1 General introduction

1.1 The bioeconomy – on the road towards sustainability

One of the key challenges in the 21st century is to establish a sustainable society on an economic, ecological, and social level. To achieve this, the United Nations developed 17 *Sustainable Development Goals* (SDGs) in 2015 that should serve as a guide for policy action and provide a general orientation for addressing global challenges (Figure 1)[1].

Figure 1 – The United Nations' Sustainable Development Goals*. This figure is reprinted with permission from the United Nations - Department of Global Communications. (Official information on the SDGs can be found at: https://www.un.org/sustainabledevelopment/; "The content of this publication has not been approved by the United Nations and does not reflect the views of the United Nations or its officials or Member States.")*

A central topic in the SDGs is the replacement of fossil resources, mainly to avoid greenhouse gas emissions and enable a society within planetary boundaries[2]. One opportunity to contribute to this goal could be the establishment of a bioeconomy. The idea of the bioeconomy is to transform the largely fossil-based economy into an economy based on renewable carbon resources, such as biomass, CO_2, or waste streams, that are transformed by biological means, to create a circular and more resource-efficient system[3,4]. Microorganisms could play a key role in forming a bioeconomy[5,6]. Their unique metabolic properties enable the utilization of a wide range of carbon feedstocks that are channeled through rigid and efficient central carbon metabolism and are then converted into a variety of industrially relevant products through anabolic reactions[7,8]. In addition, the potential range of substrates and products can be further expanded through metabolic engineering strategies. Thus, the use of microbes can enable the production of a variety of industrially important chemicals with high specificity and under mild reaction conditions that are traditionally derived from petroleum and other fossil resources[9]. In this way, using microbes in a bioeconomy can contribute to the successful completion of several SDGs, the most obvious of which are Goal

13: *Climate Action*, Goal 12: *Responsible Consumption and Production*, and Goal 9: *Industry, Innovation and Infrastructure*.

1.2 C1 molecules as feedstocks for the bioeconomy

An essential aspect to consider when using microbes in the bioeconomy is which substrates will be applied as feedstocks. Currently, many microbial production processes on an industrial scale rely on glucose, starch, and other sugars as carbon sources, which are derived from potential food sources such as sugarcane or wheat[10]. This is because sugars are readily assimilated by many microbes and enable high growth rates and yields. However, producing these sugars requires arable land. Using increasing amounts of land for sugar production to replace bulk chemicals with bio-based products could lead to an intensification of agriculture potentially resulting in eutrophication, deforestation, or even competition with food production[11,12]. Therefore, it is important to explore alternative feedstocks that would allow an environmentally friendly and socially responsible production of large quantities of industrially relevant chemicals. In recent years countless studies have been published, evaluating the suitability of certain alternative carbon sources, among the most promising ones are waste streams, such as plastic waste or lignocellulosic waste streams[8,13,14]. One-carbon (C1) molecules could pose another alternative to traditional feedstocks. These C1 molecules include the potent greenhouse gases carbon dioxide (CO_2) and methane (CH_4). There have been many efforts to sequester them into value-added products with the help of microorganisms instead of releasing them into the atmosphere and some of these processes have already reached a commercial scale in recent years[15]. However, both CO_2 and CH_4 are gaseous at ambient temperature, which oftentimes makes the low mass transfer between gas and liquid and the resulting low dissolved gas concentrations a limiting factor in these gas fermentations[16–18]. One solution to this might be the use of C1 molecules that can be stored and transported in liquid form at ambient temperature and are thus more accessible to traditional fermentation approaches. This includes formic acid (CHOOH) and methanol (CH_3OH). At the moment, the global methanol demand of 98 Mt is almost entirely (99.8 %) produced from natural gas or coal[19]. However, it is possible to generate *green* methanol derived from CO_2 via photo- or electrocatalytic CO_2 reduction or via the gas-phase reaction of CO_2 and hydrogen using heterogeneous catalysts[20], the latter of which is a fully commercial technique that is also applied in the methanol production from fossil resources[21]. If hydrogen derived from renewable energies is used for this process, the produced methanol has the potential to become a sustainable feedstock that could allow carbon-neutral or even carbon-negative biomanufacturing of several essential platform chemicals (Figure 2).

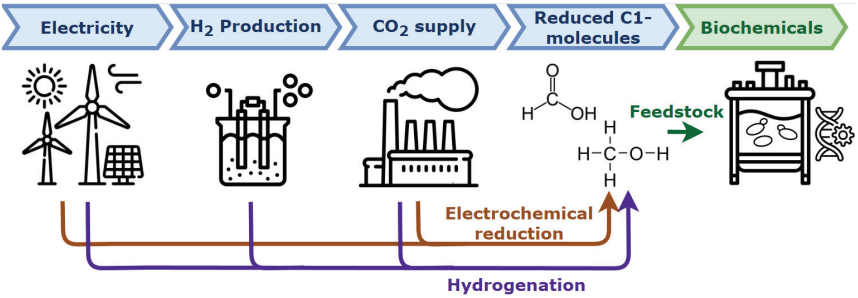

***Figure 2** – Producing CO_2-derived C1 molecules as feedstocks for microorganisms to produce industrially relevant chemicals.*

In fact, a so-called methanol economy has already been proposed[22]. In such an economy, methanol could be produced whenever renewable energy generation exceeds consumption during peak production periods. In this way, methanol would additionally serve as an energy storage molecule, that could buffer fluctuations in electricity production and thus prevent overloads of the power grids[23]. This *power-to-liquid* approach based on methanol has an approximate overall efficiency of 45 %[24] and is often regarded as superior to commonly proposed *power-to-gas* approaches based on methane (synthetic natural gas) or hydrogen due to the higher energy density of methanol and easier storage and transportation[25]. As a result, interest in the production of CO_2-derived methanol has increased in the past years (Figure 3). Recently, the largest commercial-scale production plant was constructed by Carbon Recycling International and commissioned in 2022 with a production capacity of 110.000 $t_{Methanol}/yr$[26]. In addition, major maritime corporations have committed to incorporating methanol-fueled cargo ships into their fleets, and the shipping company Maersk recently commissioned its first methanol-fueled container ship[27].

Another C1 feedstock that could be a potential alternative to methanol is formic acid[28]. Like methanol, it is completely miscible with water and can be produced via hydrogenation of CO_2[29]. It has attracted less attention compared to methanol due to its higher market price and its lower energy density of 6.4 MJ/L (compared to 17.6 MJ/L for methanol)[30]. However, in contrast to methanol, formic acid has the advantage that it is non-volatile, does not form explosive mixtures with oxygen, and is less toxic to humans[31]. Moreover, the technology for the electrochemical conversion of CO_2 to formate, the use of which would bypass the need for *green* hydrogen, is already more mature than the electrochemical conversion

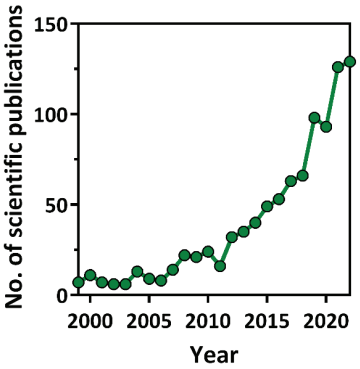

Figure 3 – ***Publication trend in research on CO_2-derived methanol.*** *The number of scientific publications was retrieved from the database* Web of Science™ *until the year 2022 by searching for documents containing the keyword "CO_2 derived methanol" in their title, abstract or the article keywords.*

to methanol[32,33]. Therefore, it is also worthwhile to investigate formic acid as a potential C1 substrate for the bioeconomy.

Using methanol or formate as substrates in microbial production processes could kill two birds with one stone, as the emission of greenhouse gases could be avoided, and at the same time, essential platform chemicals would be produced. Currently, *green* methanol prices are estimated to range between 800-1600 USD/t, which is more than double the current market price of methanol (app. 500 USD/t)[19,34]. However, with an expected decrease in the prices for renewable energy together with a potential increase in taxes on CO_2 emissions and unpredictable fluctuations in the prices for fossil resources, the costs for renewable methanol production are expected to drop to 250-630 USD/t by 2050[19]. However, compared to a ton of formate, a ton of glucose has three times as many usable electrons, which greatly facilitates cultivation processes and makes sugar a difficult microbial substrate to replace[28]. Nevertheless, it should be taken into account that sugar prices on the European market have surged to new highs of over 800 USD/t in recent times[35]. At the same time, bioprocesses based on glucose will always be limited by the availability of agricultural land. These are only some of the reasons why potential alternative substrates should be explored. Only electricity and CO_2 as substrates offer the possibility of theoretically unlimited scalability that could enable the replacement of fossil fuels in the production of bulk chemicals[28]. Therefore, it is worthwhile to explore the production of crucial platform chemicals using C1 molecules as substrates.

1.3 Methanol utilization by microorganisms

It is possible to convert reduced C1 molecules to longer-chain hydrocarbons by purely physicochemical processes, e.g. by a Fischer-Tropsch process[36]. However, these processes are often limited by their low selectivity in producing specific molecules, often requiring complex downstream processing steps, such as multiple distillation steps for product purification and further synthesis steps[37]. The corresponding plants usually require large areas, and the reactions have to take place under extreme conditions such as high pressures and temperatures[38]. Here, microorganisms have a clear advantage, as they can produce a large number of industrially relevant products with high specificity even under mild reaction conditions, in plants that require comparatively little land[39]. Consequently, it has been suggested that C1 compounds produced physicochemically and then upgraded via microbial fermentations could become key intermediates in the valorization of CO_2 into commodity chemicals[28,40,41].

There is a large variety of microorganisms that can natively use methanol as their sole carbon and energy source (methylotrophs). This ability evolved due to their adaptation to specific niche habitats where methanol is available. For example, methanol is produced during the decomposition of the plant cell wall component pectin and is also present in marine sediments as an exudate byproduct of phytoplankton metabolism[42,43]. The key challenge of growth on C1 molecules is the ability to form biomass without the availability of carbon-carbon bonds, the cleavage of which typically supports microbial growth on sugars and other substrates with

multiple carbon atoms[44]. Methylotrophic organisms can be found in all three domains of life. While prokaryotic methylotrophs are phylogenetically diverse and can be found among both bacteria and archaea, eukaryotes have only developed methylotrophy among a small group of yeasts, including, e.g., the genera *Candida, Komagataella, Ogataea*, and *Pichia*[45,46]. The most efficient way to assimilate methanol has evolved among anaerobic acetogenic bacteria, which can convert methanol via the Wood-Ljungdhal pathway (also referred to as reductive acetyl-CoA pathway, Figure 4) with up to 90 % energetic efficiency[47].

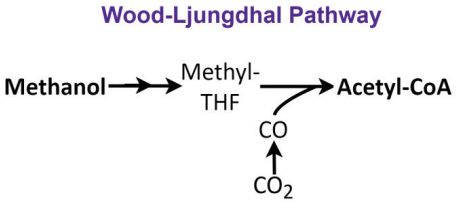

Figure 4 – Wood-Ljungdhal pathway *enabling the anaerobic utilization of methanol as a carbon source. Used abbreviations: THF: Tetrahydrofolate, CoA: Coenzyme A. Multiple arrowheads on one line indicate multiple reaction steps.*

In contrast, the conversion of carbon monoxide or hydrogen/CO_2 by acetogens via the Wood-Ljungdhal pathway, which is already performed on an industrial scale, only reaches energetic efficiencies of around 60 % and 75 %, respectively[48,49]. This highlights the unexploited potential of methanol as a substrate. However, despite these high energetic efficiencies, anaerobic methanol assimilation with acetogens has the disadvantage that the potential product spectrum is limited to molecules that generate ATP during their biosynthesis, such as ethanol, isopropanol, or n-butanol[50,51]. Additionally, the potential productivity of acetogens is often constrained by their limited genetic accessibility, typically low growth rates, and low maximal cell densities[52]. Consequently, while aerobic methanol assimilation is energetically less efficient, it may represent an attractive alternative to explore. It allows a drastic expansion of the product spectrum since biomass and product formation can be decoupled from energy synthesis and more accessible production organisms can be used[49].
For the aerobic assimilation of methanol, an organism must accomplish three metabolic tasks that can be considered as independent modules: 1) oxidation of methanol to formaldehyde, 2) assimilation of a C1 unit, and 3) dissimilation of formaldehyde[53].

1.3.1 Oxidation of methanol to formaldehyde

In all aerobic methylotrophic organisms, the first step required for methanol assimilation is the oxidation of methanol to formaldehyde, which can be carried out by different enzymes, that can be classified based on the electron acceptors used. While Gram-positive bacteria, such as *Bacillus methanolicus* have evolved an alcohol dehydrogenase (ADH) that uses NAD^+ as cofactor[54], Gram-negative bacteria (e.g., *Methylobacterium extorquens*) use a pyrroloquinoline quinone (PQQ) dependent ADH[55]. In yeasts, the assimilation of methanol to formaldehyde occurs via an oxygen-dependent methanol oxidase (MOX), which produces hydrogen peroxide (H_2O_2) as a toxic byproduct[56]. As a result, this part of methanol assimilation is localized in the peroxisomes of the yeasts, where H_2O_2 can be detoxified to

H_2O by the catalase (CAT) enzyme[57]. At first glance, this oxidation appears to be energetically wasteful and raises the question of why alcohol dehydrogenases for methanol assimilation have not evolved in yeasts, even though these enzymes are abundant in those microbes. It is speculated that methanol oxidases evolved because of the typically very low activity of alcohol dehydrogenases toward methanol and the unfavorable Gibbs free energy of the methanol dehydrogenase reaction compared to the oxidase reaction at mesophilic temperatures[58]. Since bacteria lack peroxisomes, they never had the chance to evolve a methanol oxidase. This hypothesis is also supported by the fact that methylotrophic yeast strains lacking peroxisomes are not able to grow on methanol even when all required enzymes are present in the cytosol[59].

1.3.2 Assimilation of a C1 unit

Since formaldehyde is highly toxic to all cells as it causes unspecific crosslinking of proteins and nucleic acids, it is essential in methanol metabolism to convert it into non-toxic molecules[60]. Four natural pathways support aerobic growth on methanol by assimilating formaldehyde into C2- and C3-skeletons, which then serve as precursors to build up cell constituents[49,61]. These pathways are the xylulose monophosphate (XuMP) cycle, the ribulose monophosphate (RuMP) Cycle, the serine cycle, and the Calvin-Benson-Bassham (CBB) cycle (Figure 5, Table 1). All of these pathways share a cyclic structure.