1. General Introduction

1.1. Towards a circular bioeconomy based on renewable carbon

1.1.1. Climate Change and the urge for a circular bioeconomy

The topic of Climate change has matured over the past decades as a possible concern in the distant future, mainly defended by environmentalists, to an imminent major threat to human society and organic life now acknowledged by the United Nations. Climate change, defined as a long-term change in the average weather patterns, observed in the past decades has been linked with a 95% probability to human activities in the Fifth Assessment Report of the Intergovernmental Panel on Climate Change (IPPC) [1]. Such Climate Change is directly related to global warming resulting from a human expansion of the greenhouse effect, warming resulting from the entrapment of the radiating heat leaving from Earth. Gases that contribute to the greenhouse effect and which are produced by human activity are mainly, nitrous oxide (N_2O), methane (CH_4), and carbon dioxide (CO_2). Nitrous oxide is a powerful greenhouse gas arising from commercial and organic fertilizers, fossil fuel combustion, nitric acid production, and biomass burning. Methane, on the other hand, is produced in landfills through the decomposition of wastes, agriculture (especially rice cultivation), and domestic livestock, and, on a molecular base, methane entraps 25 times more heat than a carbon dioxide molecule [2]. Carbon dioxide is released through natural events, such as respiration and volcanic activity but also through human activities such as deforestation and the combustion of fossil fuels. Since the Industrial Revolution, the CO_2 concentration in the atmosphere increased by 47% and is one of the main driving forces for the incremental temperature increase of the atmosphere and consequent climate change (Figure 1) [3]–[5].

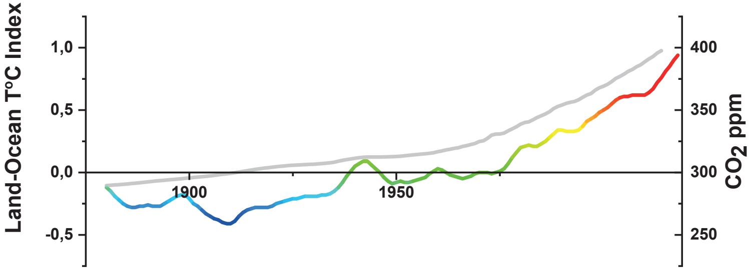


Figure 1- Interrelation of Carbon Dioxide levels in the atmosphere and Land-Ocean temperature index over the past decades [3]–[5].

The increase of the Land-Ocean temperature index, which indicates the global temperature increase on land and water bodies, has led to undeniable consequences to the diverse planetary habitats. Evidence of these consequences is the continued shrinking of ice sheets, as recorded by NASA's Gravity Recovery and Climate Experiment which shows an average yearly loss of 279 billion tons of ice in Greenland since 1993 while Antarctica lost around 148 billion tons of ice per year, the global Sea level rise, which rose 20 centimeters in the last century or the increase of extreme weather events [6]–[8].

The temperature rise in a future scenario in which no global environmental concerns are emphasized and future social-economic development is accompanied by higher greenhouse gas emissions (scenario B in Figure 2) can reach a maximum of 8ºC as extrapolated by the National Climate Assessment (NCA) from the U.S. Global Change Research Program (USGCRP) [9]. In this scenario, the temperature rise would lead to a decrease of precipitation volumes during spring times in the western part of the USA of up to 25%, accompanied by a respective increase of rain in Antarctica and northern Canada. The precipitation anomalies would lead to a decrease in soil moisture, which would increase the probability of a megadrought, a drought lasting more than three decades, from the current 12% to 80% [10]–[12].

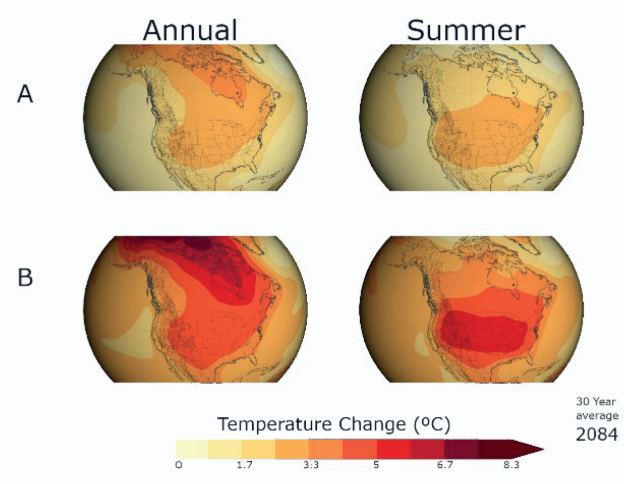
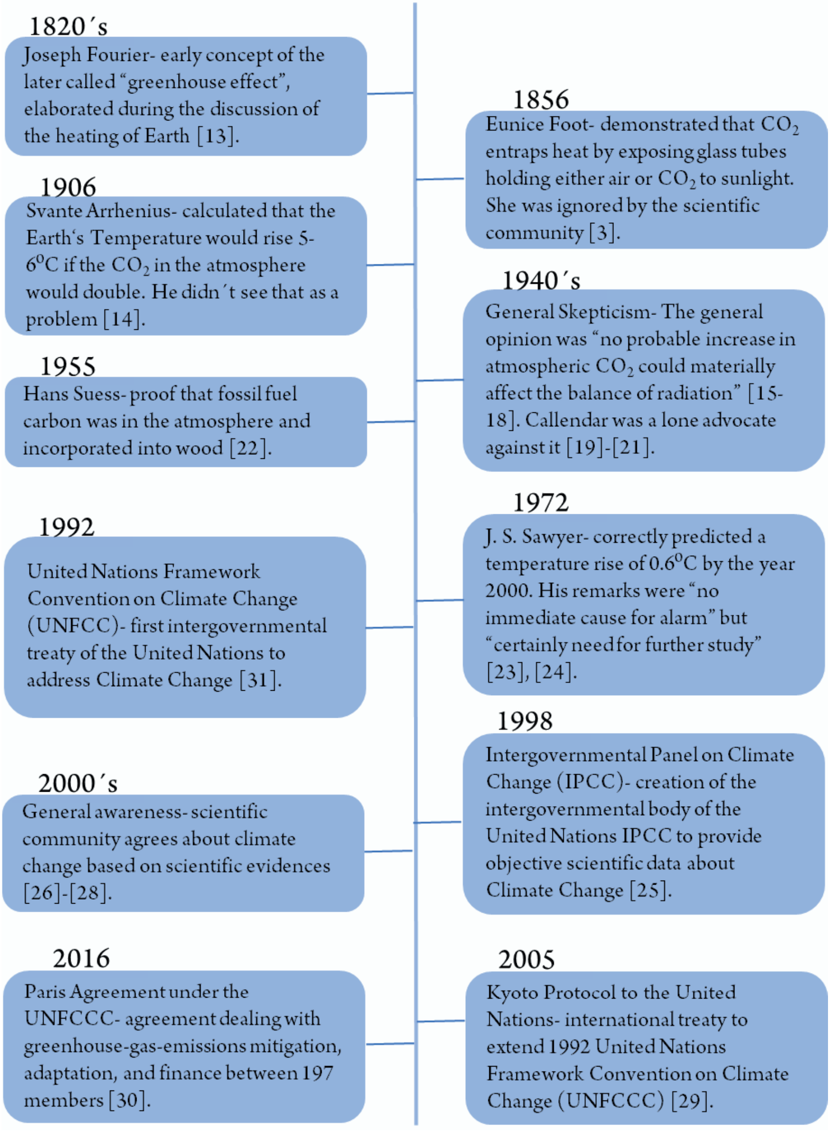


Figure 2- Temperature anomalies of a model projected 30-year temperature average. of scenario A (lower greenhouse emissions) and scenario B (higher greenhouse emissions) with the 1970-1999 average. Adapted from [9].

On the other hand, if greenhouse gas emissions are tackled and efforts to a carbon-neutral economy are put in place the temperature rise would be halted which would diminish the effects of climate change (scenario A in Figure 2).

The topic of climate change might seem to only have arisen during the last decade, however, the early concept of the Greenhouse effect was postulated in the 1820s by Joseph Fourier and since then several warnings have been sent by the scientific community about the negative effect of fossil fuel combustion on large scale. A summary of the warnings and evolution of the scientific evidence supporting the contribution of carbon emissions towards global warming is summarized in Table 1.

Table 1. Timeline of the evolution of the scientific evidence supporting the contribution of carbon emissions towards global warming and the most recent measures taken to tackle this threat.



1820´s
Joseph Fourier- early concept of the later called "greenhouse effect", elaborated during the discussion of the heating of Earth [13].

1856
Eunice Foot- demonstrated that CO_2 entraps heat by exposing glass tubes holding either air or CO_2 to sunlight. She was ignored by the scientific community [3].

1906
Svante Arrhenius- calculated that the Earth's Temperature would rise 5-6^{o}C if the CO_2 in the atmosphere would double. He didn´t see that as a problem [14].

1940´s
General Skepticism- The general opinion was "no probable increase in atmospheric CO_2 could materially affect the balance of radiation" [15-18]. Callendar was a lone advocate against it [19]-[21].

1955
Hans Suess- proof that fossil fuel carbon was in the atmosphere and incorporated into wood [22].

1972
J. S. Sawyer- correctly predicted a temperature rise of 0.6^{o}C by the year 2000. His remarks were "no immediate cause for alarm" but "certainly need for further study" [23], [24].

1992
United Nations Framework Convention on Climate Change (UNFCC)- first intergovernmental treaty of the United Nations to address Climate Change [31].

1998
Intergovernmental Panel on Climate Change (IPCC)- creation of the intergovernmental body of the United Nations IPCC to provide objective scientific data about Climate Change [25].

2000´s
General awareness- scientific community agrees about climate change based on scientific evidences [26]-[28].

2005
Kyoto Protocol to the United Nations- international treaty to extend 1992 United Nations Framework Convention on Climate Change (UNFCCC) [29].

2016
Paris Agreement under the UNFCCC- agreement dealing with greenhouse-gas-emissions mitigation, adaptation, and finance between 197 members [30].

Public awareness of climate change did increase over the past years but still encompasses a percentage of indifferent or non-believers [32]–[35]. On an intergovernmental level, the threat of climate change is taken as a high-priority concern and discussions emphasize which steps are necessary to halt its effects. The necessary steps to combat climate change can be crystalized into two approaches: adaptation and mitigation [36]. Adaptation encompasses all actions to reduce the susceptibility to the harmful consequences of climate change, like sea-level rise, extreme weather episodes, and food insecurity, but also encloses taking advantage of beneficial opportunities, such as harnessing the energy of stronger winds or longer growing seasons which could lead to increased yields. Simultaneously, mitigation efforts should be made to halt climate change by reducing the number of greenhouse gases in the atmosphere arising from the combustion of fossil fuels or by enhancing the natural resources which capture the heat-trapping gases.

Mitigation efforts cannot only rely on stopping the use of fossil fuels but also need to present sustainable alternatives which maintain the social requirements. Almost all relevant social and economic sectors are heavily dependent on fossil fuels and greenhouse gas emissions (Figure 3).

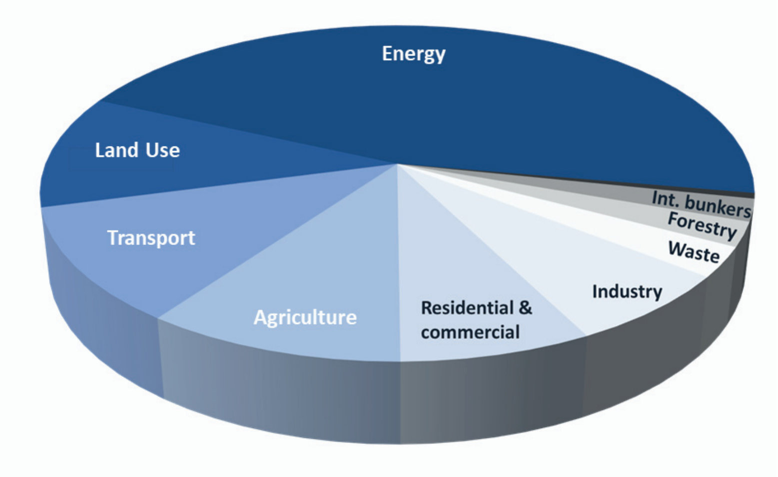


Figure 3- Percentage of greenhouse gas emissions by social and economic sectors by 2017 [37].

For societal sectors, which mainly require the combustion of fossil fuels, like energy and transport, the carbon-neutral alternatives mainly focus on using sustainable energy sources like solar, wind, hydro, tidal, geothermal, and biomass energy. To tackle the greenhouse gas emissions emitted in agriculture the solutions focus more on adopting a plant-rich diet and avoiding especially bovine meat since it is one of the major methane sources. Other solutions, which are not directly related to greenhouse gas emissions but still have an impact on climate change, are the adoption of vertical farms or the use of insect-based protein to reduce the required farm areas, water and energy requirements and, optimize the crop productivities.

Fossil resources are also used to retrieve all the chemicals and molecules required to produce daily used products. The usage and processing of these fossil resources are based on a take-make-waste extractive industrial model which frames a linear economy. Such a linear economy leads to major amounts of waste which end up in the environment disrupting the habitats and contributing to climate change. The global population is predicted to increase to 9.7 billion by 2050, leading to a further increase in energy and resource demands on a finite-resource planet, making it necessary to find a sustainable alternative [38]. A sustainable alternative is the so-called circular economy which entails the decoupling of economic activity from the consumption of finite resources and where regeneration is key [39]. The circular economy model holds two types of cycles, biological and technical. The biological cycle engulfs consumption products like food and biologically based materials which are after consumption fed back into the system through fermentation, composting, or anaerobic digestion. Technical cycles hold materials that can be recovered by strategies based on reuse, repair, remanufacture, or recycling. The circular economy strategy was embraced by the European Commission through its action plan ´Closing the loop´ in 2015 which emphasized ´The transition to a more circular economy, where the value of products, materials, and resources is maintained in the economy for as long as possible, and the generation of waste minimized, is an essential contribution to the EU's efforts to develop a sustainable, low carbon, resource efficient and competitive economy´.

1.1.2. Integration of bioeconomy and circular economy- the concept of a circular bioeconomy

The circular economy concept focuses on maintaining the value of products, materials, and resources for as much as possible in the economy and thus reducing the amount of waste, but it does not emphasize the generation of new renewable resources or the exploration of alternative raw material sources to fossil-based ones.

A sector that aims for the obtention of raw materials sustainably is the so-called bioeconomy which pursues to substitute fossil carbon with renewable biomass carbon arising from agriculture, forestry, and marine sources. Bioeconomy and circular economy are different but complementary approaches that share the common traits of attempting to improve resource and eco-efficiency, lowering the greenhouse gas footprint, reducing the dependency on fossil carbon, and valorizing waste [40]. Rather than focusing on recycling and reuse approaches, bioeconomy handles product or service functionality such as new chemical building blocks, processing routes, and, the identification of new functionalities and properties of products. Bioeconomy activities can be categorized into the direct exploitation of biological resources (such as the primary sectors of agriculture, fishery, and forestry), the further processing of biomass (like food or wood processing), or novel activities that process biomass and biomass residues into bioenergy or bio-based chemicals [41]. Examples of how these goals are achieved are precision and vertical farming allied to gene editing for efficient agriculture and forestry developments, new processing pathways with lower toxicities and reduced use of harsh chemicals, biotechnology to produce sustainable bulk and fine chemicals, and, healthier and nature-compatible bio-based products [40]. In the past decade the bioeconomy concept has been on the policy agenda on a global scale, such as the National Bioeconomy Blueprint from the United States of America, the European Green Deal, and European Bio-Based Industries Joint Undertaking, or the implementation of bioeconomy in the growth strategies of emerging economies [42]–[45].

However, bioeconomy and circular economy are per se not necessarily sustainable and require a symbiosis between the two, the circular bioeconomy, to come closer to that end. For instance, biobased products should not harm the ecosystem nor incite the linear economy model whereas the circular economy should reduce its dependency on fossil resources. The definition of circular bioeconomy can be crystallized into an economy mainly fueled by bio-based products with properties to be able to be reused, remanufactured, or recycled to foment a cascading use until the greenhouse gas emissions required for the cycling are lower than the ones to produce virgin bio-based products [40], [46]. The concept of circular bioeconomy is already adopted and promoted by industrial associations such as CEPI (Confederation of European Paper Industries) and EuropaBio (The European Association for Bioindustries) [47], [48].

However, certain regulatory challenges are still in place which hinders the development of a circular bioeconomy. From a European perspective, several regulatory blocks are still present such as the heavy regulation on the use of CRISPR/Cas technologies for plant breeding (in contrast to the United States Department of Agriculture that since 2018 does not regulate genome-edited plants if they could have been developed through traditional breeding), the longer and more expensive approval of new products and the tendency for a court ruling on a process-based approach rather than the regulation of the resulting product as it is followed by other nations [49]–[52].

Despite these hindrances for a circular bioeconomy, several indicators show that relevant steps are being taken to reach this goal. One indicator is the increase in public and private investment in biotechnology, more specifically in cell factories and synthetic biology. Cell factories and synthetic biology play a crucial role in the challenge to reduce the dependency on fossil fuels since they allow the obtention of a wide spectrum of chemicals ranging from fine and bulk chemicals to bioactive molecules from renewable sources. The development of cell factories and respective synthetic biology tools will be discussed in this work.

1.2. Cell factories and their development

Modern society is only able to thrive on the base of materials and chemicals that fulfill all its demands. These chemicals and materials have until recently either been directly extracted or converted from unrenewable sources. As discussed in the previous section, the need for a sustainable alternative is urgent since the foundations that made modern society thrive might become the same which will make it crumble. Biologically sustainable alternatives are already present as shown by the estimation of Carlson that 25% of the chemicals produced in the USA are biologically based [53], [54]. Microbial cell factories harness the capability of obtaining most of these materials and chemicals from renewable feedstocks and further contribute to the increase of this percentage [55]. One successful example is the industrial applicable bio-based production of 1,3-propanediol in *E. coli* with a titer of 135 g/L requiring 42% less energy consumption and 56% fewer greenhouse gas releases than the respective fossil fuel alternative [56]. However, being proven that these chemicals can be produced at large scale by cell factories, the strain development pipeline to reach capabilities for industrial scale production still requires further breakthroughs. For some target chemicals, it is possible to find natural producers which do not require extensive engineering to achieve industrial-relevant production levels since they already possess high tolerance and robust metabolic fluxes towards the target product. Some examples are *Mannheimia succiniciproducens* for succinic acid, *Corynebacterium glutamicum* for amino acids; *Clostridium* sp. for butanol, and *Yarrowia lipolytica* for oleochemicals [57]. Rational approaches to boost the production of native metabolites can be summarized into the following strategies: a) pathway overexpression: this strategy encompasses the increase of activity by either overexpressing the biosynthetic activity or engineering the enzymes to have higher specific activities. One example is the improved production of ganoderic acid in *Ganoderma lucidum* [58]; b) transporter engineering: product yields can be affected by the intracellular accumulation of product due to interferences in enzyme kinetics, feedback inhibition or cellular toxicity making the overexpression of efflux pumps a very important strategy for improved productivities as shown by Dunlop et al for the production of biofuels in *E. coli* [59]; c) de-branching: branching or competing pathways tend to reduce productivities due to decreased carbon flux towards the product. Deletion of non-lethal competing pathways or decreasing the activity of lethal knockouts by knockdown, tunable promoters, or mRNA silencing is a common approach in metabolic engineering and a rather relevant one as shown for isoleucine production in *E. coli* [60]; d) product degradation: the arguably most intuitive strategy from the ones described here is the deletion of non-essential reactions which consume the desired product being one example the deletion of threonine dehydrogenase for the production of L-threonine [61]; e) co-factor engineering: in biosynthetic pathways were intermediate steps require co-factors (NADH/NAD+, NADPH2, NADP+, acetyl-CoA, etc.) their limitation can become the bottleneck for enhanced productivities [62], [63]. To overcome this bottleneck two strategies that can be followed to increase the available amount of co-factors are deleting non-essential enzymes which consume the required co-factor or replacing enzymes with ones that have an alternative co-factor dependency. Some successful examples of co-factor engineering are the substitution by an enzyme with an alternative co-factor requirement for sesquiterpene production [64] or in the specific case of NADH and NADPH the overexpression of an *E. coli* transhydrogenase for interconversion of these co-factors [65]; f) removal of feedback inhibition: some biosynthetic pathways are tightly regulated by feedback inhibition, for example, amino acids, which hampers the possibility to achieve desired high carbon fluxes. Random or targeted mutagenesis of feedback-inhibited enzymes allied to screening methodologies based on irreversible binding analogs of the product do sometimes allow to find variants that lost this property as shown for the production of different amino acids in *E. coli* [66]–[68]; g) de-regulation of carbon catabolism: some of the required biosynthetic pathways for a given product are under the control of general metabolic regulators which hamper the possibility to achieve higher fluxes. Disruption of such global regulators has been reported to lead to higher productivity as shown for ethanol production in *Pichia guilliermondii* [69] the last strategy is the signal transduction engineering in which the production is not regulated by carbon or nitrogen sources but by micronutrients or other steps within the pathway which once engineered can retrieve higher productivities [70].

For the majority of the desired target molecules, however, no natural producers can be found, or have been found yet, imposing the introduction of heterologous pathways to well-known organisms and further engineer them to achieve industrial-relevant production levels. One example of a successful heterologous expression in non-native producing strains with further strain engineering is the production of astaxanthin in *E. coli*, an antioxidant molecule with pharmaceutical and cosmetic applications, in which the *crt* and *trC* operons from *Pantoea anantis* and *Chlamydomas reinhardtii* where respectively introduced and further engineered with signal and solubility tags to improve their expression. Besides the heterologous pathway expression, the native methylerythritol phosphate pathway metabolism was engineered based on *in silico* metabolic flux analysis leading to the production of 430 mg/L in fed-batch fermentation. [71]. Furthermore, for some of the required molecules, no natural pathways have been found yet, and therefore non-natural occurring enzymatic activities have to be generated, or novel synthetic pathways have to be assembled. Due to the combinatorial complexity of pathway assembly and the growing amount of known enzymatic reactions, computational model-based pathway assembly has been used to explore this vast potential for novel metabolic pathways of products. Several prediction tools have been developed like BNICE, DESHARRY, RetroRules, RetroPath, GEM-Path, or SimPheny and, despite the peculiarities of each, most are based on generalized reaction rules and enzymatic reaction databases exploration. Successful examples of how these prediction tools lead to the production of compounds that are not naturally produced are the conversion of syngas to monoethylene glycol based on BNICE analysis or the identification of a novel biosynthetic pathway using the SimPheny BioPathway Predictor for the synthesis of 1,4-butanediol [72], [73]. In the example of the production of 1,4-butanediol, another type of model-driven cell factory engineering principle was applied which consists of Flux Balance Analysis (FBA) of genome-scale reconstructions of metabolic networks. These models allow to identify possible flux bottlenecks in the pathway and suggest knockouts to increase the productivity of the desired target compound. In the example of 1,4-butanediol, several knockout targets were identified through OptKnock simulations, and once these were applied a final production of 18 gL of 1,4-butanediol was achieved [72], which was further commercialized by Genomatica (USA). Another example of FBA-driven knockout identification, which leads to a relevant increase of production titers is the synthesis of methyl ketones by *Pseudomonas taiwanensis* VLB120 [74]. The above-mentioned engineering strategies for improving natural producers are also applicable to cell factories that rely on heterologous pathways. Further required strategies for heterologous pathway expression aim to allocate the enzymes to the proper cellular compartment and transcription engineering to assure that the genes are properly expressed in the selected host.

The previously described rational approaches to engineer cell factories are sometimes not applicable for some target molecules due to a lack of prior knowledge between genotype and phenotype. In such cases, resorting to evolutionary engineering is a resourceful alternative since it explores either the natural capabilities of cells to adapt to their environment or the potential of mutagenesis. Two disciplines can be delineated in evolutionary engineering, directed evolution and adaptive laboratory evolution (ALE). Directed evolution is initiated with mutagenesis of proteins or cells to create mutants which are further screened for the desired traits. In the protein engineering realm, it is an important approach to achieve enhanced catalytic activities, desired substrate/product specificity, and protein stability which might be further integrated into cell factories, whereas directed evolution of cells by chemical and/or physical mutagenic treatments allows the identification of mutants with higher resistance levels towards chemicals, higher productivities or enhanced growth on specific carbon sources [75]. The keystone of the directed evolution cycle is the screening methodology to identify the beneficial variants, which can be further mutagenized until the desired trait is reached. Examples of successfully directed evolution workflows are the doubling of L-arginine production titers in *C. glutamicum* after an N-methyl-N-nitro-N’-nitroguanidine (NTG) and ultraviolet treatment or the evolution of cytochrome C from *Rhodothermus marinus* to produce chiral organoboranes on a gram-scale through whole cell biosynthesis [76], [77]. The second discipline, ALE, does not rely on forced mutagenesis as in directed evolution but rather on natural mutagenic rates during cell growth under the conditions which are targeted. ALE is a powerful application to increase growth efficiency on specific carbon sources or adaptation to either external toxic compounds or self-produced metabolic by-products. In both cases, the cells are grown in the presence of these compounds and re-inoculated until the growth rate achieves a defined threshold. For the increase of resistance levels towards toxic compounds, once the growth rate threshold is achieved the concentration of the toxic compound is increased and further adaptation cycles are performed. Once the desired growth rates are achieved in both application cases, substrate adaptation and resistance acquirement, cells are harvested and sequenced to identify the genetic modifications which lead to the phenotypic upgrade. Examples of increased growth efficiency achieved through ALE are the increased growth of *E. coli* with glycerol, glucose, and citrate or the metabolization of ethylene glycol by *P. umsongensis* GO16 as the sole carbon source, a major step in the upcycle of post-consumer polyethylene terephthalate to medium chain-length polyhydroxyalkanoates (PHA) and a novel bio-based polyamide urethane (bio-PU) [78]–[81]. Successful examples of *E. coli* ALE to toxic compounds are the adaptation to ethanol, iso- and n-butanol, and ionic liquids [82]–[84].

All the so far mentioned approaches to improve cell factories are powerful synthetic biology tools that could allow modern society to obtain most of the required chemicals from renewable sources using microorganisms.

The awareness of the potential of synthetic biology for cell factory development such as for other sectors like diagnostics and healthcare has already been translated to an increase in small and medium enterprises and has also gained interest from investors [85], [86]. In 2018, SYNBIOCHEM, the University of Manchester-based SBRC, summarized guidelines for the development of materials using synthetic biology showing the growing potential and interest in this field [87].

1.3. *Pseudomonas taiwanensis* VLB120 as a cell factory

Microorganisms have been used by humans for several millennia as catalysts to produce bioactive molecules, way before the existence of unicellular organisms was even known to mankind. The use of yeast to modify food and beverages has been over the past millennia the major fermentative process to occur on earth and its leading position is enforced by the use of these fungi to produce bioethanol, the most important biomolecule by mass of annual production [88]. The designation of microorganisms as cell factories for biotechnological use to produce diverse molecules and materials has only been coined in the 1990s with its adoption as a Key Action in the 5th Framework Programme of the European Union [89]. This boosted the interest in biotechnological processes for the biomanufacturing of molecules and materials using mainly model organisms such as *E. coli* and *S. cerevisiae* [90]. The designation of *E. coli* and *S. cerevisiae* as model organisms have emerged due to their recurrent use which increased the understanding of their metabolism and further developed their respective genetic toolboxes. However, the interest in alternative organisms harboring biotechnological advantageous traits has been increasing since the development of genetic tools for these organisms has been facilitated by the CRISPR/Cas system such as the increased amount of annotated genomes [91]–[95]. Some of the most promising microorganisms harboring unique traits are *Acinetobacter baylyi*, due to their natural competence for synthetic biology, *Shewanella* species for their electro-active properties, *Vibrio natriegens* for its rapid-growth properties, *Halomonas* for its ability to grow in saline media under non-sterile conditions and, the species on which this work will focus on, *Pseudomonas* for their stress-resistant properties that can be exploited for the production of new-to-nature products [96]–[100].

Pseudomonads are Gram-negative, rod-shaped bacteria belonging to the class of Gammaproteobacteria usually featuring fast growth, low production of by-products, and low nutritional demand [101]. These soil-dwelling bacteria are usually found in challenging ecosystems which bolster the evolution of complex genetic repertoire granting them for example the capability to degrade toxic xenobiotic aromatics like benzene, toluene, styrene, and phenol [102], [103]. Besides the ability to degrade toxic compounds, this genus has also been used for the synthesis of bulk and fine chemicals like aliphatic alcohols [104], prodiginines [105], polyhydroxyalkanoate (PHA), polyesters [106], rhamnolipids [107], and methyl ketones [74].

The *Pseudomonas* strain on which this work focuses on is *Pseudomonas taiwanensis* VLB120, a strictly aerobic gram-negative bacterium isolated as a styrene degrader in a German forest and with its genome being sequenced in 2013 [108]. This non-pathogenic bacterium, with a safety level 1 status, harbors several industrial-relevant traits since it can grow on D-xylose via the Weimberg pathway and shows an inherent tolerance towards inhibitors from biomass hydrolysates [109], [110]. Besides its industrial applicability for cellulosic feedstocks, this strain has metabolic peculiarities which broaden its industrial relevance. This strain does not contain the gene encoding the 6-phosphofructokinase (Pfk) in its genome, which halts the glycolytic compounds to be catabolized via the Emden-Meyerhof-Parnas (EMP) route but mainly diverts them to the Entner-Doudoroff (ED) pathway and to a minor extent to the pentose phosphate pathway (PPP). This peculiarity results in a lower ATP generation but allows higher reduction rates of NADP+ by the partial recycling of triose-phosphates [111]. The lower ATP generation rate and also the necessity for even higher reduction rates of NADP+ have been shown in a phylogenetical proximal strain, *Pseudomonas putida* KT2440, to be satisfied with an increased carbon source uptake rate [112]. This higher reducing power grants the strain a tolerance towards oxidative stress and fitness for redox-intensive pathways [113], [114]. It was also shown that NADH-dependent processes can also profit from this high NADPH regeneration through the activity of pyridine nucleotide transhydrogenases [115]. Besides the glycolytic metabolic peculiarity, *P. taiwanensis* VLB120 also contains mechanisms that confer resistance towards solvents with a $\log P_{o/w}$ (the logarithmic partition coefficient in a 1:1 mixture of octanol and water) between 2.11 (toluol and styrene) and 4, which is generally linked to cytotoxicity since the solubility of these molecules allows direct contact with the cellular membrane [116]–[120]. One of the mechanisms which grant the solvent-resistant phenotype to this strain is the expression of resistance-nodulation-cell division (RND)-type efflux pumps (TtgGHI localized on the pSTY megaplasmid) which are driven by proton motive force to expel solvents from the cytoplasm [108], [121]. A second mechanism that contributes to the solvent-resistant phenotype of this strain is the ability to reduce the membrane fluidity by enforcing a denser lipid packing. This denser lipid packing is achieved by changing the unsaturated fatty acids in the membrane from *cis* to *trans*, by incorporating saturated fatty acids in the membrane, and by changing the phospholipid hydrophilic group [122]–[124].

1.4. Scope and outline of the thesis

This work aimed to further develop *Pseudomonas taiwanensis* VLB120 as a cell factory by further expanding the genetic tools portfolio of this strain for heterologous gene expression and providing an insight into how certain genetic parts function in this organism and the model organism *E. coli*. Besides the insight into the genetic tools of this strain, emphasis was laid on the creation of a chassis strain to expand the possible product range of this strain.

Chapter 3.1 focuses on the creation of a σ_{70} promoter library expressing the reporter gene *msfGFP* which was further integrated as a single copy into the genome of *P. taiwanensis* VLB120 and *E. coli* TOP10. The promoter library was further characterized in both organisms through online measurements of cellular density and fluorescence during exponential growth. The performance of each promoter in both organisms was described by a specific *msfGFP* expression rate in which the specific growth rate was considered. This allowed insight into how specific genetic parts behave between these two organisms.

Chapter 3.2 describes the evaluation of a previously proposed optimized gene expression architecture to achieve higher gene expression by focusing on mRNA stability and translation initiation rather than reaching high gene expression using strong promoters. This optimized gene expression architecture was tested on a plasmid basis and after single genomic integration using different promoters and reporter genes to evaluate the applicability of this optimized gene expression architecture in different scenarios.

Chapter 3.3 aims to extend the acyl-CoA product portfolio of *Pseudomonas taiwanensis* VLB120 to odd-chain products by enabling the production of propionyl-CoA. Propionyl-CoA production was enabled by expressing the sleeping beauty mutase operon from *E. coli* as a single integration copy. Assessment of this propionyl-CoA chassis strain for odd-chain products was done by expressing an acyl-CoA hydrolase enabling the synthesis of propionate. Fermentation strategies were evaluated during bioreactor fermentations to evaluate further the potential of this strain for the synthesis of odd-chain products.

The achievements and outcomes of the above-mentioned chapters are discussed in chapter 4 and debated from a broader perspective.