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Effect of specific growth rate in continuous cultures of acetogen gas fermentation

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Abstract:

The global demand of fossil fuel and petroleum is on rise. Extensive use of fossil fuel has led to global warming. Gas fermentation is an emerging attractive microbial process which uses acetogen (an anaerobic bacteria) that can convert carbon-containing waste feedstocks (e.g. gasified solid waste, steel-mill gas) into valuable biofuels and chemicals. The general aim is to study the effect of dilution rate in continuous culture acetogen gas fermentation grown under both CO and syngas. *C. autoethanogenum* produces acetate, ethanol, 2,3-butanediol (2,3-BDO) in continuous culture chemostat. In the steady-state continuous culture of Syngas-fermenting acetogen higher the specific growth rate of the culture results in higher products concentration. The performance of Syngas fermentation was superior then CO fermentation at higher dilution rate as ethanol and 2,3 BDO production increased significantly at a higher special growth rate.

Keywords:

Gas fermentation, Acetogen, Wood-Ljungdahl Pathway, *Clostridium autoethanogenum*, biofuel, Gas fermenting acetogen, Syngas.

CERCS:

T490 Biotechnology

Kasvuerikiiruse mõju atsetogeense gaasfermentatsiooni pidevkultuuri protsessile

Lühikokkuvõte:

Fossiilkütuste ja nafta ülemaailmne nõudlus on kasvamas. Fossiilkütuste laialdane kasutamine on kaasa toonud globaalse soojenemise. Gaasfermentatsioon on atraktiivne ning tõusev mikroobne protsess, mis kasutab atsetogeene (anaeroobseid bakterid), mis võimaldavad konverteerida jäätmetes peituva süsiniku (nt gaasitatud tahked jäätmed, terasetehase gaas) väärtuslikeks biokütusteks ja kemikaalideks. Töö üldeesmärk on uurida lahjenduskiiruse mõju atsetogeense gaasfermentatsiooni pidevkultiveerimisel kasutades nii CO-d kui ka

sünteesgaasi. *C. autoethanogenum* toodab kemostaat pidevkultuurides atsetaati, etanooli, 2,3-butaandiooli (2,3-BDO). Sünteesgaasi puhul põhjustas kõrgem kasvuerikiirus ka kõrgema produktide kontsentratsiooni stabiilses steady-state seisundis. Sünteesgaasi protsessi tulemuslikkus oli parem kui CO puhul kõrgema lahjenduskiiruse korral, etanooli ja 2,3 BDO tootmine suurenes märkimisväärselt kõrgema kasvuerikiiruse juures.

Võtmesõnad:

Gaasi kääritamine, atsetogeen, Wood-Ljungdahl Pathway, *Clostridium autoethanogenum*, biokütus, Gaas kääritamine atsetogeen, Syngas.

CERCS:

T490 Biotehnoloogia

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TERMS, ABBREVIATIONS AND NOTATIONS

ABBREVIATIONS	TERM
FTP	Fischer-Tropsch process
WLP	Wood–Ljungdahl pathway
2,3-BDO	2,3-Butanediol
C. autoethanogenum	*Clostridium autoethanogenum*
C. ljungdahlii	*Clostridium ljungdahlii*
A. woodii	*Acetobacterium woodii*
AOR	aldehyde ferredoxin oxidoreductase
ACS	acetyl-CoA synthase
CODH	carbon monoxide dehydrogenase
Nfn	transhydrogenase
PFOR	pyruvate ferredoxin oxidoreductase
Rnf	Rhodocbacter nitrogen fixation
THF	Tetrahydrofolate
WGS	water-gas shift reaction
OD	Optical density
BC	Biomass concentration
DCW	Dry cell weight
MS	Mass spectrometer
HPLC	High-performance liquid chromatography
Eg.	For example

INTRODUCTION

The global demand of fuel and petroleum is on rise. In recent times, the world is highly reliable on fossil fuels. Therefore, the over-reliance and extensive use of petroleum and fossil fuel have led to global warming. The countries are looking for alternative option for fossil fuel. Once such option is biofuel. In this an innovative and full of potential technology for CO_2 valorization and waste recycling is acetogen gas fermentation that uses gasified municipal or industrial off-gas waste as a feedstock and produces useful biofuels and chemicals. Many technologies have developed new ways to produce biofuel from different energy sources, nevertheless, no technology has shown as much potential as gas fermentation acetogen that solves a unique problem for waste management and extensive use of fossil fuel.

Gas fermentation is a microbial process which uses acetogen that can convert carbon-containing waste feedstocks (e.g. gasified solid waste, steel mill gas) into valuable industrial-scale fuels chemicals. In modern times, Gas fermentation has emerged as an appealing option for renewable and sustainable production of high-quality fuels and chemicals from abundant, cost-effective and non-food-based feedstocks e.g. industrial waste gases, syngas (CO/ CO_2 and H_2) and CO. Acetogens are unique type of anaerobic bacteria that utilize the wood-Ljungdahl pathway (most simple and efficient pathway to produce acetate) to fix simpler carbon compounds and converts them into biofuel and other valuable products. Gas fermentation has potential to become profitable industrial scale process.

In Gas fermentation, acetogens are grown under steady-state continuous culture because it provides a higher degree of control than a batch culture. In continuous culture fermentation one the most important aspect is that by changing the dilution rate, biomass concentration can be controlled. The most interesting aspect to study is the effect of specific growth rate on gas fermenting acetogens and how it effects the growth of microbe and to investigate its performance under different dilution rate and acetogen metabolism has not been investigated yet. The study of systems-level quantification of acetogen is necessary for design-build-test-learn bioprocess optimization by metabolic engineering (Valgepea et al., 2017).

1. LITERATURE REVIEW

1.1 General introduction and challenges

The global economy of the world is highly reliable on fossil fuels. Modern-day industries over-reliance and extensive use of oil and fossil fuel have led to global warming. One of the top contributors to global warming is the human-generated greenhouse gases, especially CO and CO_2. To overcome these challenges, On 4 November 2016, the group of 195 countries signed the "Paris Climate Change Agreement". The Paris agreement "aims to improve the global response to the threat of climate change and global warming" and maintain "the increase in the global average temperature to well below 2°C above pre-industrial levels". (United Nations, 2015) This underlines the urgency to restrict the amount of greenhouse gas emission into the atmosphere. To achieve the goal of staying within the above mentioned 2°C target may require leaving a third of oil reserves, half of the gas reserves, and over 80% of current coal reserves unused until 2050 (Friedlingstein et al., 2014; McGlade and Ekins, 2015).

The timespan to reach the mammoth goal is too short to switch away and eliminate the demand for fossil-based transportation fuels. Apart from reducing the reliance on fossil fuel for transportation and other necessities, the present-day world is facing a significant challenge in handling and recycling waste. As the population of the world is on the rise thus resulting in the generation of more waste. One of the prime examples of increasing waste accumulation is municipal and plastic waste. One of the most predominant methods used to decompose plastic and municipal waste is bearing the waste in landfills, to recycle waste using this method takes a lot of time. In the case of plastic waste, it takes about 300 to 400 years. To cope with the need for fossil fuel, technologies for CO_2 capture and recycling of waste are needed.

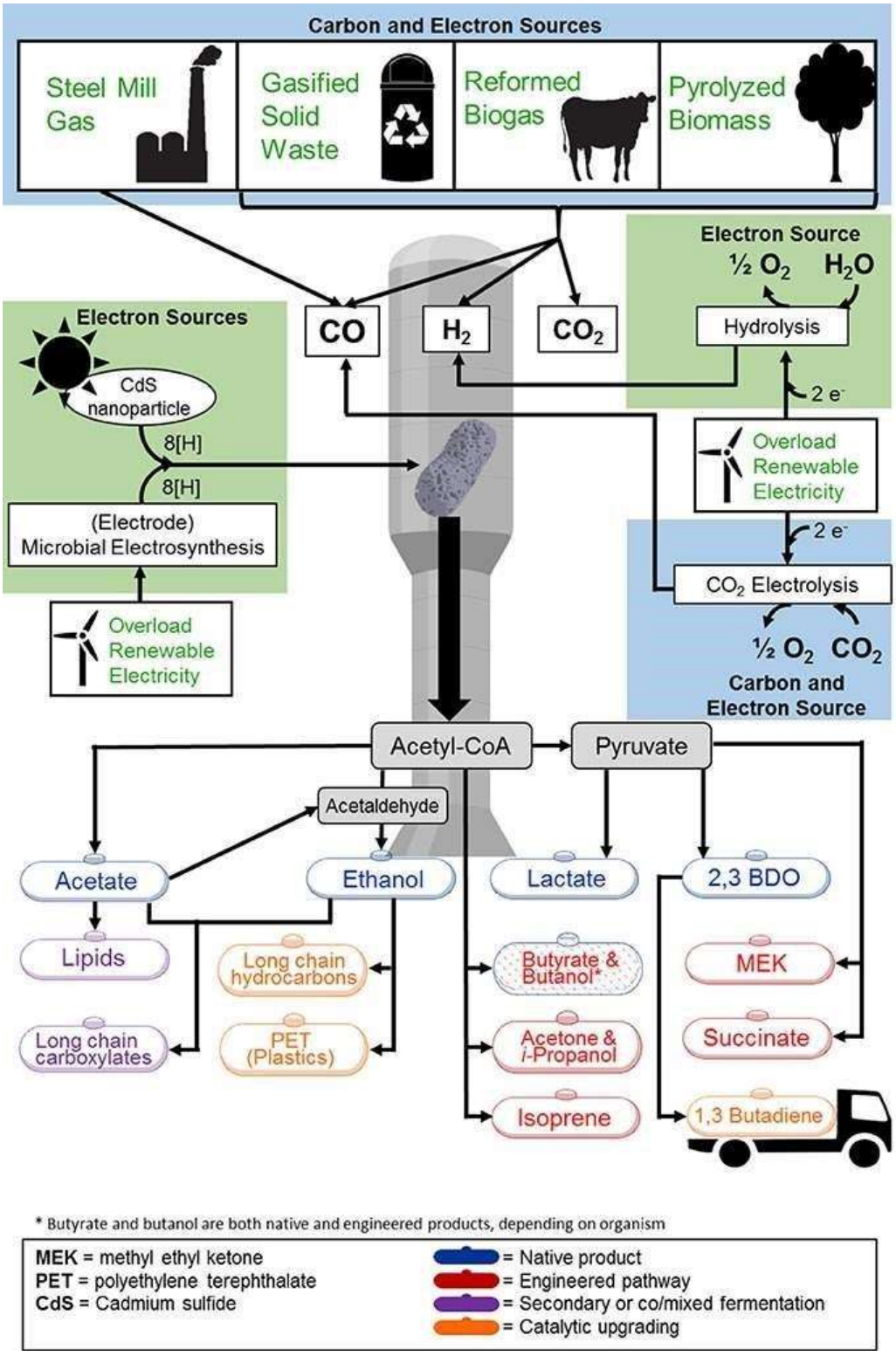
An innovative and full of potential technology for CO_2 valorization and waste recycling is acetogen gas fermentation that uses gasified municipal or industrial off-gas waste as a feedstock and produces useful biofuels and chemicals. Many technologies have developed new ways to produce biofuel from different energy sources, nevertheless, no technology has shown as much potential as gas fermentation acetogen.

1.2 Overview of gas fermentation

Gas fermentation is a microbial process which uses acetogen that can convert carbon-containing waste feedstocks (e.g. CO_2+H_2, syngas, industrial waste gases) into valuable industrial-scale fuels chemicals. In modern times, Gas fermentation has emerged as an appealing option for renewable and sustainable production of high-quality fuels and chemicals from abundant, cost-effective and non-food-based feedstocks e.g. industrial waste gases, syngas (CO/ CO_2 and H_2) and methane. (Amador-Noguez et al., 2011; Bengelsdorf et al., 2018; Bertsch & Müller, 2015b) Gas Fermenting acetogen does not compete with sustainable farming neither it uses sugar as a feedstock. It simply utilizes the CO_2 and H_2 emitted in the atmosphere or capturing industrial waste gases from chimneys or gasifying solid municipal waste thus enabling the fermentation to produce a wide range of fuels and chemicals. Using syngas (a unique mixture of carbon monoxide (CO), carbon dioxide (CO_2) and hydrogen (H_2) at a certain composition) as a gas feedstock which is cost-effective and energy-rich offers high product versatility and a potential to scale gas fermentation industrially.

Gas fermentation has striking advantages against traditional methods such as the Fischer-Tropsch process (FTP) that uses gases to convert them into useful products. Converting syngas to a low-carbon liquid hydrocarbon that is used in the plastic industry with short to medium range carbon chains have been achieved by FTP, A technique that has been developed since 1925 which operates under high temperature (150–350 °C), elevated pressures (30 bar), and complex catalysts such as cobalt, ruthenium, and iron (De Klerk et al., 2013). In contrast to gas fermentation which takes place at 37 °C and atmospheric pressure, which presents significant energy and inexpensive as compared to FTP. FTP, unlike gas fermentation, also requires a fixed H2: CO ratio of ideally ~2:1. (De Klerk et al., 2013). Nevertheless, syngas derived from biomass has usually a lower H_2:CO ratio (van der Drift et al., 2001; Datar et al., 2004; Barrister and Rauch, 2005; Boateng et al., 2007; Piccolo and Bezzo, 2009; Zheng et al., 2016). Habitually, Chemical processes are regarded as faster processes as compared to biological however enzymatic specificities of biological conversions results in more product selectivity and fewer by-products.

Figure 1: Synopsis of all the possible feedstock and product possibilities for acetogen gas fermentation



Modified from Liew et al. 2016

Figure description 1: (1) The feedstocks of acetogen gas fermentation are highlighted in light blue color (mainly carbon and electron sources) and green text (only electron sources for energy conversion). (2) Commercial deployment several feedstocks are displayed on the top-most side of the figure. (3) Formation of all products displayed (3.1) all the native products (blue text), (3.2) synthetic products formed via genetic modification (red text), (3.3) products generated via secondary fermentation of co/mixed cultures (purple text), and (3.4) products produced through additional catalytic reactions (orange text). Abbreviation used: 2,3-BDO: 2,3-Butanediol.

1.3 Introduction to acetogens

Acetogens are the distinctive bacteria that utilize the wood-Ljungdahl pathway to produce acetyl-CoA and consequently for biofuels, for example, ethanol, 2,3-butanediol, acetate and a small amount of lactate. Acetogen uses syngas (a gas mixture of hydrogen, carbon monoxide and carbon dioxide at a certain composition) as a carbon source and an energy source. According to the research paper of (Drake et al., 2006), the standard definition of an acetogen is as follows: an anaerobe that can use the acetyl-CoA pathway (widely known as Wood-Ljungdahl pathway) as a (1) mechanism for the reductive synthesis of acetyl-CoA from CO_2, (2) terminal electron-accepting, energy-conserving process, and (3) mechanism for the fixation (assimilation) of CO_2 in the synthesis of cell carbon. In acquiescence with this definition, acetate formation is irrelevant, only the process of acetyl-CoA. Acetogens are facultative autotrophs that can grow by the oxidation of a large variety of organic substrates, including pentoses, alcohols, methyl groups and formic acid, or by the oxidation of inorganic substrates such as hydrogen (H_2) or carbon monoxide (CO), which is usually coupled to the reduction of CO. (Schuchmann & Müller, 2014).

The pathway that acetogenic bacteria uses is speculated to be the prime candidate for the earliest autotrophic pathway that resulted in the origin of life.(Fuchs, 2011) The pathway is known to be the only linear CO_2 fixation pathway and regarded as the most thermodynamically efficient pathway in acetate synthesis. (Drake et al., 2006; Fast and Papoutsakis, 2012; Fuchs, 2011). Acetogens play a major role in the global carbon cycle of the earth by fixing approximately 20% of carbon and producing billions of tons of acetic acid. (Drake et al., 2006; Ljungdahl, 2009).

One of the special features of acetogens is the ability to fix C1 gases to produce chemicals and fuels thus making them commercially attractive bacterial organisms. There has been an increasing interest in using gas fermentation acetogens as cell factories for producing sustainable fuels and chemicals. In recent times, LanzaTech (one of the leading synthetic biotech and cell factory companies) commercialized the first waste gas-to ethanol process, efficiently consolidating the carbon from steel mill off-gas into industrial quality ethanol via *Clostridium autoethanogenum* acetogen. *Clostridium autoethanogenum* is a special one of a kind anaerobic bacterium that has the potential to produce high-quality ethanol from carbon gases such as carbon dioxide, carbon monoxide and syngas that can be acquired from the chimneys of industrial steel-mill gas or solid municipal waste that can be gasified and used as a carbon source, it is amongst fewest known microorganisms to do so. *Clostridium autoethanogenum* can be genetically modified to increase the efficiency of the production of valuable fuels and chemicals such as ethanol, butanol and 2,3-butanediol.

1.4 Description of *Clostridium autoethanogenum* strain

Clostridium autoethanogenum was first discovered in the year of 1994. It is a gram-positive anaerobic bacterium which was acquired from Rabbit faeces. In earlier stages of the logarithmic phase cell shape is usually rod-like, during the late of the growth phase the shape of the cell is long filamentous and while forming colonies the shape of cell changes to roll-tube with the distinct round center. The name *Autoethanogenum* indicates that microorganism uses inorganic carbon, specifically carbon monoxide (as well as carbon dioxide) as possible sole source of carbon for growth, thereby producing ethanol. (Abrini, Jamal; Naveau, Henry; Nyns, Edmond-Jacques (1994). The strain strictly grows on anaerobic conditions and It can grow without a yeast extract, but essential vitamins are required for growth. *C. autoethanogenum* can grow from 20 °C until 44 °C and pH between 4.5 - 6.5. The maximum growth occurs at optimal temperature of 37 °C.

Clostridium autoethanogenum has a close relative strain which was discovered a few months before discovery of *C. autoethanogenum* and it is known as *Clostridium ljungdahlii*. It was isolated from chicken yard waste (Tanner et al., 1993). Both strains produce significant amounts of acetate, ethanol and minor amounts of 2,3- butanediol as well as lactate (Kopke et al., 2011; Kracke et al., 2016)

1.5 Brief description of the Wood-Ljungdahl pathway and energy conservation model

Gas fermenting acetogens undergo metabolic pathway of reductive acetyl-CoA, better known as Wood-Ljungdahl pathway (WLP) (Drake et al., 2008), a non-photosynthetic C1-fixation metabolic pathway with the highest-known theoretical thermodynamic efficiency (Fast and Papoutsakis, 2012; Schuchmann and Müller, 2014; Müller, 2019). A model acetogen such as *C. autoethanogenum* undergo WLP, Wood-Ljungdahl pathway is the only known linear pathway for CO_2 fixation. ATP is the universal energy carrier in biology and every living cell has to conserve energy in this energy equivalent. There are two modes of known energy conservation: 1) substrate-level phosphorylation (SLP): In substrate-level phosphorylation, the pathway couples with a chemical reaction directly to the phosphorylation of ADP, which results in the generation ATP. 2) Chemiosmotic energy conservation: In chemiosmotic energy conservation the pathway couples with an exergonic reaction electron transfer reaction (mostly exergonic reaction) to the translocation of ions across a membrane, which results in the generation of an electrochemical ion gradient across the membrane, which encourages the ATP synthesis. The reactions which are involved in WLP and result in substrate-level phosphorylation (SLP) achieve no net ATP formation. Thus, the model of energy conservation in WLP highly relies on the establishment of a chemiosmotic energy conservation mechanism.

Gas-fermenting acetogenic bacteria use Wood–Ljungdahl pathway (WLP) to produce acetate from two molecules of carbon dioxide (refer to figure 2). During the process of acetogenesis under heterotrophic circumstances, glucose ($C_6H_{12}O_6$) is oxidized into two different compounds two molecules of acetate and two molecules of carbon dioxide via a combination of glycolysis, pyruvate: ferredoxin oxidoreductase, acetate kinase and phosphotransacetylase.(Schuchmann & Müller, 2014) In this process the reducing equivalents are oxidized again by the reduction of the two molecules carbon dioxide to acetate in the WLP.

During the process of acetogenesis under autotrophic circumstances, acetate is formed from hydrogen and carbon dioxide source. In the carbonyl branch of the Wood-Ljungdahl pathway (refer to the bottom right section of figure 2), one molecule of CO2 is reduced to carbon monoxide via CO dehydrogenase/acetyl-CoA synthase (CODH/ACS). In the methyl branch of the Wood-Ljungdahl pathway (refer to the top left corner of the figure 2), one molecule of carbon dioxide is reduced to formate via formate dehydrogenase and bound to the cofactor THF, resulting in the formation of formyl-THF via the formyl-THF synthetase. Formyl-THF is utilized by the formyl-THF cyclohydrolase to to form methenyl-THF, thus it is converted to methylene-THF via the methylene-THF dehydrogenase.

In the final stages of the pathway, the Methylene-THF is converted into methyl-THF via the methylene-THF reductase. Lastly, a methyltransferase transfers the methyl group from methyl-THF via a corrinoid Iron-Sulphur protein (CoFeSP) to the CODH/ACS (Schuchmann & Müller, 2014). The bifunctional enzymes (refer to the top right corner of figure 2), reduces carbon dioxide to carbon monoxide in the carbonyl branch and combines it with the methyl group from methyl branch and helps in formation of acetyl-CoA, which is then used by other enzyme namely phosphotransacetylase to generate acetyl phosphate, and then it is later turned into acetate by the help of an acetate kinase. Converting the acetyl-CoA into acetate requires a lot of energy with regards to WLP. Therefore, there must be mode of energy production to support the formation products and cell growth. In recent times a membrane based mechanism for the model of energy conversation was proposed but the discovery of an important Rnf complex in *A.woodii* changed everything.(Biegel et al., 2011; Schuchmann & Müller, 2014).

The Rnf complex was originally identified in Rhodobacter the complex was helping in the process of nitrogen fixation. Rnf complex can build up a transmembrane electrochemical sodium ion gradient which enables coupling electron-transfer reaction which the reaction is exergonic. The electrochemical Na^+ gradient can then drive ATP synthesis by a membrane-bound F1FO ATPase (Biegel and Müller, 2010; Biegel et al., 2011; Hess et al., 2016;). This same mechanism was also identified *in C. ljungdahlii* and *C. Autoethanogenum*. (Köpke et al., 2010; Mock et al., 2015; Hess et al., 2016) Not every acetogen has this Rnf complex but those who have Rnf complex shows promising hopes to commercial biofuel sector.

Figure 2: Description of Wood-Ljungdahl pathway (WLP) and energy conservation model of acetogen *C. autoethanogenum*.

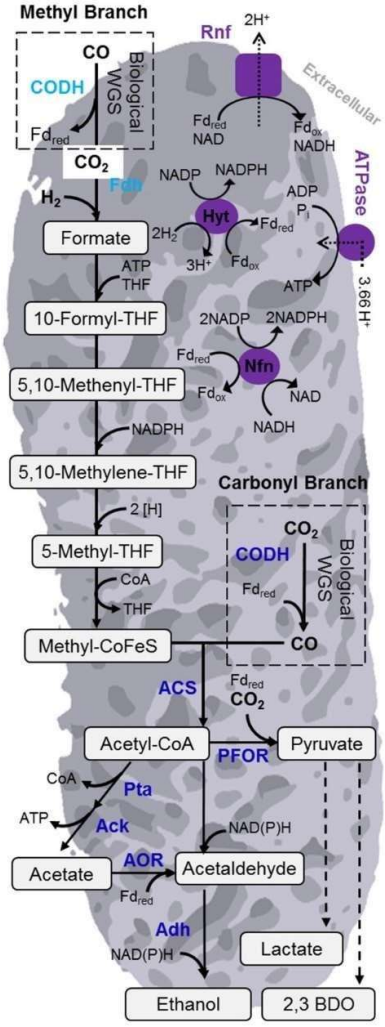


Figure description 2: Gas-fermenting acetogenic bacteria use Wood–Ljungdahl pathway (WLP) in order to produce acetate from two molecules of carbon dioxide. The Wood-Ljungdahl pathway (WLP) consists of two branches: (A) a methyl branch (located at the top left section of the diagram) and (B) a carbonyl branch (located at the bottom right section of the diagram).

(1) The enzymes are labelled in blue color text. (2) The enzymes which are associated with the energy conservation model are represented in purple color. Abbreviation used for chemical compounds : 2,3-BDO: 2,3-butanediol; AOR: aldehyde ferredoxin oxidoreductase; ACS: acetyl-CoA synthase; CODH: carbon monoxide dehydrogenase; Nfn, transhydrogenase; PFOR: pyruvate ferredoxin oxidoreductase; Rnf: Rhodocbacter nitrogen fixation; THF: Tetrahydrofolate; WGS: water-gas shift reaction.

Modified from Liew et al. 2016

1.6 Advantages of continuous culture gas fermentation

In Gas fermentation, acetogens are grown under steady-state continuous culture because it provides a higher degree of control than a batch culture. Growth rates can be maintained for extended intervals. On the other hand, in batch cultures have to be re-grown after each the fermentation run, before they become productive again. In terms of syngas fermenting acetogens-growing can take a very long time because of the longer doubling time in the case of commercial batch system that downtime of the bioreactors would in weeks hence not economically viable solution. In steady-state continuous culture, mixed cultures can be sustained using chemostat cultures – unlike in a batch process where one organism usually outgrows one another.

In continuous culture fermentation one the most important aspect is that by changing the dilution rate, specific growth rate of the cells can be controlled. The most interesting aspect to study is the effect of specific growth rate on gas fermenting acetogens and how it effects the growth of microbe and to investigate its performance under different dilution rate and acetogen metabolism has not been investigated yet. The study of systems-level quantification of acetogen is necessary for design-build-test-learn bioprocess optimization by metabolic engineering (Valgepea et al., 2017). Hence, collecting quantitative datasets from steady state chemostat cultures, whose analyses are comparable between experiments, is important for the development of these systems (Bertsch & Müller, 2015a).

2. THE AIMS OF THE THESIS

The aims of the thesis are as follows:

- To investigate the effect of different dilution rates D = 1.0 day^{-1} and D = 2.0 day^{-1} on *C. autoethanogenum* grown under both CO and syngas in continuous cultures bioreactor gas fermentation.
- Comparing the performance of gas fermenting acetogen under CO and Syngas.

3. EXPERIMENTAL PART

3.1. MATERIALS AND METHODS

3.1.1. Bacterial strain and storage condition

The bacterial strain of *C. autoethanogenum* DSM 23693 obtained from biotech company Lanzatech was used throughout the experiments and the strain were stored as a glycerol stock at -80°C.

3.1.2. Growth media

The pre-cultures cells were grown in Schott bottles on standard PETC-MES media (Valgepea et al., 2017) with yeast extract (YE), bottles were pressured to 140 kPa and the media were carried out with a starting volume of 50 ml in 250 ml Schott bottles under strict anaerobic conditions at 37°C with orbital shaking at 200 rpm. The experiments were carried out under strict anaerobic conditions and Cells were grown on Syngas (∼50% CO, ∼20% H_2, ∼20% CO2 and ∼10% Ar) and CO (∼60% CO and ∼40% Ar). After observing the initial growth of cells at optical density of ~ 0.2 at 600 nm in Schott bottles the experiment moves to the next phase which was growing the cell inside a continuous chemostat bioreactor .The bioreactor media which was used in this experiments was a chemically defined medium (1561 UT medium) without yeast extract (YE) containing per liter: 0.5 g $MgCl_2 \cdot 6H_2O$, 0.2 g NaCl, 0.1 g $CaCl_2$, 2.65 g $NaH_2PO4 \cdot 2H_2O$, 0.5 g KCl, 2.5 g NH_4Cl, 0.017 g $FeCl_3 \cdot {}_6H2O$, 0.5 g cysteine-$HCl \cdot H_2O$, 1 mL of 2 g/L resazurin, 10 mL trace metal solution (TMS) and 10 mL B-vitamin solution. The TMS composition consist of per liter: 1.5 g nitrilotriacetic acid, 3 g $MgSO_4 \cdot 7H2O$, 0.5 g $MnSO_4 \cdot H2O$, 1 g NaCl, 0.667 g $FeSO_4 \cdot 7H2O$, 0.2 g $CoCl_2 \cdot 6H_2O$, 0.2 g $ZnSO4 \cdot 7H_2O$, 0.02 g $CuCl_2 \cdot 2H2O$, 0.014 g $Al_2(SO_4)_3 \cdot 18H_2O$, 0.3 g H_3BO_3, 0.03 g $NaMoO_4 \cdot 2H_2O$, 0.02 g Na_2SeO_3, 0.02 g $NiCl_2 \cdot 6H_2O$ and 0.02 g $Na_2WO4 \cdot 2H_2O$. The B-vitamin solution contained per liter: 20 mg biotin, 20 mg folic acid, 10 mg pyridoxine hydrochloride, 50 mg thiamine-HCl, 50 mg riboflavin, 50 mg nicotinic acid, 50 mg calcium pantothenate, 50 mg vitamin B12, 50 mg 4-aminobenzoic acid as well as 50 mg thioctic acid. All cultures were carried out using standard anaerobic techniques and procedures.

3.1.3 Growth Conditions

All the experiments were running under strict anaerobic conditions. The cells were grown inside of chemostat bioreactor at optimal temperature of 37°C and at standard pH of 5 which was maintained by base 5M NH_4OH. The bioreactor has capacity of 1.4L but for this experiment working volume of the bioreactors was 750 mL. Initially, the continuous culture at the dilution rate of D = 0.5 day-1 in order to reach the goal of desired steady-state the dilution rate of D=1 day-1 and 2 day-1 gradual ramping which is increase in media flow rate happened periodically. All the Multifors bioreactors were controlled and monitored by the bioreactor control software called Eve (Infors AG) and. The bioreactor system was well-equipped with peristaltic pumps; mass flow controllers (MFCs); pH, ORP and temperature sensors and to a Hiden HPR-20-QIC mass spectrometer (Hiden Analytical) for online high-resolution off-gas analysis was attached to the system. Antifoam (435530; Sigma-Aldrich) was continuously added to the bioreactor using a syringe pump at a rate of 10 µL/h to avoid foaming. Different steady-state gas-liquid mass transfer rates were used for different experiments (refer to Table 1) resulting in the same biomass composition (BC) 1.5 - 1.8 (gDCW/L). The steady-state results reported below in the result section were collected after optical density (OD), gas uptake and production rates had been stable in chemostat mode for 3–5 working volumes.

Table 1: Operating conditions of CO and Syngas-fed bioreactors.

Gas	Dilution rate (day^{-1})	Gas flow (mL/min)	Media flow rate RPM	Biological replicates
Syngas	1	50	650	4
Syngas	2	72	815	3
CO	1	50	665	4
CO	2	72	815	3

3.1.3. Biomass concentration analysis

The cell growth on liquid medium was measured spectrophotometrically by measuring the optical density of the cell culture at 600 nm (OD_{600}).Biomass concentration (gDCW/L) for syngas and CO cultures was determined by the optical density of cell culture at OD_{600} using relationship between the correlation coefficient (K=0.23) and culture OD which is equal to dry cell weight (BC = OD × K), according to the research paper of (Peebo et al. 2014) .The average coefficient variation for technical replicates for the determination of biomass concentration was 1.2 - 1.5%.

3.1.4. Analytical procedures

The sampling of metabolites was performed using filtered broth samples and this samples were stored in a refrigerator at the temperature of -20 °C until further experimental analysis. About 1ml of filtered sample was taken from each bioreactor from which 600 µl of sample from each bioreactor was taken for analysis. The analysis was mainly done to identify and quantify the organic acids and alcohols produced during the experiment and it was performed by High-performance liquid chromatography HPLC (Shimadzu Prominence-I LC-2030 plus) using a column (Aminex HPX-87H 300*7.8 mm, 9µm, Bio-Rad, Munich, Germany). Twenty microliters of sample were injected and eluted isocratically with 0.5 mM H2SO4 at 0.6 mL/min for 30 min at 45 °C and detected by a refractive index detector (RID-20A, Shimadzu, Japan). Bioreactor off-gas analysis was performed by an on-line Hiden HPR-20-QIC mass spectrometer using a Faraday Cup detector monitoring the intensities of H2, CO, ethanol, H2S, Ar, and CO2. Syngas and CO gas were used for on-line gas calibration for each mass-spectrometer MS- cycle to achieve reliable off-gas analysis data. Regular volume check and dilution rate check of each bioreactor were done before sampling in order to get more reliable and accurate readings.

3.1.5. Gas uptake and production rates

Gas uptake rate (mmol/min) for CO, CO2 and H_2 and production rate (mmol/min) were determined by MS-cycle for each bioreactor using a subsequent 'on-line calibration' of the mass-spectrometer for analyzing syngas and CO gas directly from the cylinder. Specific rates (mmol/gDCW/h) were determined by taking into account the exact composition of syngas and CO gas, actual bioreactor working volume, feeding gas flow rate, off-gas flow rate based on the fractional difference of the inert gas Ar in the feeding and off-gas composition, the molar volume of ideal gas as well as the steady-state biomass concentration (gDCW/L).

3.1.6. Carbon balance Analysis

Carbon balance analysis was carried out to determine the carbon recovery of system. Carbon recovery is the total fractional sum of C-mol products from net C-mol substrates. To achieve accurate carbon balance, the net soluble CO2 fraction in cell culture were taken into consideration based on the off-gas data analysis. The possibility of ethanol stripping was considered. Henry's law was applied to estimate the net soluble CO2 fraction. After calculating the carbon recoveries for the different dilution rates the data was normalized to 100% to get the fair comparison between different dilution rates.

3.1.7. Statistical analysis and formulas

Type of analysis / rate	Formula	Unit
Biomass concentration	BC =OD * K OD : optical density K: correlation coefficient	X (gDCW/L) gram dry cell weight per liter
Volumetric substrate uptake rate	$q_S = -D*(S_F - S)$ S_F – feed substrate concentration (mmol/L) S – residual substrate concentration (mmol/L) D – dilution rate (day-1)	q_S (mmol/L/day)
Volumetric production rate	$q_P = D*P$ P – product concentration (mmol/L) D – dilution rate (day-1)	q_p (mmol/L/day)
Volumetric gas uptake rate	q_{gas} = Gas%*Fgas*(Ingas%F/Ingas%)))*1440)/GasMV*1000/V) Gas%F – specific gas fraction in feed gas (unitless) Gas% – specific gas fraction in bioreactor off-gas (unitless) Ingas%F – inert gas fraction in feed gas (unitless) Ingas% – inert gas fraction in bioreactor off-gas (unitless) Fgas – flow rate of feed gas (L/min; at STD T and P) GasMV – molar volume of ideal gas (L/mol) V – bioreactor liquid working volume (L)	q_{gas} (mmol/L/day)
Carbon balance (C-recovery %)	Cmol substrates – Cmol products = 0	Percentage (unitless)

All the graphs and tables in the results section were made in Microsoft Excel with accordance to the formula mentioned above.

3.2 RESULTS AND DISCUSSION

C. autoethanogenum cells were grown on a chemically defined medium (1561 UT medium) in chemostat bioreactors at optimal temperature of 37°C and at constant pH of maintained by base 5M NH_4OH. (refer to table 1) on Syngas and CO. The first 2 experiments were carried out in continuous culture CO-fermenting *C. Autoethanogenum* chemostat. The aim of this experiments was to successfully run and achieve two different dilution rates D = 1 day-1 and D = 2 day-1. The second lot of experiments were carried in Syngas and aim was to achieve three different dilution rate D = 1 day-1, D = 2 day-1 and D = 2.83 day-1. Dilution rates D = 1 day-1 and D = 2 day-1 were achieved successfully in both Syngas and CO. The results from D = 2.83 day^{-1} are omitted out of this paper because of its unreliability. CO gas experiments were carried by my colleague and group members and results of this experiments are included in this paper for analytical purposes . I participated extensively involved in the second set of syngas experiments which were performed. I was actively involved in media preparation, ramping of gas and media intake throughout the experiment. I also took part in the steady-state sample analyses under the guidance of my direct supervisor. The results of both CO and syngas experiments for product concentration, gas uptake rate and carbon balance are shown graph 5, 6 and 7 respectively.

Figure 3: Steady-state biomass concentrations at dilution rate D = 1 day-1 and D = 2 day-1 in syngas-fermenting *C. autoethanogenum* chemostats. The data for high biomass concentration chemostats in syngas (~ 1.65 gDCW/L) are displayed and represented as the average ± standard deviation between biological quadruplicates at dilution rate D = 1.0 day-1 and triplicates at dilution rate D = 2.0 day-1. During the steady-state the average biomass of the amongst various biological replicates was 7.02 ± 0.2 gDCW/L at dilution rate of 1.0 day-1 and 7.00 ± 0.3 gDCW/L at dilution rate of 2.0 day-1. Abbreviation used: BR bioreactor

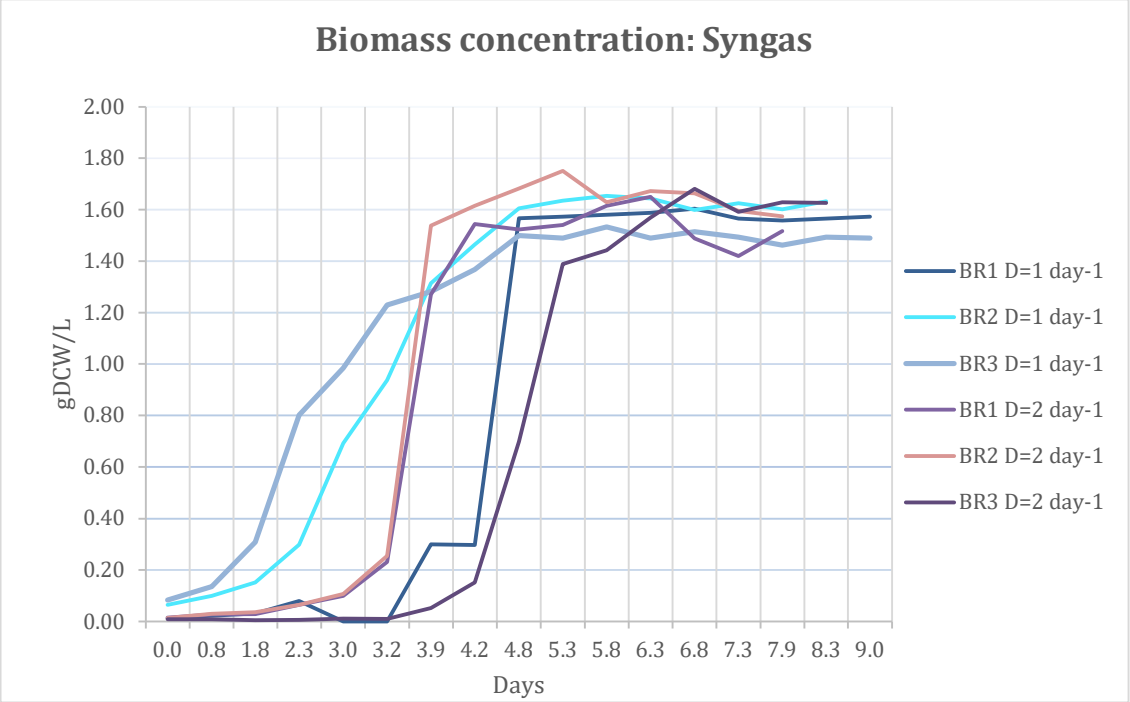


Figure 4: Steady-state biomass concentrations at dilution rate D = 1 day-1 and D = 2 day-1 in CO fermenting *C. autoethanogenum* chemostats. The data for high biomass concentration chemostats in CO (~ 1.98 gDCW/L) are shown and represented as the average ± standard deviation between biological quadruplicates at dilution rate D = 1.0 day-1 and triplicates at dilution rate D = 2.0 day-1. During the steady-state the average biomass of the amongst various biological replicates was 1.67 ± 0.5 gDCW/L at dilution rate of 1.0 day-1 and 1.64 ± 0.05 gDCW/L at dilution rate of 2.0 day-1 Abbreviation used: : BR bioreactor.

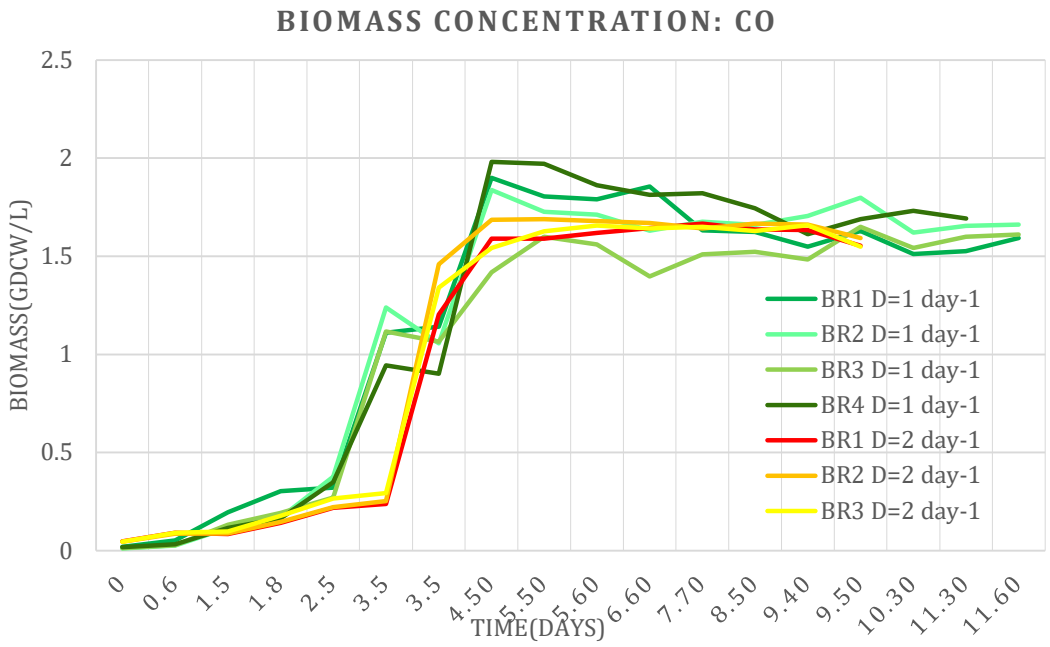
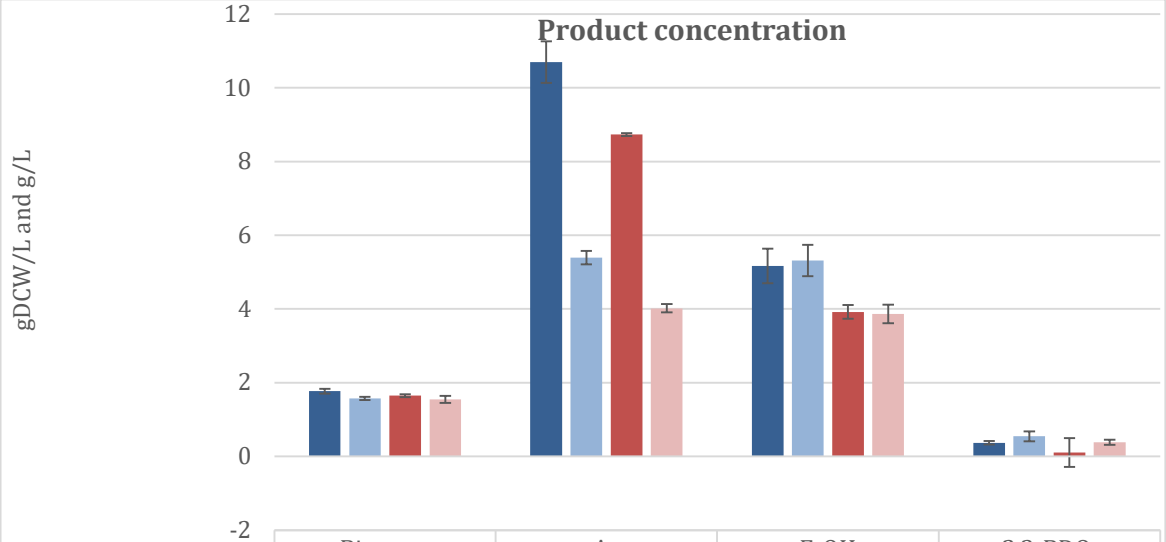


Figure 5: Steady-state product concentrations at dilution rate D = 1 day-1 and D = 2 day-1 in Syngas and CO fermenting *C. autoethanogenum* chemostats. The data for high biomass concentration chemostats in Syngas (~ 1.65 gDCW/L) and in CO (~ 1.98 gDCW/L) are shown and represented as the average ± standard deviation between biological quadruplicates at dilution rate D = 1 day-1 (CO and Syngas) and triplicates at dilution rate D = 2 day-1 (Syngas and CO). Abbreviation used: Ac acetate, EtOH ethanol, 2,3-BDO 2,3-butanediol.

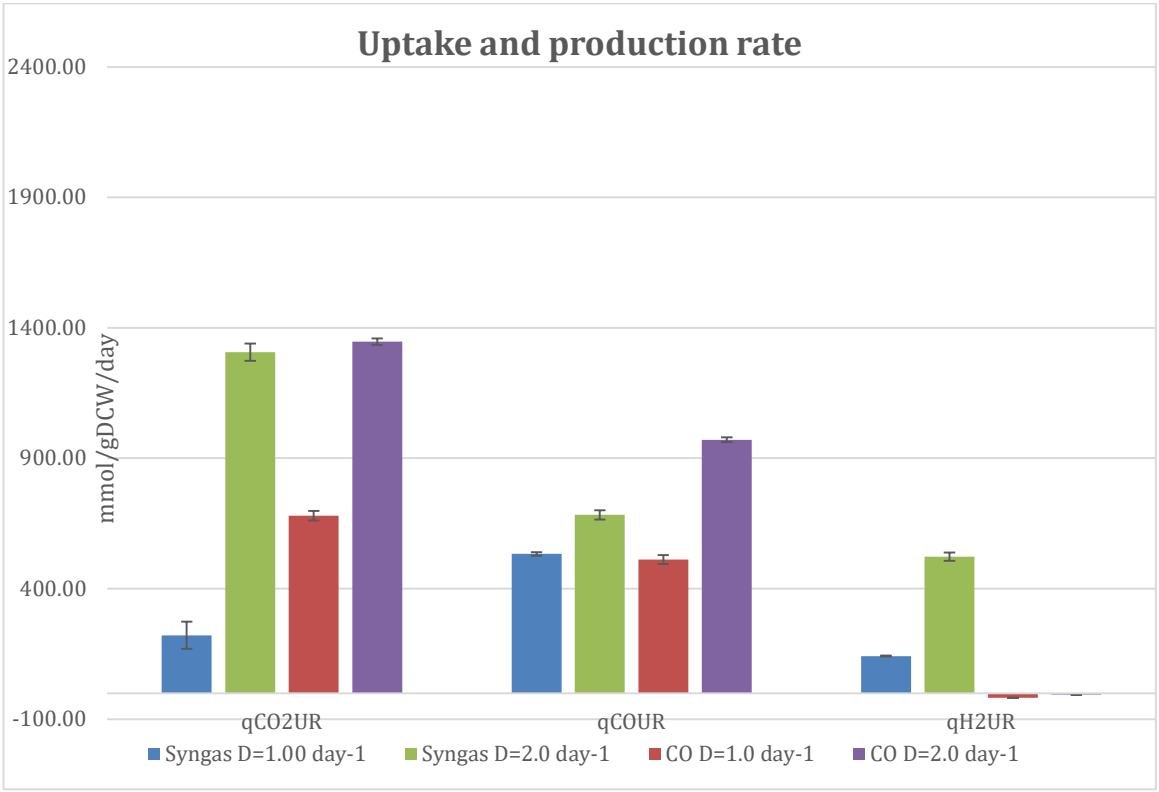


	Biomass	Ac	EtOH	2,3-BDO
Syngas D=1.0 day-1	1.77073	10.69453334	5.169368896	0.371215012
Syngas D=2.0 day-1	1.57297	5.392793845	5.31500661	0.547143795
CO D=1.0 day-1	1.64726	8.731641115	3.920606709	0.104975655
CO D=2.0 day-1	1.54634499	4.018874357	3.863939069	0.382907138

Ethanol and 2,3 BDO production increases at higher dilution rate

The continuous culture *C. autoethanogenum* chemostat produces acetate, ethanol, 2,3-butanediol (2,3-BDO). At a lower dilution rate of 1 day-1 production of ethanol is 5.16 g/L (syngas) and 3.92 g/L (CO). At high dilution rate of 2 day-1 production of biomass and acetate decreases and production of ethanol and 2,3 BDO increases in syngas 5.32 g/L and 0.55 g/L also the production of 2,3 BDO grown under CO gas increases but the production of ethanol remains approximately the same. At higher dilution rate in syngas the production of ethanol increases thus running the syngas fermentation at D = 2.0 day-1 is more economically viable. The molar acetate/ethanol ratio at D = 1.0 day-1 ~ 2.06 (syngas) and at a higher specific growth rate the major acetate/ethanol ratio decrease by 1-fold ~1.01 (syngas).

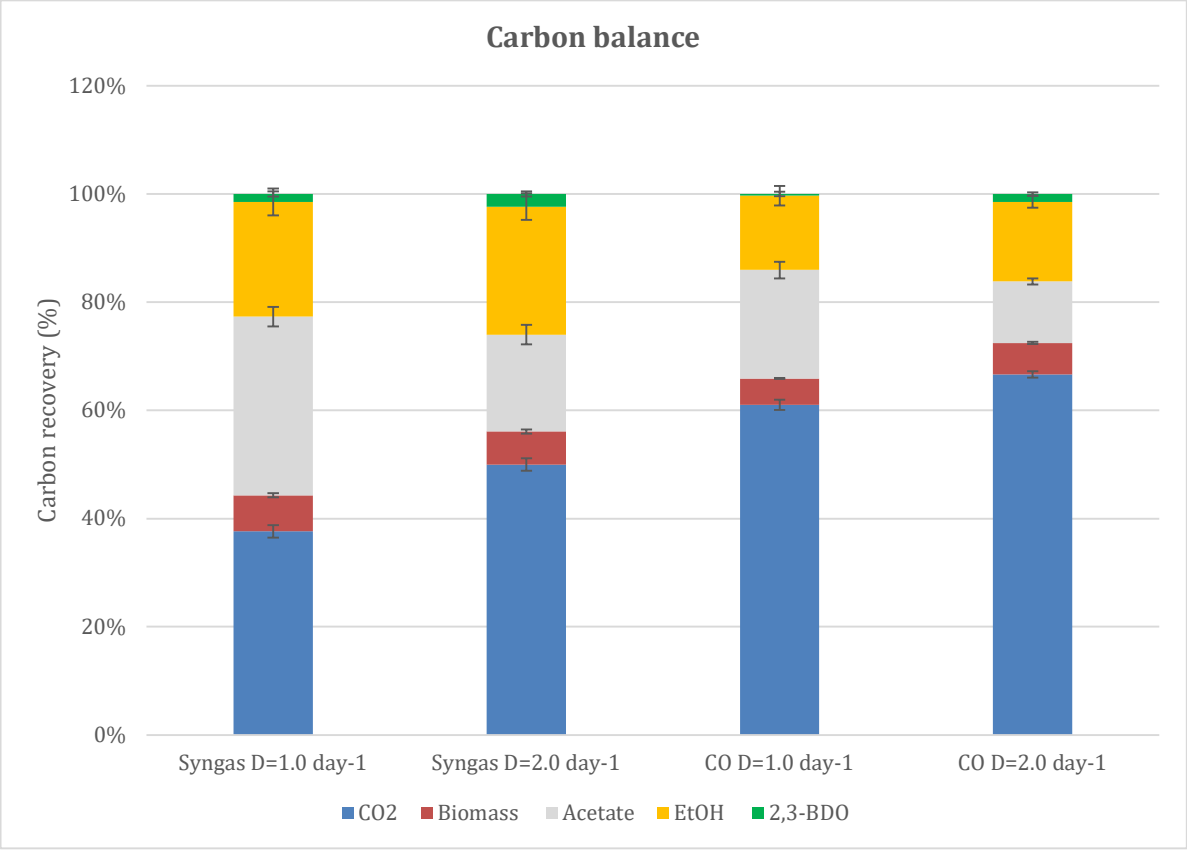
Figure 6: Steady-state gas uptake and production at dilution rate D = 1 day-1 and D = 2 day-1 in Syngas and CO fermenting *C. autoethanogenum* chemostats. The data for high biomass concentration chemostats in Syngas (~ 1.65 gDCW/L) and in CO (~ 1.98 gDCW/L) are shown and represented as the average ± standard deviation between biological quadruplicates at dilution rate D = 1 day-1 (CO and Syngas) triplicates at dilution rate D = 2 day-1 (Syngas and CO). Abbreviation used: qCO_2 specific CO_2 production rate, qCO and qH_2 specific CO and H_2 uptake rates, respectively.



Gas uptake and production rates analysis

Analysis of gas uptake rates and production rates by an autotrophic continuous culture is vital for identifying and comparing the flow of carbon and redox between cells grown on syngas and CO gas. Off-gas analysis shown that the specific CO uptake rate (q_{COUR}) is higher at higher specific growth rate. At a lower dilution rate, specific CO uptake rate (q_{COUR}) is almost similar in both CO and Syngas. Noteworthy, that at a higher dilution rate, specific CO_2 uptake rate (q_{CO2UR}) is higher than usual. At D = 1.0 day-1 in CO gas q_{CO2UR} value is the highest. q_{H2UR} value D = 1.0 day-1 in CO is identical to (Valgepea et al., 2018).

Figure 7: Carbon balances at dilution rate D = 1 day-1 and D = 2 day-1 in Syngas and CO fermenting *C. autoethanogenum* chemostats. Carbon recoveries (refer to 3.1.6 text) were normalized to 100% to have a fairer comparison of carbon distributions between two different dilution rate and two different gas mixes. The data for high biomass concentration chemostats in Syngas (~ 1.65 gDCW/L) and in CO (~ 1.98 gDCW/L) are displayed and represented as the average ± standard deviation between biological quadruplicates at dilution rate D = 1 day-1 (CO and Syngas) triplicates at dilution rate D = 2 day-1 (Syngas and CO). Abbreviation used: EtOHethanol,2,3-BDO:2,3-butanediol.



Carbon balance reveals that carbon flux to ethanol increases at higher dilution rate

The gas analysis was coupled with extracellular metabolomics in continuous culture to get accurate carbon balancing, also it allows experimental validation of theoretical stoichiometric calculations of product yields. The carbon recoveries were normalized to 100% to have a fairer comparison of carbon distributions between two different dilution rates and two different gas mixture. The carbon recovery at higher dilution rate produces higher percentage of carbon flux to ethanol and CO_2 as shown in the figure 6. Percentage of carbon flux to acetate decreases at dilution rate 2.0 day-1 in both CO and syngas thus resulting in the increase of product formation. It validates the previous claim of higher production rate at higher specific growth rate.

Steady-state Syngas-fermenting *C. autoethanogenum* D ~1,0 day-1 (Valgepea et al., 2017), the carbon recovery for dilution rate D = 1 day-1 was identical to the precious Syngas-fermenting results. The product concentration of ethanol and 2,3 BDO was higher than the pervious syngas experiment D ~1,0 day-1 (Valgepea et al., 2017). Overall, In terms of performance and product profile Syngas is better then CO gas.

In future, more experiments can be conducted in which the focus should be on changing different biochemical parameters such as pH and temperature. The mentioned before *C. autoethanogenum* can grow between pH 4.5- 5.5 and temperature 20°C - 44°C (refer to the text 1.4). It would be interesting to investigate at different pH 4.5 and pH 5.5. It would be fascinating to achieve the steady-state of D = 2.83 day-1 in syngas and push the boundries of Gas-fermenting *C. autoethanogenum* bacteria to achieve maximum product profile.

Conclusion

C. autoethanogenum produces acetate, ethanol and 2,3-butanediol in continuous culture chemostat. The change in special growth rate results in the change in the production of acetate, ethanol and 2,3-butanediol. In steady-state continuous culture of Syngas-fermenting acetogen, higher specific growth rate of the culture resulted in higher product concentration thus subsequently decreasing the production of acetate in order to production of ethanol.

The productivity of ethanol and 2,3-butanediol at D = 2.0 day-1 in Syngas was highest amongst the other different dilution rates and gas mixture. In terms of product profile, the performance of Syngas-fermenting acetogen was superior CO-fermenting acetogen.

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