# SYNGAS FERMENTATION FOR EFFICIENT CONVERSION OF LIGNOCELLULOSE TO BIOETHANOL

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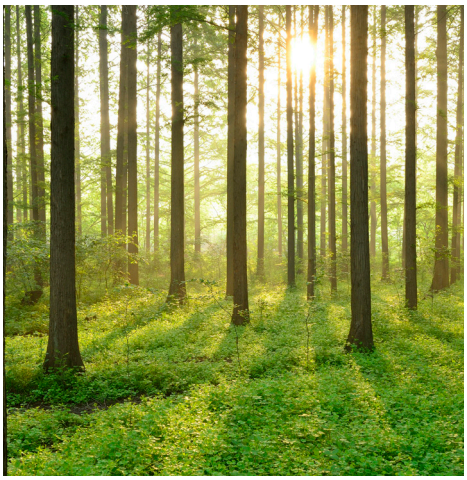

BIODRIVMEDEL FÖR SVERIGE 2030

Energiforsk

# Syngas Fermentation for Efficient Conversion of Lignocellulose to Bioethanol

## Syngasfermentering för effektiv omvandling av lignocellulosa till bioetanol

**YVONNE NYGÅRD**

**PAWEL PIATEK**

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Energiforsk AB | Phone: 08-677 25 30 | E-mail: kontakt@energiforsk.se | www.energiforsk.se

# Foreword

**The project has been conducted within the Energiforsk programme Biofuels for Sweden 2030 (Biodrivmedel för Sverige 2030), with the goal to contribute to the development of biofuels for the transportation sector and a fossil free transportation fleet by 2030.**

The programme has been financed by EON Gas Sverige AB, Gasnätet Stockholm AB, Göteborg Energi AB, Neste AB and the Region Skåne.

Within this project, the potential for syngas fermentation for production of biofuels, has been evaluated. The report has been produced by Yvonne Nygård and Pawel Piatek from the Division of Industrial Biotechnology at Chalmers University of Technology. Yvonne Nygård has led the project. The project has been performed in collaboration with Henrik Thunman at The Division of Energy Technology at Chalmers. This work was also financed by the Swedish Energy Agency. This work was also made possible by financial support from Chalmers Area of Advance Energy.

The reference group for the project had the following members: Mats Rydehell (University of Skövde), Paul Christakopoulos (Luleå University of Technology), and Bertil Wahlund/Anton Fagerström (Energiforsk). The reference group is gratefully acknowledged for invaluable contribution to the project.

Stockholm december 2019

Bertil Wahlund
Energiforsk AB

# Sammanfattning

**Bakteriell syngasfermentering är ett radikalt nytt koncept för att producera förnybara bränslen av hög betydelse för den svenska energisektorn. Syngas är en blandning som huvudsakligen består av CO, $CO_2$ och $H_2$ som produceras genom förgasning av kolhaltiga råmaterial (t.ex. biomassa, kommunalt avfall och utsläpp från industriella processer). Förgasning gör det möjligt att konvertera bokstavligen allt kolhaltigt material till syngas och syngasgasfermentering med hjälp av biokatalysatorer (mikrobiella celler) kan fullt utnyttja kolet i råmaterialet. Däremot kvarstår många vetenskapliga utmaningar för att göra processen mer effektiv.**

Under detta projekt inrättades en plattform för att arbeta med acetogener, bakterier som naturligt kan omvandla syngas till etanol och andra biokemikalier, vid avdelningen för Industriell bioteknik på Chalmers Tekniska Högskola. En gedigen litteraturstudie följt av experimentell utvärdering ledde till valet av acetogen som biokatalysator för pågående och framtida syngas och elektrosyntesbaserat arbete. Eftersom det långsiktiga målet är att etablera en plattform där industriell syngas används som råmaterial utfördes en kartläggning av syngaskompositionen i syngas från flera industriella anläggningar. Detta ledde till en identifiering av typiska gaskompositioner såväl som kartläggning av de vanligt förekommande föroreningarna. Kemiska föreningar som finns i små mängder i syngas kan ha en hämmande effekt på syngas-jäsande bakterier.

Fokus för detta projekt var att övervinna utmaningar relaterade till processhämning. De föroreningar som finns i syngas (inhibitorer), antogs störa celltillväxt och leda till en lägre produktivitet. Genom detta projekt har vi utvecklat en propageringsmetod som möjliggör bakteriell tillväxt och produktion av etanol från CO (den energiinnehållande komponenten i syngas) i närvaro av hämmande BTX-föreningar (bensen, toluen och xylen), med samma effektivitet som med rent CO. Detta är ett stort framsteg mot jäsning av industriell syngas. Potentialen för syngasfermentering är betydande eftersom det är en ny strategi för att fånga kol, minska utsläppen av växthusgaser och producera hållbara kemikalier och biobränslen. Syngas fermentering är ett speciellt attraktivt alternativ för omvandling av utsläppsgaser från små eller medelstora förgasningsanläggningar som innehåller stora mängder kväve.

# Summary

**Bacterial syngas fermentation is a radically new concept for producing renewable fuels of high importance to the Swedish energy sector. Syngas is a mixture consisting primarily of CO, $CO_2$, and $H_2$ that is produced through gasification of many carbon-containing materials (e.g.: biomass, municipal waste and from heavy industrial processes). Gasification allows the conversion of literally any carbon-containing material into syngas and syngas fermentations by biocatalysts (microbial cells) can fully exploit the carbon but many scientific challenges persist.**

During this project, a platform for working with acetogens, bacteria that can naturally convert syngas into ethanol and other biochemicals, was set up at the division of Industrial Biotechnology at Chalmers University of Technology. A thorough literature study followed by experimental evaluation led to the selection of an acetogen as biocatalyst for current and future syngas end electrosynthesis-based work. As the long-term aim is to establish a platform where industrial syngas is used as a raw material, a mapping od syngas composition from several industrial plants was performed. This led to the identification of typical gas compositions as well as mapping of the commonly found impurities, chemical compounds found in small amounts in the syngas, that may have an inhibitory effect on the syngas fermenting bacteria.

The focus of this project was on overcoming major obstacles related to process inhibition, as impurities found in syngas, were hypothesized to disrupt cellular growth and lower productivity. Through this project we have developed a propagation scheme, that allows the production of growth and production of ethanol from CO (the energy containing component of syngas) in the presence of inhibitory BTX compounds (benzene, toluene and xylene), with similar efficiency as with pure CO. This is a major advancement towards fermentation of industrial syngas. The potential of syngas fermentation is substantial as it could prove to be a novel strategy in capturing carbon, reducing greenhouse gas emissions and producing sustainable chemical and fuel bio-commodities. Syngas fermentation is a particularly attractive alternative for converting emissions gases from small or medium-sized gasification plants containing large amounts of nitrogen.

# List of content

# 1 Introduction

An increased production of biofuels, needed to reach the target of a fossil free vehicle fleet by 2030, demands a broader raw material basis and novel technologies for more efficient energy generation. Conventional fermentation of biomass residues, degraded to so-called lignocellulosic hydrolysates remains complex, timely and costly. Full conversion of the biomass is never achieved and a large part of it is still left as lignin (up to 25% of the carbon and 35% of the energy content in biomass). In comparison, gasification is fast, can handle very heterogeneous substrates, and is very efficient in carbon use and recovery. Current syngas production is traditionally followed by the thermochemical Fischer-Tropsch process that produces liquid based hydrocarbons. However, this method is sub-optimal in smaller, decentralized scales that need to access local raw materials (e.g. forest biomass residues and municipal solid waste) and achieve sustainable transportation costs and carbon footprint.

The use of microbial catalysts that convert (ferment) the gaseous mixtures into ethanol or other products is an alternative to Fischer-Tropsch synthesis. These biocatalysts can handle a wider spectrum of gases and work in relatively simple reactor systems. Merging these thermochemical and the biochemical routes (Figure 1) will help to reach strict environmental goals on carbon utilisation and greenhouse emissions, while satisfying our ever-increasing needs for energy and chemicals.

This project was conducted at the Division of Industrial Biotechnology in collaboration with the Division of Energy Technology at Chalmers University of Technology. The project was led by Asst. Prof. Yvonne Nygård and most of the practical work was performed by Dr Pawel Piatek. Other participants in this project were Prof. Henrik Thunman, Prof. Lisbeth Olsson and Dr Nikolaos Xafenias. Prof. Paul Christakopoulos from Luleå University of Technology and Dr Mats Rydehell, from the University of Skövde participated in the project reference group.

The project supported the project 44730-1, led by Prof. Lisbeth Olsson and funded by the Swedish Energy Agency. Some details of the results presented in this report have been omitted and will be presented in coming scientific publications.

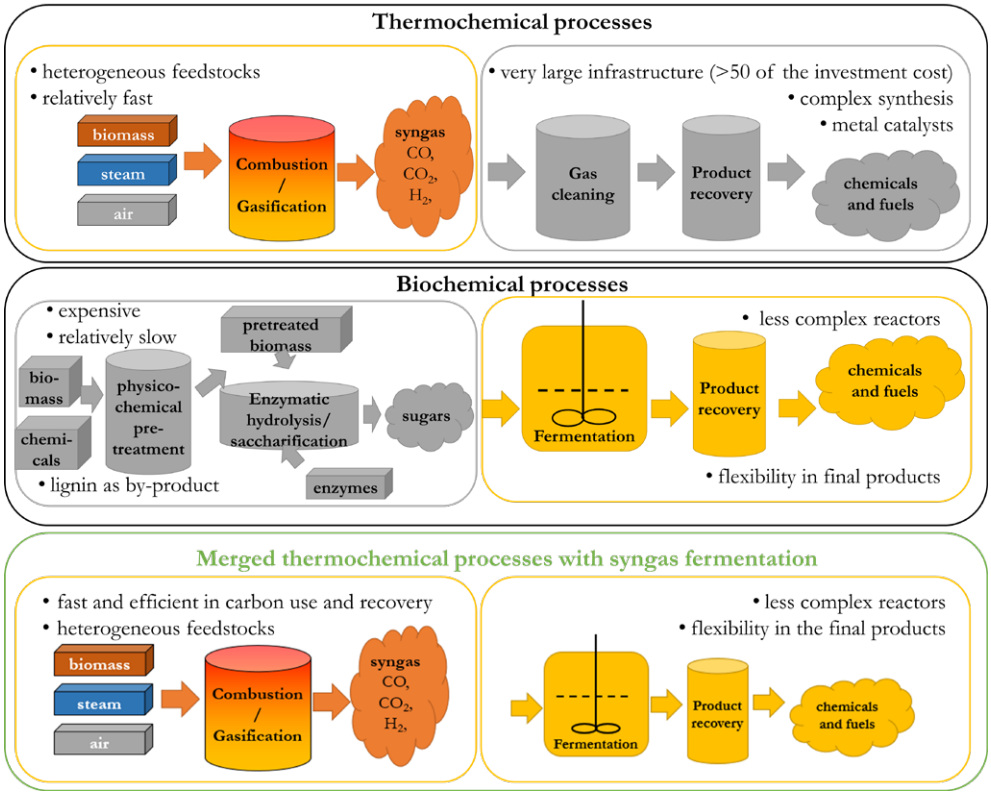


**Figure 1. An overview of how thermochemical and biochemical processes are merged in syngas fermentation processes.**

# 2 Background

According to the Intergovernmental Panel on Climate Change (IPCC), extensive changes will be necessary in energy systems to replace fossil fuels with sustainable energy sources, if we are to limit global warming to less than 2° C. This will include the application of technologies to remove $CO_2$ from the atmosphere, i.e., carbon capture and storage. It is expected that the demand for food, bioenergy and other biobased products will increase dramatically in the future. Current land use is often unsustainable, and society faces the challenges of addressing this while increasing biomass production to meet growing demands. It is expected that the use of organic waste and residues and the cultivation of lignocellulosic plants will be important in this respect. System transformation will involve tightening the cap on $CO_2$ emissions. Ideally, all the carbon entering a production process should be incorporated into the final product. Furthermore, technologies and systems that can use diverse sources of feedstock are desirable.

Microbial fermentation processes are used to produce many chemicals that are important today and will be tomorrow. These processes currently use sugars as feedstock, utilizing only approximately half the available carbon. The use of synthesis gas, syngas, as feedstock (containing mainly CO, $CO_2$, $CH_4$ and $H_2$ gases) and employing electrochemical methods would make it possible to convert all the carbon in a broad range of feedstocks, such as biomass and industrial and municipal effluents, into valuable products such as fuels and commodity chemicals. Microbial fermentation processes thus have great potential in our efforts to reach ambitious climate targets and contribute to a sustainable transportation sector and a sustainable production of chemicals. Syngas fermentation is not yet established in the Swedish industrial sector, where waste biomass and formed syngas is commonly burned for heat and energy, leading to large $CO_2$ emissions (Avfall Sverige 2018). Syngas fermentation thus offers new important advantages to chemical conversion of syngas. Many different products can be produced from syngas, the production scale used can be much smaller than chemical conversion routes, which improves the flexibility.

However, several challenges must be addressed before gaseous substrates can be used as sources of carbon and energy in industrial microbial fermentation processes. These include selection or construction of efficient syngas-fermenting organisms and developing novel fermentation approaches. Moreover, even though microorganisms are more tolerant towards impurities in the syngas compared to chemical processes, some compounds in raw syngas may inhibit the growth of microorganisms. In this project, the main focus was to study how microorganisms interact with these impurities and to improve their tolerance in order to improve the syngas conversion rate.

## 2.1 PREREQUISITES FOR SYNGAS FERMENTATION IN SWEDEN

Several Nordic countries have adopted strict environmental policies whereby municipal solid waste (MSW) incineration and gasification have become the primary means of non-recyclable waste management. In Sweden 34 incinerator

facilities annually handle 2.2 – 2.5 million tons of MSW and recover it into energy and heating (Sundqvist 2017). In 2017, more than 18.3 TWh of energy was produced from this form of recovery which included district heating for housing and electricity generation (Avfall Sverige 2018). Therefore, MSW incineration is a crucial part of managing waste as well as supporting energy production within Sweden.

Within this same concept, gasification is also a widely adopted process within Sweden that aims to handle forest waste residue, agricultural waste and industrial waste. Although it is technologically similar to incineration, it is arguably superior as it maximises waste conversion into a wider variety of high-value flue gases such as CO, $H_2$, $CO_2$, and $CH_4$ of which are the key components of syngas (Arena 2012). Syngas has been utilised for decades as a feedstock for many thermochemical processes that include the Fischer–Tropsch process which enables the production of chemicals and fuels (Dry 2002). However, significant disadvantages of Fischer–Tropsch have included the need for high purity syngas, high temperatures and expensive running costs, which have prevented it from being widely established in many countries. The answer to this problem has been to turn towards microbially fermenting syngas which does not require stringent gas ratios or high temperatures, and irreversible biocatalytic reactions ensure a steady unidirectional product output. As a result, it is not only possible but it may be economically viable to tap into industrial waste-gas streams (Daniell et al. 2012; Liew et al. 2016).

The syngas fermentation technology has already been put into commercial practice by the multinational company Lanzatech in cooperation with two Chinese steel mill plants producing 0.1 million gallons of ethanol per year (Liew et al. 2016). New schemes in Europe are currently underway with a € 150 Million Lanzatech facility development in Belgium in partnership with ArcelorMittal (Dürre 2017).

This similar success could be positively envisioned within Sweden, that has a longstanding tradition of MSW incineration processes, and a growing gasification sector aiming to produce considerable levels of syngas. Combining these opportunities with already well-established microbial fermentation knowledge, may contribute towards a sustainable Swedish chemical and fuel circular economy, whilst mitigating greenhouse gas emissions.

## 2.2 BIOELECTRICAL FERMENTATION

Recently, bioelectrochemical systems (BESs) have received a lot of attention as BESs are new techniques for storing energy or converting chemical energy present in biomass/waste streams to more valuable forms such as electricity, fuels and biogas. BESs are relying on microorganisms and their abilities in electron transfer as a vital process for sustaining their viability. Microorganisms can gain energy by oxidizing compounds with low reduction potential (electron donors) and reducing compounds with high reduction potential (electron acceptors). The capability of microorganisms to use a solid-state electrode as electron donor or electron acceptor has been demonstrated (Logan et al. 2006; Rabaey et al. 2011). Such an ability of

microorganisms can be used in BESs for catalysing different reactions on electrodes.

Microbial electrosynthesis cells (MECs) are one type of BESs, which could be used for recovering the biochemical energy present in waste streams (Modin and Gustavsson 2014). In MECs, the electrons are given an extra energy boost by applying an external input voltage, then, microorganisms present on the cathode harvest these electrons to reduce soluble compounds such as carbon dioxide. MECs can produce valuable bio-gases, such as hydrogen (Escapa et al. 2016; Liu et al. 2005; Oh and Logan 2005; Rozendal et al. 2006) and methane (Cheng et al. 2009; Villano et al. 2011) as well as valuable chemicals such as ethanol (Steinbusch et al. 2010), acetate (Marshall et al. 2012; Marshall et al. 2013; Nevin et al. 2010), and caproate (Van Eerten-Jansen et al. 2013).

Recently, microbial fermentation of industrial wastes such as syngas into more valuable chemicals become a hot topic among researchers both in academia and in industry. In syngas fermentation, microorganisms use carbon monoxide and hydrogen as an electron donor and carbon dioxide as an electron acceptor for producing chemicals such as acetate. In the presence of a solid electrode, the CO fermentation was shown to be enhanced due to better availability of electrons (Im et al. 2018). Combining microbial fermentation of syngas with BES technology would help us build a platform for sustainable production of chemicals such as ethanol.

## 2.3 ROLE OF ACETOGENIC BACTERIA IN SYNGAS FERMENTATION

Studies into microbial gas fermentation have mainly involved a group of anaerobic, carbon monoxide and hydrogen-utilising species that can grow in moderate or high temperatures (Henstra et al. 2007). This collection of bacteria are commonly referred to as acetogenic bacteria, or "acetogens", that use $CO_2$ and $H_2$ for growth and energy respectively, and to which the final acetogen metabolic products are acetate and ethanol (Drake et al. 2008). Acetogens play an integral role in the global carbon-assimilation cycle as they are extremely common in nature and can be found in soil sediments, intestinal tracts of humans and mammals, and form symbiotic relationships with insects (Drake et al. 2006). Therefore, the importance of acetogens cannot be understated. Within the known 22 genera of acetogens, *Acetobacterium* and *Clostridium* accommodate the most known acetogenic species (Drake et al. 2008). Several *Clostridium* species have been shown to be capable of performing syngas fermentation (Phillips et al. 2015) and catalysing cathodic reactions (Liu et al. 2018).

Converting $CO_2$ into acetate relies on the ancient Wood-Ljungdahl pathway (WL-pathway), which allows acetogens to conserve energy and produce one molecule of acetate for every two molecules of $CO_2$ assimilated. Like many bacteria, acetogens can utilise sugars as a primary growth and energy substrate but with the WL-pathway, it enables them to thrive in competitive, anaerobic environments. Within the context of industrial bioprocesses, acetogens are able to utilise mixtures of industrial waste syngas ($H_2$/$CO_2$/CO) into acetate, ethanol and other native products (e.g.: butyrate, butanol, 2,3-butanediol, hexanol, propionate), dependant

on the species (Daniell et al. 2012; Debabov 2013; Imkamp and Müller 2007; Köpke and Liew 2011; Ragsdale and Pierce 2008; Schuchmann and Müller 2014). Moreover, advances in genetic manipulation have allowed modification of acetogens towards production of a wider spectrum products that include biodegradable polymers such as 3-hydroxypropionate and isoprene or industrially important 2-butanol, butanone, acetone and isopropanol solvents (Chen et al. 2013; Köpke et al. 2013; Köpke and Liew 2012; Mueller et al. 2013). Furthermore, genetic manipulations have enabled the development of strains better able to tolerate high solvent concentrations.

# 3 Objectives

Our long-term vision is to develop sustainable biorefineries at intermediate scales (20-100 MW fuel input) that can make full use of the carbon and energy content of any feedstock for production of renewable chemicals and fuels.

Prior to inoculation of the fermentation medium, microbial cells are propagated in a cultivation step to obtain a suitable cell biomass to be used for inoculation. It was known that this propagation has a wide influence on the fermentation performance and that cells can be adapted for better tolerance during the propagation. Our hypothesis was that an efficient propagation process for the microbe would allow efficient conversion of real syngas into ethanol.

The main objective of this project was to develop an efficient propagation scheme for a selected, syngas fermenting bacteria. The following tasks were set in relation to the objective:

1. Mapping the inhibitory syngas components and conducting a survey on their microbial influence.
2. Evaluation of different microbes to be used as biocatalysts.
3. Developing an efficient propagation process for the biocatalyst.
4. Evaluation of the fermentation capacity of propagated cells.

# 4 A laboratory for working with acetogens and syngas

Acetogenic bacteria require continuous, strict anaerobic conditions in order to survive and propagate. Therefore, all handling and cultivation of the syngas fermenting bacteria was done in an oxygen-free environment (Figure 2). This was possible through the usage of a state-of-the-art anaerobic workstation that can handle a vast range of anaerobic bacteria in a highly controlled environment that maintains a set temperature and humidity.

**Figure 2. The workstation uses a special gas mix to maintain a highly anaerobic environment and is able to support two users.**

When culturing acetogens in serum flask experiments (Figure 3) using components of syngas ($CO_2$, CO and $H_2$), special care is required as some of these gases are highly toxic (CO) and explosive (CO + $H_2$). Therefore, users of the above systems must carry personal CO detectors. Our lab is also fitted with a central CO detector that monitors for leaks, but above all training ensures that users are aware of the risks present within this work.

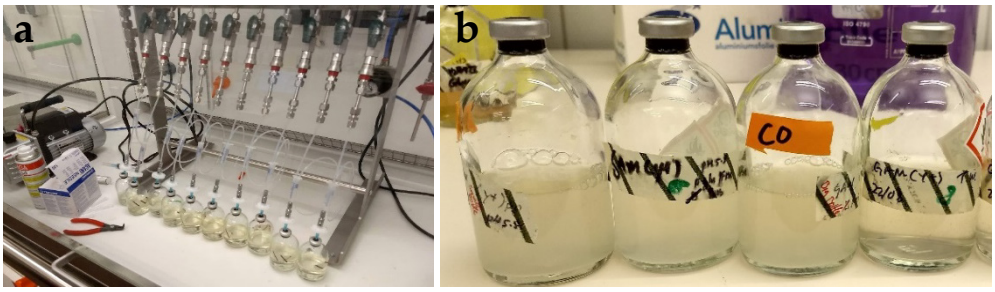


**Figure 3. a) Gas exchanger system used to apply gas into several serum flasks via a needle. b) Pressurised bottles containing bacterial culture.**

# 5 Choosing the microbe to be used as biocatalysts

A large number of microbes have been shown capable of syngas fermentation, *Clostridia* being the most widely used. The choice of microbe for this project and coming syngas fermentation activities set an important milestone for the project.

## 5.1 A LARGE NUMBER OF BACTERIA CAN GROW ON GAS

An extensive literature search was performed to map bacteria and their potential for syngas fermentation. More than 1300 bacteria capable to grow on CO were found in commercial depositories. A phylogenetic mapping of these bacteria shows that these bacteria are genetically very distant from each other (Figure 4). 98 different bacteria were compared based on published information in a BSc study performed within this project (*BBTX01-18-01, Chalmers University of Technology).*

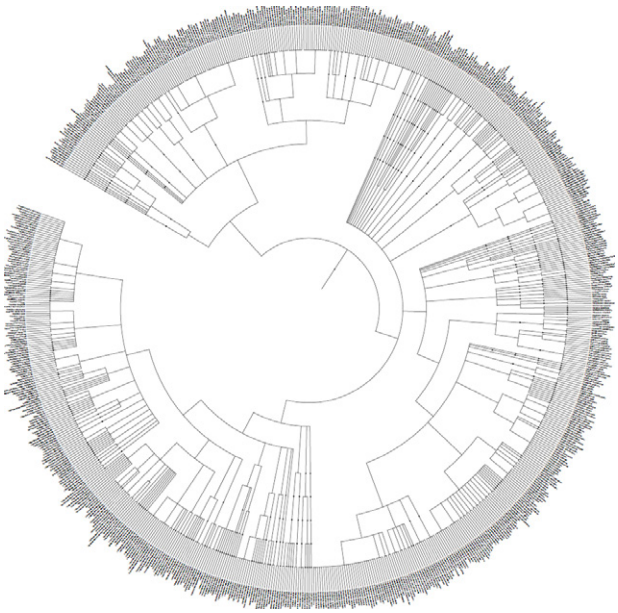

**Figure 4. Phylogenetic mapping of bacteria capable to grow on CO.**

## 5.2 SELECTION CRITERIA FOR THE SYNGAS FERMENTING BACTERIA

The selection of acetogenic microbes for our work was done based on the following criteria:

The organism is

1. capable of autotrophically using CO, $H_2$ and $CO_2$.
2. has been relatively well studied and/or is regarded as a “model acetogen”.
3. is responsive to electricity and can produce a current.
4. does not require specialised growing conditions other than anaerobic, and grows at moderate temperatures of 30 – 37 °C.

From these four points, the following acetogenic bacteria were selected for experimental work; *C. autoethanogenum, C. ljungdahlii, C. carboxidivorans*, *C. scatologenes*, *C. drakei* and *Sporomusa ovata.*

*C. autoethanogenum* and *C. ljungdahlii* are closely related and relatively well characterised in the context of syngas fermentation. Originally isolated from the rabbit gut and chicken manure respectively, studies later have highlighted their industrially important gas utilising metabolisms. Both organisms are known for producing notably high levels of ethanol when cultivated on syngas and have been genetically modified to produce products such as butanol, 2-butanol, butanone, acetone, isopropanol, 3-hydroxypropionate and isoprene products, from syngas using genes from a non-acetogenic *Clostridia* species. Both *C. ljungdahlii* and *C. autoethanogenum* have been commercially exploited by several companies to date and are regarded as model acetogens.

*C. carboxidivorans*, *C. scatologenes* and *C. drakei* were noted for their genetic similarities but have slightly different gas metabolising abilities. These organisms all have a wide spectrum of products other than the typical acetate and ethanol, which include butyrate, hexanoate, butanol and hexanol. Each compound has a high value and is regarded as a "drop-in" fuel meaning that very little further processing is needed when wanting to incorporate them in into an existing fuel infrastructure. Despite this, these organisms are not as well characterised as *C. autoethanogenum* and *C. ljungdahlii*, and do not have many examples in large scale syngas fermentation or bioelectrochemical systems.

Finally, the last organism chosen was *S. ovata*, the only non-*Clostridium* species, which has been noted for its exceptional electrochemical performance. Although unremarkable in terms of product repertoire, which is predominantly acetate, it had been shown to be able to utilise syngas alongside electrons.

## 5.3 SELECTION OF ACETOGENS FOR BIOELECTROCHEMICAL SYSTEMS

In order to enable bioelectrochemical work ability for biofilm formation on the bio-cathode is crucial. For enhancing biofilm formation, the bacteria were propagated in rich medium with the carbon felt, that was then transferred to subsequent BES experiments (Figure 5).

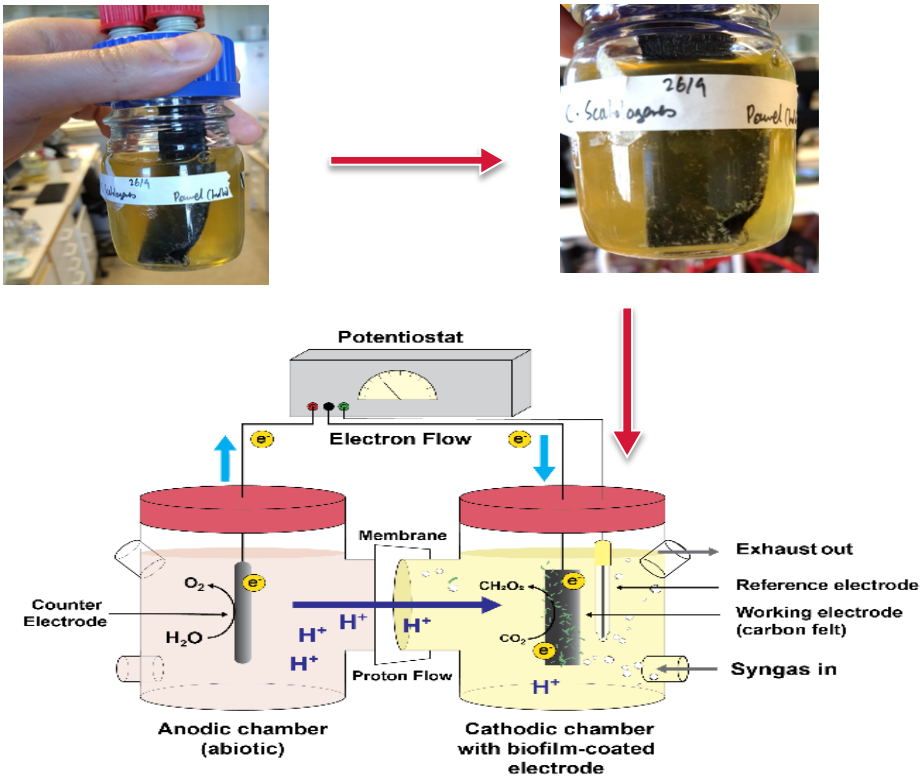


**Figure 5. Biofilm formation strategy. Biofilm cultivation took place in a separate vessel with rich medium, before the bio-electrode was transferred to a prepared MEC that contained fresh, anaerobic medium.**

A "General Acetogen Medium" was developed from literature (Groher and Weuster-Botz 2016) and adapted to our strains, in order to be able to compare the different acetogens to each other. The experimental screenings were limited by temperature as testing was conducted at room temperature (22° C), and therefore several candidates could not perform to their maximum potential. Despite this, *C. scatologenes* proved to be an excellent candidate as it is fast growing, able to propagate at room temperature and formed favourable biofilm on the cathode (Figure 6, Table 1). *C. scatologenes* was shown to be electroactive, but we also found that it did not grow on gas as only substrate.

**Table 1: Microbes tested as biocatalysts.**

| Acetogen species | Optimal Temp °C | Biofilm formation? |
|---|---|---|
| *Clostridium autoethanogenum* | 37 | Yes - low |
| *Clostridium ljungdahlii* | 37 | Yes - low |
| *Clostridium scatologenes* | 30 | Yes - visible |
| *Clostridium drakei* | 30 | Yes - low |
| *Clostridium carboxidivorans* | 37 | Yes - visible |
| *Sporomusa ovata* | 30 | No growth |

The physiological traits for evaluating cellular performance that were chosen includes; product formation through HPLC measurement, pH change over time, growth monitoring through cyclic voltammetry, morphology and viability of the cells studied via microscopy.

# 6 Syngas composition & inhibitors

Small amounts of toxic compounds in raw syngas may inhibit the growth and productivity of the microorganisms. In this project, the syngas composition mapped and the effect of certain inhibitors on the syngas fermenting microbes was studied.

The long term goal of our work is to address whether crude syngas, regardless of compositions or inhibitors is able to be applied directly into a microbial fermentation system. In the scope of this project, practical work was focused on elucidating the impact of ammonia and BTX-compounds (benzene, toluene and xylenes) on the performance of acetogens. No studies on aromatic hydrocarbons in acetogenic gas fermentations had so far been published, yet these compounds are commonly found in many gasification and petroleum refining processes.

## 6.1 SYNGAS: CO, $CO_2$ AND $H_2$ + SMALL AMOUNTS OF OTHER COMPOUNDS

Data on syngas composition was collected from different gasification companies in Sweden and from the Chalmers' gasification plant. This revealed that amounts of each inhibitor present in a specific process differs (Table 2). Although found in relatively low concentrations, the inhibiting components may pose a serious challenge to the microbe's fitness and fermentative efficiency.

**Table 2. Inhibitors in industrial syngas streams.**

| Trace Components | Relative abundance |
|---|---|
| Acetylene | ~2 % |
| Benzene | ~2 % |
| Ethylene | ~2 % |
| Hydrogen sulphide | ~0,2 % |
| Propane | ~0,01 % |
| Propene | ~0,3 % |
| Ammonia | <10 ppm |
| Carbon disulphide | <1 ppm |
| Carbonyl sulphide | <1 ppm |
| Hydrogen cyanide | <10 ppm |
| BTX (Benzene, Toluene, Xylene) | (11,06 g/m$^3$) |
| Tars | (21,33 g/m$^3$) |

Data collected at the Chalmers heating plant.

The data on the composition of inhibitors found in syngas (Table 2), shows that according to previous studies, several inhibitors are already microbially tolerated beyond these concentrations, (Gupta, Ahammad, and Sreekrishnan 2016; Oswald et al. 2018; Xu, Tree, and Lewis 2011). Cyanide in the syngas fermentation process has been reported as a serious challenge for commercial syngas fermentation

(Alberts et al. 2016). Still, studies on the matter (Oswald et al. 2018) have described acetogens tolerating levels of hydrogen cyanide much higher than found in the Chalmers heating plant.

Our original plan was to use our growth profiling system (Growth Profiler – Enzyscreen, NL) to monitor and compare growth of several acetogens using a range of inhibitor concentrations. This system is attractive as it enables real-time growth monitoring but after several attempts it was concluded that the system is not suitable for strict anaerobic fermentations, the anaerobic chambers provided was shown to allow some oxygen leakage, which hinders growth of the acetogens. Therefore, inhibition studies were performed in closed serum bottles filled with 50 mL of growth medium. This proven strategy provided fewer growth time points, but it enables analytical sampling – which is imperative to determining at which inhibitor concentration, fermentative product formation is affected.

Three different *Clostridia* species, *C. autoethanogenum*, *C. carboxidivorans* and *C. ljungdahlii* were grown in media with 0-100 mM ammonia (Figure 6). *C. ljungdahlii* was found to be the least inhibited by ammonia among the species studied, when production of ethanol was set as the selection criteria. All species tested could however tolerate ammonia concentrations that is commonly found in industrial syngas mixtures. Up to 20 mM ammonia did not influence ethanol production capacity of any of the acetogens, whereas ammonia concentrations of industrial syngas typically is below 10 ppm (Table 2).

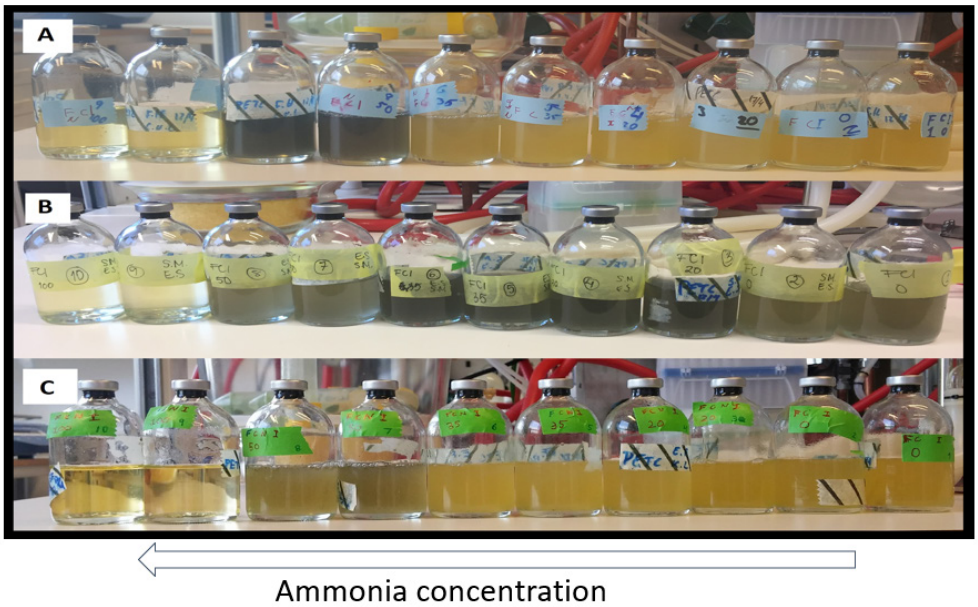


**Figure 6. Serum bottles with A) *C. autoethanogenum*, B) *C. carboxidivorans* and C) *C. ljungdahlii*, grown on general acetogen medium with 0 – 100 mM ammonia for ten days.**

## 6.2 UNDERSTANDING SYNGAS AS A MICROBIAL SUBSTRATE

Key chemical components of syngas are CO, $CO_2$ and $H_2$. The optimal mixture for microbes is crucial to guarantee efficient fermentation, and desirable product

output. However, to obtain the ideal stochiometric ratio for microbial gas fermentation outside the laboratory conditions is very difficult. We have gained an insight into different industrial syngas compositions by obtaining data from three independent sources, see Table 3.

**Table 3. Examples of industrial syngas compositions**

| Source | % $H_2$ | % CO | % $CO_2$ | % $N_2$ | % $CH_4$ | % Other |
|---|---|---|---|---|---|---|
| **Chalmers heating plant** | 42 | 17 | 28 | 4 | 7 | 2 |
| **Cortus energy AB** | 50 - 60 | 18 -30 | 8 - 17 | n/a | 1 - 3 | n/a |
| **Meva energy AB** | 10 | 20 | 12 | 50 | 3 | 5 |

This data details the syngas composition from a gasification process, e.g.: gasification of wood waste (Chalmers heating plant). Typically, when cultivating acetogens in laboratory setting, the bacteria are grown on either 100 % CO as it is both an electron and carbon source, or a ratio of 80:20 $H_2$:$CO_2$. When all three components are present, these gases may in theory pose an inhibitory effect on growth, but in practice it has been observed that this is not entirely the case – with reports of high ethanol production using mixtures of CO, $CO_2$ and $H_2$ (Liew et al. 2013; Molitor et al. 2016). Overall, the key to high productivity is a readily available source of electrons, either from CO or $H_2$, but yet again there are more challenges present, both gases may be present in constantly changing ratios in crude mixtures and both gases have poor gas to liquid solubility which impacts fermentation efficiency.

We are approaching this problem in a unique way; through the direct transfer of electrons, extra-cellularly, using a microbial electrosynthesis system. It has already been shown in several studies that it is possible to utilise such a system with an electro-active microbe, and in turn produce organic acids and alcohols. In theory, it would then be possible to utilise a highly variable mixture of syngas with a consistent electron source and obtain a steady stream of target fermentative products. To this end, we are developing a system to use acetogens as our biocatalysts, combined with electrochemically-assisted syngas fermentation.

## 6.3 INFLUENCE OF BTX COMPOUNDS AS INHIBITORS

Data provided by the Chalmers heating plant showed that the most abundant inhibitors were benzene, toluene and xylenes (BTX, Table 4; in total up to 11 mg/L). The BTX compounds are by-products of wood gasification and studies have described potentially harmful effects they may have upon microorganisms. This includes dissolving the bacteria's cell membrane, causing leakiness and ultimately cell death (Isken and de Bont 1998). No such studies have been performed yet in acetogens, therefore we set out to answer the following questions:

1. Can a model acetogen tolerate levels of BTX inhibitors as described in Chalmers heating plant data?
2. What is the maximum BTX concentration level that an acetogen can tolerate?
3. Is gas utilisation effected by BTX inhibitors?

The abundance of each component in the BTX mixture was determined to be the following: benzene 6.63 mg/L, toluene 2.79 mg/L and xylenes 0.63 mg/L, the BTX component ratio being roughly 7:3:1. The inhibitory effect of benzene alone (the main component) or the three BTX compounds in a mixture reflecting the ratio that was found in the Chalmers heating plant syngas output was tested (Table 4). The tolerance of the BTX compounds was tested in medium with fructose (Figures 7-8) and CO (Figures 9-11) as a carbon source.

**Table 4. Concentrations of BTX compounds used (in mM).**

| Condition | Benzene | Toluene | Xylenes |
|---|---|---|---|
| Control | 0 | 0 | 0 |
| 1 | 0.05 | 0 | 0 |
| 2 | 0.1 | 0 | 0 |
| 3 | 0.5 | 0 | 0 |
| 4 | 1 | 0 | 0 |
| 5 | 2 | 0 | 0 |
| 6 | 5 | 0 | 0 |
| 7 | 0.05 | 0.021 | 0.007 |
| 8 | 0.5 | 0.21 | 0.07 |
| 9 | 1 | 0.43 | 0.14 |
| 10 | 2 | 0.85 | 0.28 |

When grown in presence of only benzene (conditions 1-6, Table 4), our selected acetogen showed no differences in growth or ethanol and acetate production for concentrations up to 1 mM benzene (Figure 7). Above this concentration, with 2 mM and 5 mM of benzene no detectable growth or product formation was observed. The tolerance of our acetogen to benzene was thus much higher than concentrations reported in the Chalmers heating plant (11.06 mg/L or 0.14 mM).

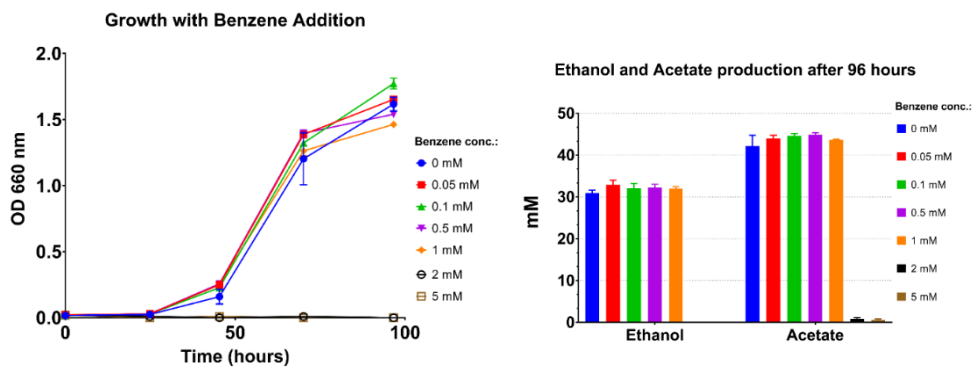


**Figure 7. Growth profiles of cultures grown on general acetogen medium with fructose, with various concentrations of benzene (left) and corresponding product formation in cultures after 96h (right).**

Even though tolerance towards benzene was remarkably high, it was hypothesized that combining several hydrocarbons may have an even more pronounced affect in comparison to benzene alone. Indeed, combining the three BTX compounds led to a greater inhibition on growth. For concentrations beyond 1 mM benzene, 0.43 mM toluene and 0.14 mM xylene, no growth was seen, no fructose was consumed, nor ethanol produced (Figure 8). Still, tolerance of up to 0.5, 0.21 and 0.07 mM of benzene, toluene and xylenes respectively, is below concentrations typically found in syngas.

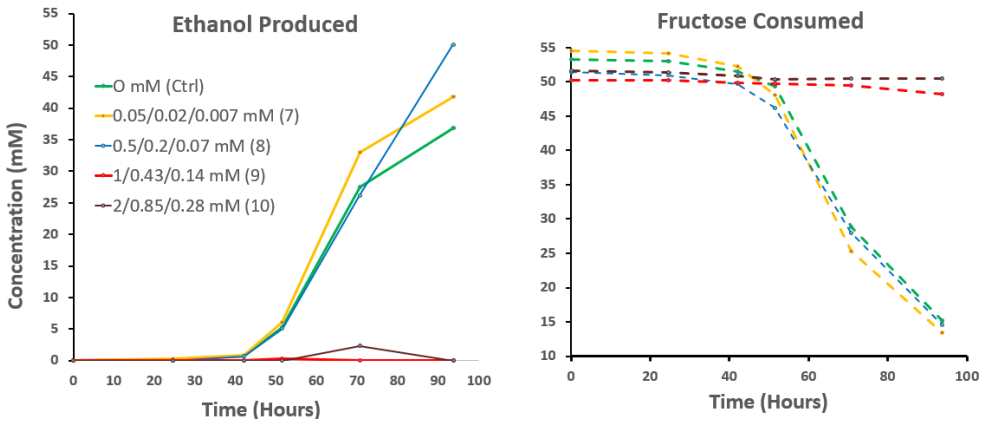


**Figure 8. Ethanol production and fructose consumption profiles of cultures grown general acetogen medium with fructose and various concentrations of benzene, toluene and xylene.**

After our chosen biocatalyst was deemed sufficiently tolerant when propagated in sugar, the next important step was to determine whether it can behave similarly when utilising a gas substrate. First, a preliminary test was done using general acetogen medium containing serum flasks filled with CO gas. The acetogen was pre-cultured on fructose to a suitable biomass density and applied to the gas culture. A similar range of concentrations of added inhibitors were added in 5 different conditions, as described in table 4, conditions 7-10, and the organisms were incubated for 30 days to allow for adaptation on a gas substrate. Results at the end of this period revealed a moderate tolerance only up to condition 7 with 0.05/0.021/0.07 mM of the BTX inhibitors. When grown on gas thus, it seemed that the tolerance towards the inhibitors was much lower compared to when grown on fructose, emphasizing the need for an efficient propagation scheme allowing for a higher tolerance.

# 7 Developing an efficient propagation process

The propagation process has been shown to greatly influence fermentation. Bacteria growing in presence of inhibitors are well known to become more tolerant to these over times, but the relation between the propagation conditions and the tolerance towards inhibitors in syngas has not been studied before. Therefore, we set out to establish a propagation scheme that would improve the tolerance of acetogens towards BTX compounds.

## 7.1 PROPAGATION AT LOW INHIBITOR CONCENTRATIONS

Low inhibitor concentrations were used to gradually propagate the acetogen. Here, we chose to work with the long-established acetogen medium PETC 1754 that will be used for coming syngas experiments. The concentration of inhibitors in the different propagation batches are described in Table 5.

**Table 5. Concentrations of BTX compounds used (in mM) in the different batches.**

| Batch | Condition | Benzene | Toluene | Xylenes |
|---|---|---|---|---|
| | Control | 0 | 0 | 0 |
| 1 | 1 | 0.05 | 0.021 | 0.007 |
| 1 | 2 | 0.1 | 0.042 | 0.014 |
| 1 | 3 | 0.2 | 0.085 | 0.028 |
| 1 | 4 | 0.4 | 0.19 | 0.056 |
| 2 | 5 | 0.1 | 0.042 | 0.014 |
| 2 | 6 | 0.2 | 0.085 | 0.028 |
| 2 | 7 | 0.3 | 0.13 | 0.042 |
| 2 | 8 | 0.4 | 0.19 | 0.056 |
| 3 | 9 | 0.2 | 0.085 | 0.028 |
| 3 | 10 | 0.3 | 0.13 | 0.042 |
| 3 | 11 | 0.4 | 0.19 | 0.056 |
| 3 | 12 | 0.5 | 0.21 | 0.07 |

After the preculture was fully adapted on CO, the acetogen was transferred into serum flask, with appropriate amount of inhibitor added, pressurised with CO gas and incubated at 37° C. The experiment took place over 7 days with 3 time point taken at the start, middle and end of the experiment. The first batch (Figure 9), showed a significant improvement over the preliminary experiments with tolerance of up to 0.2/0.85/0.28 mM (condition 3), with relatively unaffected acetate yields. Ethanol production was shown to be highest in condition 3, which was unexpected, as the presence of inhibitors slowed down the growth of the strain (Figure 9). Similarly, in condition 3 the CO consumption rates measured through lower partial pressure at the end of fermentation, was higher (Figure 9d), when compared to condition 2 (0.1/0.42/0.14 mM).

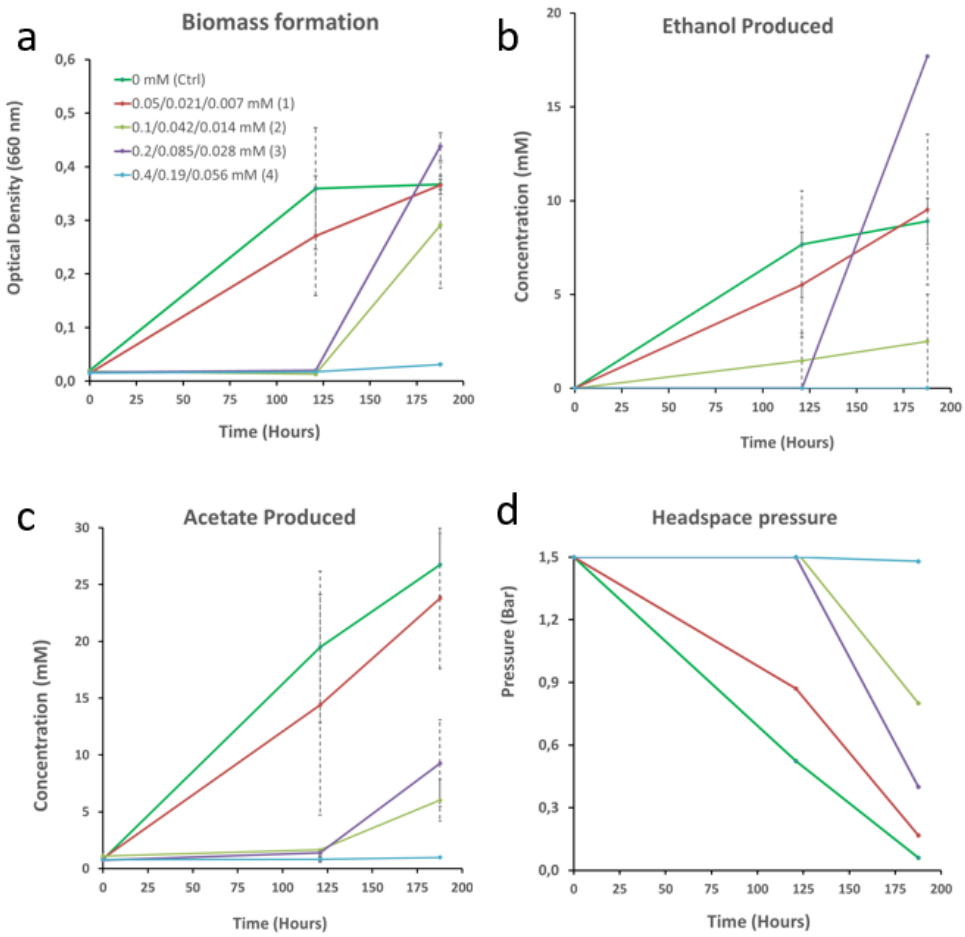


**Figure 9. Batch 1. a) Biomass formation (through optical density measurement), b) ethanol, c) acetate production and d) headspace pressure (indicating CO consumption) of cultures grown on PETC 1754 medium with CO and various concentrations of benzene, toluene and xylene. The bacteria were propagated, pre-adapted to growth on CO.**

## 7.2 A PROGRESSIVE IMPROVEMENT OF INHIBITOR TOLERANANCE

From the first batch, serum flasks containing acetogens with the highest exhibited tolerance were transferred into a fresh serum flask, making up batch 2, containing inhibitors at slightly elevated concentrations (Table 5). Starting at 0.1/0.42/0.14 mM (condition 5) and including a new concentration, 0.3/1.3/0.42 mM (condition 7), a similar experiment was conducted to assess if propagated cells can tolerate increased BTX amounts (Figure 10).

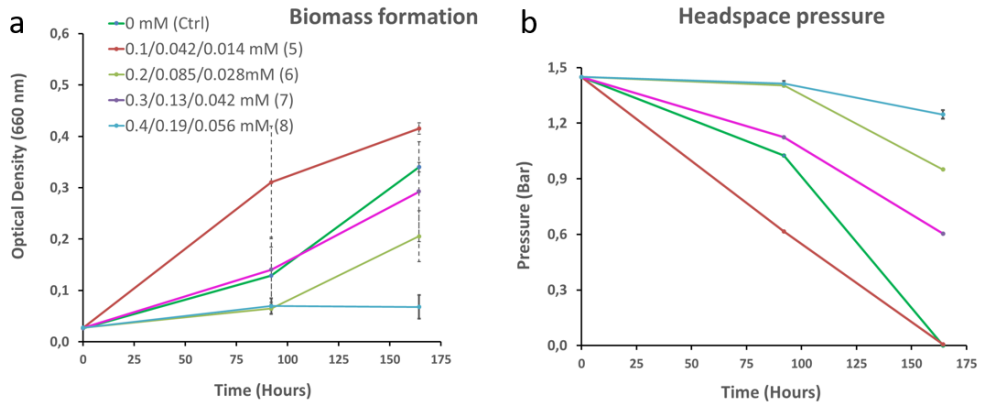


**Figure 10. Batch 2. a) Biomass formation (through optimcal density measurement) and b) headspace pressure (indicating CO consumption) of cultures grown on PETC 1754 medium with CO and various concentrations of benzene, toluene and xylene. The bacteria were propagated, pre-adapted to growth on CO and the BTX inhibitors.**

The adaptation towards the BTX inhibitors showed a clear improvement in tolerance, with growth in condition 7 (0.3/1.3/0.42 mM) exceeding the previous tolerance level (Figure 11). Gas consumption was also markedly improved, with even the highest BTX concentration treated flasks exhibited gas utilisation.

In order to determine if the adaptation could be even further improved, the acetogens with the highest exhibited tolerance were transferred into a fresh serum flask (batch 3) containing various BTX concentrations, conditions 9-12. Remarkably, the tolerance towards the BTX compounds was now gretly improved (Figure 11). The growth was high even in precense of the highest concentrations of inhibitors tested (0.5/0.21/0.07 mM, condition 12, Figure 11). Moreover, the CO consumption was complete in all conditions signifying that the acetogen is largely unaffected by the presence of the inhibitors.

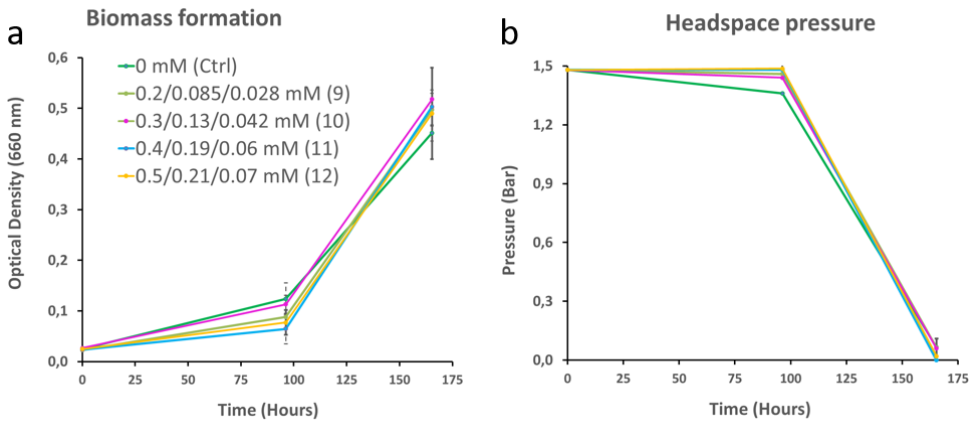


**Figure 11. Batch 3. a) Biomass formation (through optimcal density measurement) and b) headspace pressure (indicating CO consumption) of cultures grown on PETC 1754 medium with CO and various concentrations of benzene, toluene and xylene. The bacteria were propagated, pre-adapted to growth on CO and the BTX inhibitors. There were no significant differences between any of the conditions and the control.**

In conclusion, the propagation scheme developed was proven to be very efficient, allowing growth on CO at BTX concentration applied that exceeds approx. 4.5-fold of what is currently found in the Chalmers gassification plant's syngas output. A smilar gradual propagation on syngas can be expected to allow efficient fermentation of industrial syngas.

# 8 Summary of results

The following acetogens were evaluated as biocatalysts to be used in the project: *C. autoethanogenum, C. ljungdahlii, C. carboxidivorans*, *C. scatologenes*, *C. drakei* and *S. ovata.* A "General Acetogen Medium" was developed in order to be able to compare the different acetogens to each other. Product formation, pH alteration over time, growth monitoring through cyclic voltammetry, morphology and viability were used as evaluation criteria when determining the best suited biocatalyst. The biocatalyst used and experimental details for studying inhibitor tolerance and developing the propagation process is not disclosed here as the publication of the work is pending.

Data on syngas composition was collected from different gasification companies in Sweden and from the Chalmers' gasification plant. Although found in relatively low concentrations, inhibiting components may pose a serious challenge to the microbe's fitness and fermentative efficiency. Several inhibitors were already known to be microbially tolerated beyond the concentrations typically found in industrial syngas, while the influence of other inhibitors remained to be explored. Here we studied the effect of ammonia, benzene, toluene and xylene on the hetero- or lithotrophic growth of our chosen biocatalyst. Up to 20 mM ammonia or 0.5, 0.21 and 0.07 mM of benzene, toluene and xylene respectively, did not influence ethanol production during heterotrophic growth. These concentrations of inhibitors are way above what is typically found in industrial syngas. On the contrast, during lithotrophic growth (on gas), the tolerance towards the inhibitors was much lower and beyond concentrations in industrial syngas, emphasizing the need for an efficient propagation scheme allowing for a higher tolerance.

Through this project a very efficient propagation scheme was developed. The propagation process was shown to greatly influence fermentation and microbes growing in the presence of inhibitors became more tolerant to these over times. We showed that by progressively adapting acetogens to higher concentrations of benzene, toluene and xylene, tolerance beyond concentrations find in industrial syngas could be achieved. After three gradual adaptation steps, the biocatalyst produced ethanol from CO (the energy containing component of syngas) in the presence of benzene, toluene and xylene exceeding concentrations found in industrial syngas by 4.5-fold, with similar efficiency as with pure CO. This is a major advancement towards fermentation of industrial syngas.

# 9 Outlook

The project aimed at developing a methodology for producing bioethanol from syngas, produced by gasification of biomass. The focus was on overcoming major obstacles related to process inhibition, as impurities found in syngas, were hypothesized to disrupt cellular growth and lower productivity. Through this project we have developed a propagation scheme, that allows the production of growth and production of ethanol from CO (the energy containing component of syngas) in the presence of inhibitory BTX compounds (benzene, toluene and xylene), with similar efficiency as with pure CO. This is a major advancement towards fermentation of industrial syngas.

Future work will focus on 1) elucidating mechanisms for the acquired tolerance 2) further developing the propagation strategy and 3) adaptation of the developed propagation strategy to a continuous syngas fermentation.

This work has been part of a larger effort of setting up syngas fermentation activities at the division of Industrial Biotechnology at Chalmers. The newly established syngas laboratory and accumulated knowledge allows us to tackle the great challenge of merging microbial electrosynthesis with syngas fermentation, which is expected to greatly increase the process efficiency. The potential of syngas fermentation is substantial as it could prove to be a novel strategy in capturing carbon, reducing greenhouse gas emissions and producing sustainable chemical and fuel bio-commodities.

Syngas fermentation is a particularly attractive alternative for converting emissions gases from small or medium-sized gasification plants. Such gasification plants are commonly found in Sweden for incineration of municipal waste. Chemical conversion, which is a mature technology for producing chemicals from large gasification plants is not a viable alternative for smaller waste incineration plants due to large amounts of nitrogen in the output gas which hinders chemical conversion. Microbial production is not sensitive to nitrogen and has the added value of allowing for high specificity and production of higher value compounds. Syngas fermentation has great potential in adding value for existing gasification plants and contributing to a carbon-neutral bioeconomy.

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# SYNGAS FERMENTATION FOR FFICIENT CONVERSION OF LIGNOCELLULOSE TO BIOETHANOL

The potential of syngas fermentation is substantial as it could prove to be a novel strategy in capturing carbon, reducing greenhouse gas emissions and producing sustainable chemical and fuel bio-commodities.

Bacterial syngas fermentation is a radically new concept for producing renewable fuels of high importance to the Swedish energy sector. Syngas is a mixture consisting primarily of CO, $CO_2$, and $H_2$ that is produced through gasification of many carbon-containing materials, e.g. biomass, municipal waste and from heavy industrial processes. Gasification allows the conversion of literally any carbon-containing material into syngas and syngas fermentations by biocatalysts can fully exploit the carbon, but many scientific challenges persist.

The focus here has been to overcome major obstacles related to process inhibition, as impurities found in syngas, were hypothesized to disrupt cellular growth and lower productivity. A propagation scheme has been developed, allowing the production of growth and production of ethanol from CO in the presence of inhibitory BTX compounds with similar efficiency as with pure CO. This is a major advancement towards fermentation of industrial syngas.

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