration, space-time yield, biocatalyst yield or reaction yield, is a simple tool that does not require a big amount of data. But it can give very meaningful information on the process, which can guide research and considerably reduce development time. Hence, it is recommended to calculate process metrics at the start and throughout process research and development.

But setting targets for the process metrics for product and process development can be quite challenging, especially for novel products, such as (*R*)-perillic acid and brazzein. The demands on process metrics are dependent on the product annual production and price. Bulk chemicals, with an annual production in the range of 10^4-10^6 ton/year and a very low selling price (in the range of 1 €/kg) have much tighter demands on process metrics than for instance pharmaceutical chemicals with an annual production of 10-10^3 ton/year and a selling price that can be higher than 100 €/kg. With a selling price as low as 1 €/kg the profit margin is very low, therefore the process needs to be fully optimized in terms of reaction yield, product concentration, space-time yield or volumetric productivity and/or biocatalyst yield. But for these new natural products it can be a challenge to define a reasonable annual production and selling price. Since there are no alternative processes commercially implemented and competing with these products, there is more freedom to set the selling prices. But this freedom is always limited by the market where the product is to be inserted. In the case of these two products, it would be expectable that the fact that they are produced by biotechnological means gives some advantage for product marketing and influence positively the selling price. But overall it can be a challenge to define targets for process research and development when there are so many variables influencing annual production and selling price. This thesis proposes a methodology that can help setting a price for novel natural products and guide process development for novel products without a fixed selling price or identical competitors in the market.

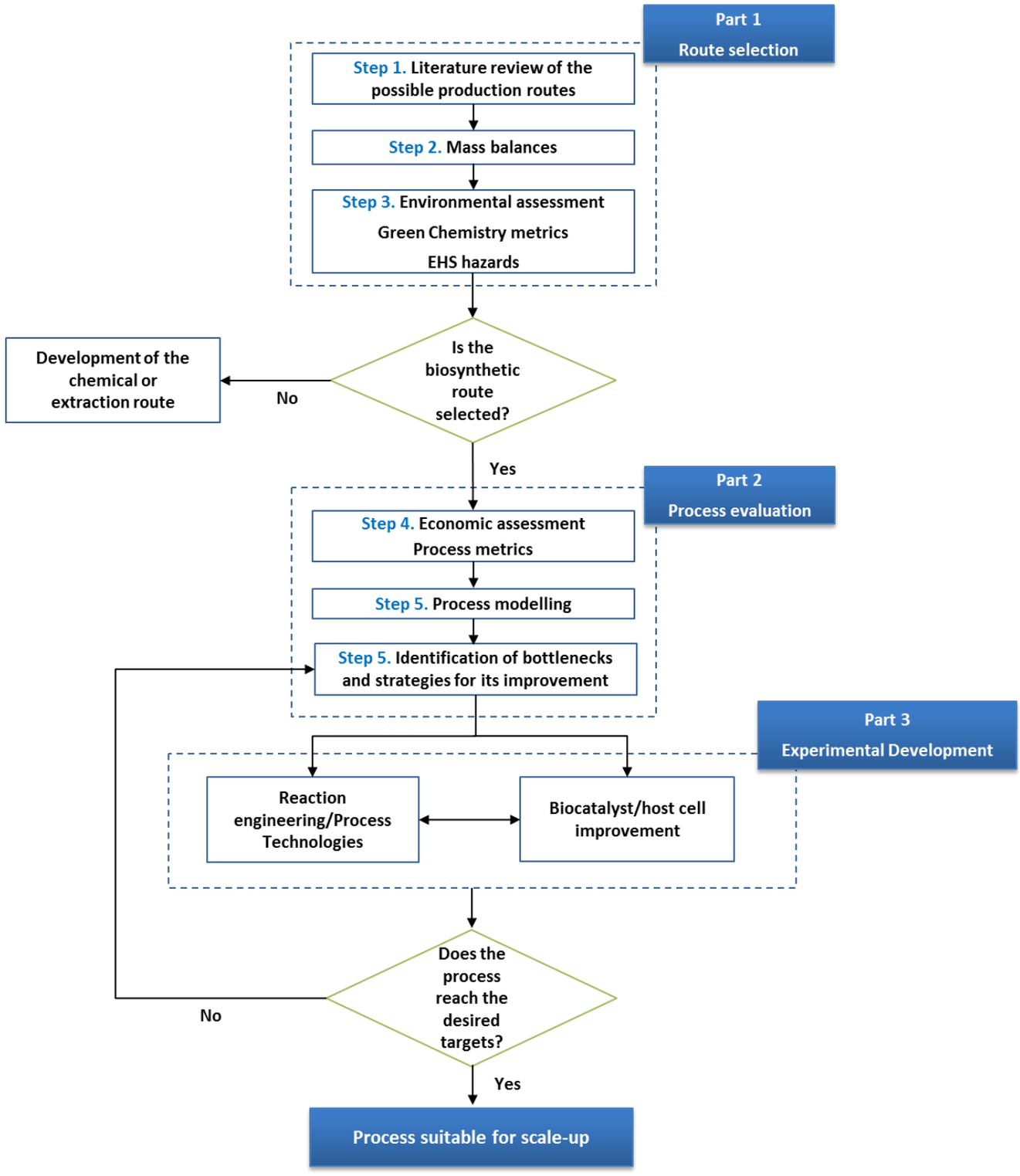


Figure 5.1 General methodology for the development of biosynthetic processes for natural products production.

Scientific work on the development of novel biosynthetic routes for natural products has been widely reported and advances on synthetic biology promise very exciting developments in this field. However, if the commercial implementation of these bioprocesses is intended, the economic demands of an industrial chemical process should be kept in mind throughout the developments stages. This thesis attempts to show how the successful implementation of a bioprocess for production of natural products is dependent on a fundamental knowledge of the biocatalyst or production host cells, reaction and process constraints that strongly influence the process viability. The application of an *in silico* process analysis can be of great benefit in guiding experimental work and suggesting guidelines for improvement of the bioprocess aiming at an industrial scale implementation of the process. The proposed systematic assessment can support the scale-up procedure and also shorten the development time by the early identification of potential bottlenecks and limitations (Heinzle and Hungerbuhler, 1997).

The development of bioprocesses for biosynthesis of natural products is a complex task, with several different bottlenecks which in most of the times requires improvements both from the biocatalyst and/or host cell and the process. Therefore, different areas of expertise are required (microbiology, protein engineering, metabolic and genetic engineering, process and chemical engineering, etc.) and the teams working in the development of these bioprocesses should be multi-disciplinary. Communication can be quite a challenge but looking at these processes from different perspectives can be the key to success.

5.2 Case-study I: Bioconversion of (*R*)-limonene to (*R*)-perillic acid by a whole-cell biocatalyst

The first case-study was the most explored in this thesis, where the whole methodology presented in Figure 5.1 was applied. In first place, the biosynthetic route for (*R*)-perillic acid production was benchmarked with a chemical synthetic route, which showed that the biocatalytic route is the most interesting one, both from an economic and environmental perspectives, to be further explored. Following this route selection, the bioconversion of (*R*)-limonene to (*R*)-perillic acid by a whole-cell biocatalyst was evaluated in an economic assessment, where key process metrics were calculated, a process model was built and a sensitivity analysis on some of the process metrics was run, which allowed the identification of the main bottlenecks of the process and different strategies for its improvement.

Calculating the key process metrics proved to be a useful tool, but it only gave information on how far the process still is from economic feasibility and industrial implementation. It did not give any insight or

guidelines on process development, meaning that it failed to rank and prioritize the different metrics. Process modelling and sensitivity analysis of the key process metrics was a very useful tool to further explore the different metrics and prioritize them.

The results emphasized that space-time yield is a metric of high relevance in whole-cell processes. This metric is very complex and it is influenced by several different parameters, which can be improved in many different ways. Whole-cell biocatalytic processes are extremely complex systems, where several different parameters are interconnected and correlated. And addressing one parameter can have positive effects in one bottleneck but it can actually aggravate others.

The overall methodology followed was a valuable tool to identify targets for improvement of the biocatalytic process, which should result in a reduced process development time. Improving the economy of the process should also be translated in terms of environmental benefits, since economic and environmental sustainability are interconnected.

Unfortunately, it was not possible to experimentally quantify any improvement on space-time yield due to a lack of stability of the system. In several fermentation batches no (*R*)-perillic acid production was observed, which revealed a lack of consistency of the strain used. Screening of a different host should also be investigated, although *Pseudomonas sp*. has an inherent competitive advantage which is its natural tolerance to solvents. (*R*)-Limonene bioconversion to (*R*)-perillic acid has also been demonstrated in yeast *Yarrowia lipolytica* (Ferrara et al., 2013), *Mycobacterium sp.* HXN-1500, *Rhodococcus sp.* ALK2-C7 (Duetz et al., 2004), recombinant *E. coli* with cym genes from *Pseudomonas putida* F1 (Mars et al., 2001) and *Pseudomonas gladioli* (Cadwallader et al., 1989), but all of them with lower productivities than *P. putida* GS1. Other oxyfunctionalizations of (*R*)-limonene with several different strains were reviewed by Duetz and co-workers (Duetz et al., 2003).

The fact that the strain used in this bioprocess is a wild-type strain also imposes some constraints in the process, since wild-type enzymes are in most cases far from being applicable in industrially relevant conditions. Product and substrate inhibition at high concentrations is one of the problems they often face. Process technologies, such as ISPR or ISSS can partially solve these problems but only to a certain extent. There is a limit on how much a process can be improved solely by process technologies and it might not be enough to achieve commercial production. Most examples of biosynthetic processes for the production of natural products that have achieved commercial scale include genetic improvement of the biocatalyst and/or strain (Paddon and Keasling, 2014). But on the other hand, the use of a GMO strain might also have some implications particularly on the marketing and consumer's acceptance of the product.

5.3 Case-study II: Fermentation of recombinant *Pichia pastoris* for brazzein production

In the second case-study only the first part of the proposed methodology, represented in Figure 5.2, was applied. In case-study II, referring to the production of brazzein by fermentation of *Pichia pastoris,* only the benchmarking of the biosynthetic route with other possible routes was addressed and the process was not further evaluated or developed.

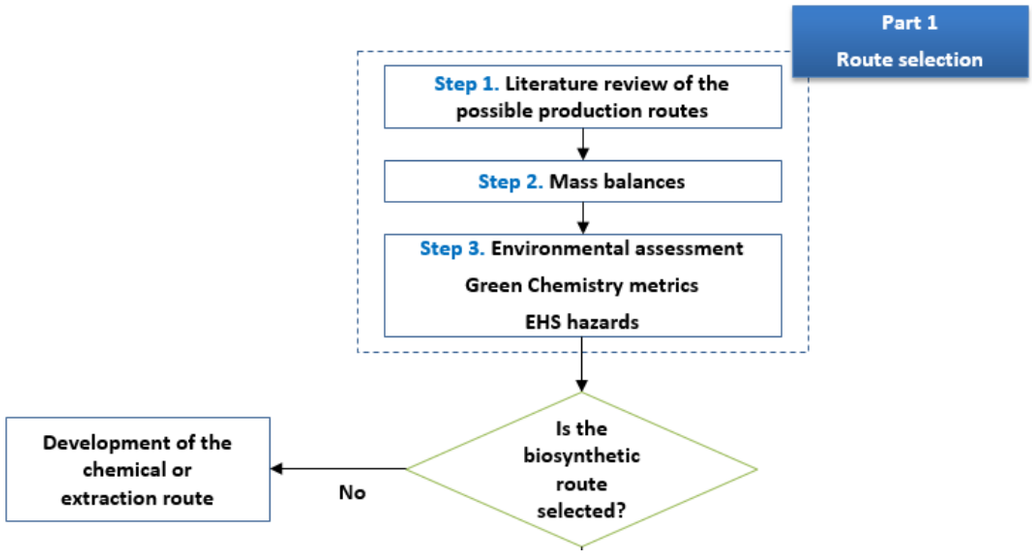


Figure 5.2 Part 1 of the proposed methodology for the development of biosynthetic processes for natural products production.

Besides benchmarking of different routes for brazzein production, the fermentation of recombinant *Pichia pastoris* was also benchmarked against processes for production of different sweeteners, namely stevia and aspartame. Even though these two processes are in different development stages than the fermentation process and are already implemented in industrial scale, using them as benchmarks allowed the definition of development targets for the teams working in process R&D. For instance, if brazzein aims to be environmentally and economically competitive with stevia, PMI of the fermentation process needs to be reduced to a value in the range of 100 kg/kg, which requires a product concentration of 35 g/L and a downstream processing efficiency of 80%. This means that product concentration achieved in the fermentation needs to be increase by approximately 17.5 fold and DSP efficiency needs to be improved from

approximately 30% to 80%. These might seem very ambitious goals, but setting this type of targets can guide and focus research, which can ultimately result in shorter development time.

The results obtained from the assessment of this case-study illustrate the importance of addressing environmental issues from an early time point on process research and development. Even though economics is what drives process development, an environmental assessment can also be meaningful and give valuable insight for process research and development. For instance, an assessment of the EHS issues of the chemicals used can give suggestions for chemicals substitution that can greatly benefit the environmental profile of the bioprocess. And this type of questions need to be addressed in an early stage of research and development, otherwise it will be costly to make any change in the process.

Reporting environmental data, such as PMI, RME or E-factor, of processes at lab-scale research can also benefit process development and research since it can provide some benchmarking examples for researchers working on similar processes. This thesis reports environmental data for two bioprocesses for biosynthesis of natural products, which is something that is not often seen in scientific literature.

The methodology followed and the metrics used to assess the environmental profile of this bioprocess proved to be simple and easy to be applied by any researcher, even researchers who are not familiar with green chemistry metrics or other tools for environmental assessment of bioprocesses in general. A full LCA is not an appropriate method for environmental assessment of bioprocesses in early stages of research since it is too complex and requires a lot of data that is difficult to obtain in early stages of research. The strength of a LCA is the fact that it provides a holistic view of the environmental profile of a given product, taking into account the raw materials production, transportation and the product use and disposal. However, from a process development perspective that might not provide any meaningful information. Very often, a full LCA "dilutes" the environmental profile of the specific process under development, since its emissions or waste produced are insignificant when compared to energy production processes or transportation of the raw materials. Therefore, in general terms LCA is not recommended for processes under development. It does not support the process developer and it is rather complex and dependent on the experience of the user.

5.4 General recommendations for implementation of processes for the biosynthesis of natural products

Estimates for the total number of living species ranges from 2 million to 100 million. Therefore, the number of natural products and enzymes remaining to be discovered is enormous and there is a great potential for discovery of molecules with very interesting properties. Synthetic biology shows the potential to map the metabolism of most species and a library of biochemical transformations could be a magnificent tool for the design and generation of new products. Just as organic chemists currently plan the total synthesis of a target molecule using established reagents and reactions, synthetic biologists might be able in a near future to use a vast array of enzymes to rationally synthesize very complex molecules. Directed evolution of enzymes or ground-breaking gene editing technologies, such as CRISPR-Cas9, can greatly favor the biosynthesis of novel natural products. The resources for natural products discovery are so vast that it is likely to see very exciting discoveries in the field of biosynthetic production of natural products (Li and Vederas, 2009).

However, the development path until industrial scale and full commercialization is rather long. This thesis proposes a methodology for addressing economic as well as environmental issues from an early stage of research and development that can greatly benefit the implementation of more of these bioprocesses for production of natural products. And knowledge sharing between different fields, such as synthetic biology and chemical/biochemical engineering, is also crucial for a successful and fast development of these biosynthetic processes.

6. Conclusions

Natural products show very interesting properties for use in several sectors of chemical industry, namely pharmaceutical, food or cosmetics. However, they are extremely difficult to synthesize given their complex structure. Synthetic biology offers tools with great potential for the synthesis of natural products and many examples of biosynthesis of natural products, either by fermentation of recombinant strains or biocatalytic reactions, have been reported in literature. But further development to industrial scale and commercialization requires high yields, productivities and product concentrations. The intensification of these bioprocesses is not straightforward and it is often a time-consuming process.

The main goal of this thesis was to establish a methodology for benchmarking of processes for the biosynthesis of natural products that can assist the development of these processes. The work developed in this thesis allowed drawing some general conclusions regarding the biosynthesis of natural products:

- In contrast to other molecules, natural products can be produced by different means – extraction of natural sources, chemical synthesis or biosynthesis. Route selection should be the first step of development of a process for natural products production.
- In case the biosynthetic route is the most promising option for production of a certain natural product, an *in silico* evaluation of the economic and environmental profile of the process can help setting targets for process development and identify the main bottlenecks of the process.
- The proposed methodology demonstrated that that space-time yield was the biggest economic constraint in the first case-study – the bioconversion of (*R*)-limonene to (*R*)-perillic acid by *Pseudomonas putida* GS1. A 4-fold improvement of space-time yield could result in a reduction of 40% on operation costs, which is a very significant reduction. The increase of space-time yield would also result in an improved environmental profile of the process given the interconnection of economic and environmental performance.

  Substrate loss by stripping on the gas stream and a reduction of reaction rate throughout reaction time due to product inhibition were identified as some of the causes for a low space-time yield.
- In the second case-study, the fermentation of recombinant *Pichia pastoris* for brazzein production was benchmarked with the production processes of two other sweeteners, stevia and aspartame, from an environmental perspective. The environmental assessment proved to be a valuable tool to define targets for the development of the fermentation process. A sensitivity analysis on some process parameters showed that if product concentration is increased to approximately 35 g/L and

DSP efficiency to 80% a PMI of 115 kg/kg could be achieved, which would make the fermentation process for brazzein production competitive with stevia production.

- The *in silico* benchmarking and evaluation of biosynthetic processes for production of natural products is a valuable tool to define a selling price for a novel product without an identical competitor in the market.

7. Future work

To further explore the process development of biosynthetic processes for natural products production, some suggestions that would improve this thesis are presented. Guidelines for the development of both case-studies are followed by some general guidelines for the development of processes for the biosynthesis of natural products.

Bioconversion of (*R*)-limonene to (*R*)-perillic acid by a whole-cell catalyst

- The reaction system should be further explored experimentally in order to understand the reason(s) for the reduction in reaction rate that was observed. There are a lot of factors possibly influencing the reaction rate (cofactor regeneration problems, loss of enzyme activity, transport of substrate and product across the cell membrane, etc.) and they should be tested experimentally.
- The operating mode of the biocatalytic reaction should be further explored.
- The ISPR operating mode should be further developed.
- The application of a second organic phase that can be used as a substrate reservoir should be tested. This could diminish the substrate loss through stripping on the gas phase and potentially also enable ISPR. Examples where this was successfully applied are available in literature for similar systems (Schewe et al., 2009). Cornelissen and co-workers showed an improvement in productivity when running the bioconversion of (*S*)-limonene to (*S*)-perillyl alcohol by a recombinant *Pseudomonas putida* strain in a two-liquid-phase system (Cornelissen et al., 2011).
- Since the biocatalyst is a whole-cell there might be some transport limitations of the substrate and/or product across the cell membrane. The reaction rate inside the cell might actually be higher than what was measured experimentally. If there are transport limitations in the membrane a lot of product might be held inside the cell. This should be tested by disrupting the cells after the bioconversion and measure (*R*)-perillic acid concentration in the cell lysate.
- The process model could be improved with real price quotes from suppliers. Since facility-dependent costs were one of the biggest contributors to the costing of the first case-study, it would be interesting to get a real quote from a manufacturing facility for renting their equipment, thereby improving the accuracy of the economic assessment and sensitivity analysis.

Fermentation of recombinant *Pichia pastoris* for brazzein production

- In this case-study both upstream and downstream processing need to be improved. Since it is a recombinant strain that is used further metabolic engineering of the strain needs to be done in order to increase the product concentration in the fermentation.
- Optimization of the operating conditions in DSP should be done in order to improve the efficiency of DSP. Design of experiments is a tool that can help and accelerate this experimental work.

General guidelines for the development of processes for the biosynthesis of natural products

- The development of more *in silico* tools for predicting properties at operating conditions could reduce experimental work and facilitate and accelerate process development. Natural products are complex molecules, whose properties, e.g. solubility, pK_a or log P, are not easily found in scientific literature. Experimentally measuring all these physicochemical properties is a time-consuming process. Therefore, having predictive tools for prediction of pKa, partition coefficients, etc. could reduce experimental work and assist process design and development.
- The application of design of experiments tools can also be a valuable way to acquire more process knowledge and could benefit the development of these biosynthetic processes.

8. References

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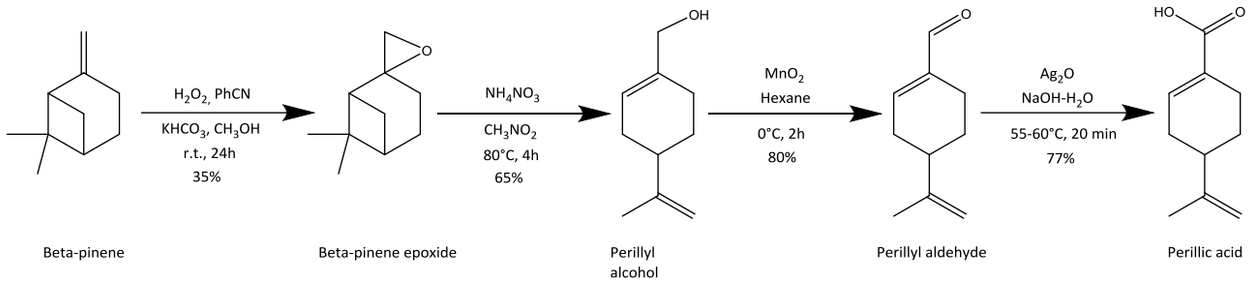
Appendices

Mass balances for the chemical route for (*R*)-perillic acid synthesis from β-pinene



- The data on reagents consumption for the chemical synthesis of perillic acid was based on (Wang et al., 1993).

Reaction Step 1

Reaction yield: 35% (based on benzonitrile)

Input	Mass (g)	Output	Mass (g)
β-pinene	5.88	β-pinene epoxide	2.24
H_2O_2 (30%)	9.52	Unreacted reagents	17.44
Benzonitrile (PhCN)	4.28		
Sum	19.68	**Sum**	19.68

Reaction Step 2

Reaction yield: 65%

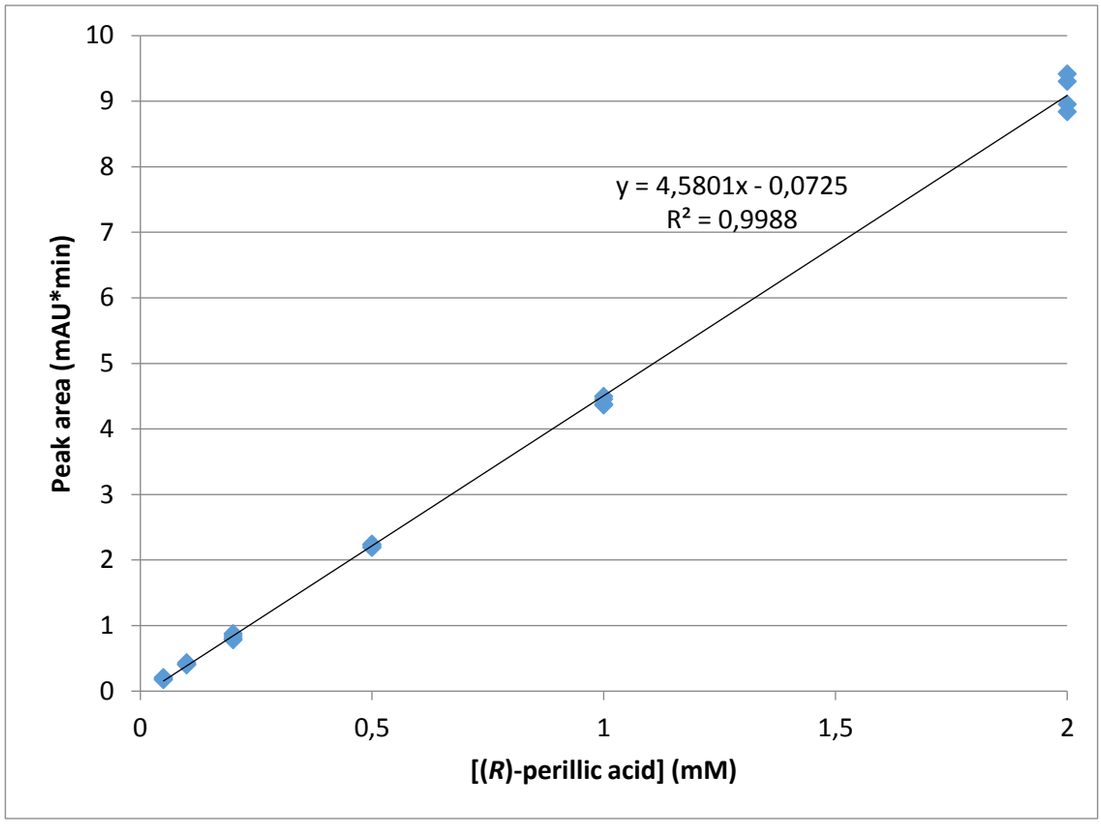
Input	Mass (g)	Output	Mass (g)
β-pinene epoxide	2.24	Perillyl alcohol	1.52
NH_4NO_3	0.15	Unreacted reagents	0.87
Sum	2.39	**Sum**	2.39

Reaction Steps 3 and 4

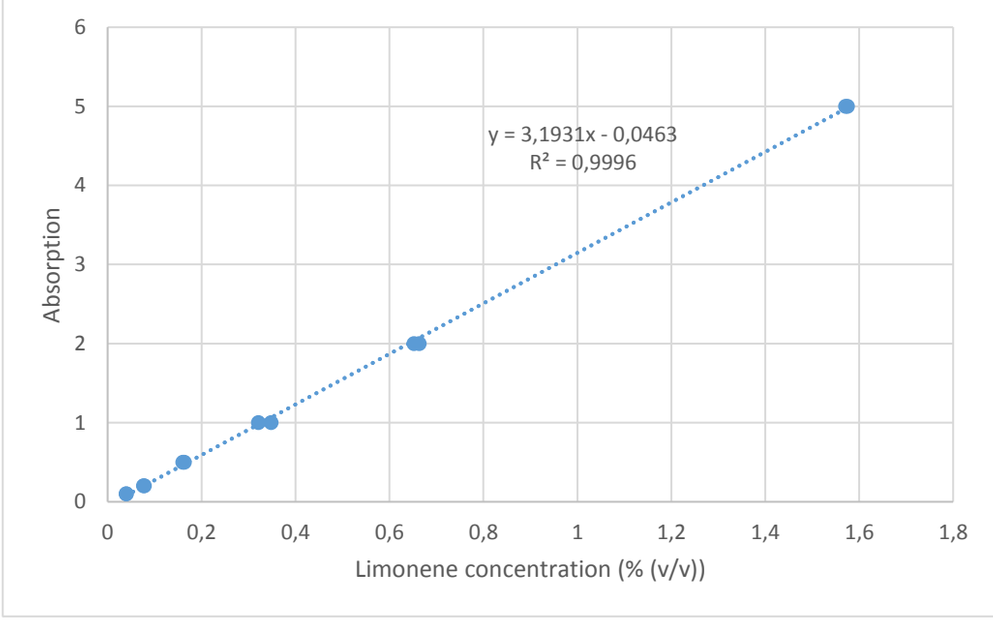
Overall reaction yield of 77% from perillyl alcohol

Input	Mass (g)	Output	Mass (g)
Perillyl alcohol	1.52	Perillic acid	1.28
MnO_2	17.4	Unreacted reagents	17.64
$AgNO_3$	1.7		
NaOH (aq.)	0.40		
Sum	18.92	**Sum**	18.92

Calibration curve for HPLC measurements of (*R*)-perillic acid



Calibration curve for the UV-VIS measurements of (*R*)-limonene at 254 nm



Mass balances for the processes assessed in case-study II

1. Brazzein extraction from the fruits of *Pentadiplandra brazzeana* Baillon

Separation of the pulp from the epicarp and seeds and solid-liquid extraction

Input	Mass (g)	Output	Mass (g)
Fruits of Pentadiplandra brazzeana	2,000	Solution after the extraction	43,364
Sodium acetate	164	Fruit pulp (Waste)	1,000
Dithiothreitol	0.6		
Glycerol	2,000		
Polyvinylpyrrolidone (PVP)	200		
Water	40,000		
Sum	44,364.6	**Sum**	44,364

Purification of the extraction solution

Input	Mass (g)	Output	Mass (g)
Extraction solution	43,364	Product	0.9
Ammonium sulfate	24,682	Waste	70,914.6
Sodium hydroxide	1.4		
Sodium chloride	51.6		
Sodium acetate	3		
Tris hydrochloride	0.5		
Water	1,813		
Sum	70,915.5	**Sum**	70,915.5

2. Brazzein fermentation

Fermentation

Input	Mass (kg)	Output	Mass (kg)
Glycerol	770.7		
Methanol	2,313		
Monopotassium phosphate	236	Fermentation broth	10,304.6
Ammonium sulfate	27.5		
Calcium chloride	5.5		
Potassium sulfate	78.7		
Magnesium sulfate	64.4		
Copper sulfate	$1.1x10^{-2}$		
Sodium iodide	$4.4x10^{-4}$		
Manganese sulfate	$1.65x10^{-2}$		
Sodium molybdate	$1.1x10^{-3}$		
Boric acid	$1.1x10^{-4}$		
Calcium sulfate	$2.75x10^{-3}$		
Cobalt chloride	$2.75x10^{-3}$		
Zinc chloride	$3.85x10^{-2}$		
Iron sulfate	0.121		
Biotin	$1.1x10^{-3}$		
Sulfuric acid	$9.9x10^{-6}$		
Inoculum	500		
Phosphoric acid	8.6		
Ammonia	154		
Antifoam (Propylene glycol)	5		
Water	6,141.2		
Sum	10,304.6	**Sum**	10,304.6

Cell removal

Input	Mass (kg)	Output	Mass (kg)
Fermentation broth	10,304.6	Cells	4,120.5
		Supernatant	6,184.1
Sum	10,304.6	**Sum**	10,304.6

Clarification

Input	Mass (kg)	Output	Mass (kg)
Supernatant	6,184.1	Waste	156.8
Water	250	Clarification solution	6,275.3
Sum	6,434.1	**Sum**	6,434.1

Concentration

Input	Mass (kg)	Output	Mass (kg)
Clarification solution	6,275.3	Waste	4,215.7
		Concentration solution	2,059.6
Sum	6,275.3	**Sum**	6,275.3

Buffer exchange

Input	Mass (kg)	Output	Mass (kg)
Concentration solution	2,059.6	Waste	18,094.9
Sodium acetate	14.76	Solution	2,032
Sodium chloride	52.56		
Water	18,000		
Sum	20,126.9	**Sum**	20,126.9

Chromatography

Input	Mass (kg)	Output	Mass (kg)
Solution	2,032	Waste	6,062.4
Sodium acetate	4.2	Elution solution	1,300
Sodium chloride	126.1		
Water	5,200		
Sum	7,362.4	**Sum**	7,362.4

Freeze Drying

Input	Mass (kg)	Output	Mass (kg)
Elution solution	1,300	Product	6
		Waste	1,294
Sum	1,300	**Sum**	1,300

3. Brazzein extraction from GMO maize

Cultivation of 1 hectare of maize

Input	Mass (kg)	Output	Mass (kg)
Nitrogen fertilizer	30	Maize	3,500
Lime	400		
Manure	170		
Pesticides	0.9		
Sum	600.9	**Sum**	3,500

Dry milling

Input	Mass (kg)	Output	Mass (kg)
Maize	3,500	Maize middlings (waste)	2,800
		Germ-rich fraction	700
Sum	3,500	**Sum**	3,500

Solid-liquid extraction

Input	Mass (kg)	Output	Mass (kg)
Maize, gem-rich fraction	700	Extraction solution	4,211.8
Sodium acetate	5.7		
Sodium chloride	6.1		
Water	3,500		
Sum	4,211.8	**Sum**	4,211.8

Purification

Input	Mass (kg)	Output	Mass (kg)
Extraction solution	4,211.8	Product	0.403
Sodium chloride	205	Waste	11,440.4
Water	7,024		
Sum	11,440.8	**Sum**	11,440.8

4. Stevia production

Cultivation of stevia in one hectare of land in one year

Input	Mass (kg)	Output	Mass (kg)
Nitrogen fertilizer	40	Dried stevia leaves	1800
Phosphorus fertilizer	20		
Potassium fertilizer	30		
Sum	90	**Sum**	1800

Solid-liquid extraction

Input	Mass (kg)	Output	Mass (kg)
Dried leaves	1,800	Extraction solution	19,800
Water	18,000		
Sum	19,800	**Sum**	19,800

Preliminary purification

Input	Mass (kg)	Output	Mass (kg)
Extraction solution	19,800	Product powder 1	220
Calcium hydroxide	43.2	Waste	19,719
Iron hydroxide	95.4		
Sum	19,939	**Sum**	19,939

Preparation of Stevioside

Input	Mass (kg)	Output	Mass (kg)
Product powder 1	220	Product powder 3	129
Methanol	867	Waste	1227
Ethanol	242		
Water	26.9		
Sum	1356	**Sum**	1356

Preparation of Rebaudioside A

Input	Mass (kg)	Output	Mass (kg)
Water	909.4	Product powder 6	52
Ethanol	255.3	Waste	1113
Sum	1165	**Sum**	1165

5. Aspartame production

Preparation of L-Aspartic acid

Input	Mass (kg)	Output	Mass (kg)
L-Aspartic acid	319.4	Aqueous ZA solution	2185.5
Sodium Hydroxide	324	Waste	1261.5
Benzyloxycarbonyl chloride	480		
Toluene	1154		
Water	1170		
Sum	3447	**Sum**	3447

Preparation of DL-Phenylalanine

Input	Mass (kg)	Output	Mass (kg)
DL-Phenylalanine	991.5	Aqueous DL-PM solution	1007.1
Methanol	3320	Waste	6501
Hydrogen chloride	437.4		
Sodium hydroxide	124.3		
Water	2634.7		
Sum	7508	**Sum**	7508.1

Condensation

Input	Mass (kg)	Output	Mass (kg)
ZA aqueous solution	192.43	Z-APM.D-PM	1217
Aqueous DL-PM solution	250.98		
Thermolysin	7.2		
Calcium acetate monohydrate	1.3		
Water	765		
Sum	1217	**Sum**	1217

Purification

Input	Mass (kg)	Output	Mass (kg)
Z-APM.D-PM	0.267	Aspartame	0.265
Hydrogen chloride	0.04		
Ammonium formate	0.126		
Methanol	2.4		
Catalyst Pd/C	0.05		
Water	28		
Sum	30.88	**Sum**	30.62

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