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Biomethanation of syngas: identification of metabolic pathways from CO in a natural anaerobic consortium

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RÉSUMÉ

Au cours des dernières décennies, l'intérêt pour la gazéification de biomasses a considérablement augmenté, notamment en raison de la grande efficacité de recouvrement énergétique de ce procédé par rapport aux autres procédés de génération de bioénergies. Les composants majoritaires du gaz de synthèse, le monoxyde de carbone (CO) et l'hydrogène (H_2) peuvent entre autres servir de substrats à divers microorganismes qui peuvent produire une variété de molécules chimiques d'intérêts, ou encore produire des biocarburants, particulièrement le méthane. Il est donc important d'étudier les consortiums méthanogènes naturels qui, en syntrophie, serait en mesure de convertir le gaz de synthèse en carburants utiles.

Cette étude évalue principalement le potentiel de méthanisation du CO par un consortium microbien issu d'un réacteur de type UASB, ainsi que les voies métaboliques impliquées dans cette conversion en conditions mésophiles. Des tests d'activité ont donc été réalisés avec la boue anaérobie du réacteur sous différentes pressions partielles de CO variant de 0.1 à 1,65 atm (0.09 à 1.31 mmol CO/L), en présence ou absence de certains inhibiteurs métaboliques spécifiques. Dès le départ, la boue non acclimatée au CO présente une activité carboxidotrophique relativement intéressante et permet une croissance sur le CO. Les tests effectués avec de l'acide 2-bromoethanesulfonique (BES) ou avec de la vancomycine démontrent que le CO est majoritairement consommé par les bactéries acétogènes avant d'être converti en méthane par les méthanogènes acétotrophes. De plus, un plus grand potentiel de méthanisation a pu être atteint sous une atmosphère constituée uniquement de CO en acclimatant auparavant la boue. Cette adaptation est caractérisée par un changement dans la population microbienne désormais dominée par les méthanogènes hydrogénotrophes. Ceci suggère un potentiel de production à large échelle de biométhane à partir du gaz de synthèse avec l'aide de biofilms anaérobies.

Mots clés : Monoxyde de carbone, gaz de synthèse, conversion anaérobie, biométhanisation, méthanogènes hydrogénotrophes, méthanogènes acétotrophes, boue granulaire, UASB.

ABSTRACT

Syngas produced through the thermal gasification of biomass for energy recovery has received increased attention in the past decades due to its higher efficiency compared to other bioenergy processes. The gas components of syngas, CO and H_2, can serve as substrates for the conversion of desirable chemicals and fuels, namely methane, by a wide range of microorganisms. Meanwhile, anaerobic wastewater-treating sludges have been reported as good sources of carboxidotrophic microorganisms which can be exploited for methane production. Thus it is important to investigate existing methanogenic consortiums which, in syntrophy, are able to convert syngas into useful fuels.

This study is mainly focused on the assessment of the carboxidotrophic methanogenic potential present in a natural consortium of microorganisms from a UASB reactor and the identification of CO conversion routes to methane under mesophilic temperatures. To achieve this, a series of kinetic-activity tests with the anaerobic sludge were performed under CO partial pressures varying from 0.1 to 1.65 atm (0.09-1.31 mmol/L) in both the presence and absence of specific metabolic inhibitors. The non-adapted sludge presented an interesting carboxidotrophic activity potential for growing conditions on CO alone. Inhibition experiments with 2-bromoethanesulfonic acid (BES) and vancomycin showed that CO was converted mainly to acetate by acetogenic bacteria, which was further transformed to methane by acetoclastic methanogens. Moreover, it was possible to achieve higher methanogenic potential under 100% CO by acclimation of the sludge. This adaptation led to a shift in the microbial population predominated by hydrogenophilic methanogens. This suggests a possible enrichment potential with anaerobic biofilms for large scale methane production from CO-rich syngas, and further advances the knowledge base for anaerobic reactor development.

Key words: Carbon monoxide, synthesis gas, anaerobic conversion, biomethanation, hydrogenophilic methanogens, acetoclastic methanogens, granular UASB sludge.

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LIST OF ABBREVIATIONS

ACS:	Acetyl-coenzyme A synthase
AD:	Anaerobic digestion
atm:	Atmosphere
ATP:	Adenosine triphosphate
BES:	2-bromoethanesulfonic acid
BLAST:	Basic Local Alignment Search Tool
bp:	Base pair (s)
˚ C:	Degree Celsius
CH_4:	Methane
CH_3COOH:	Acetate
CH_3OH:	Methanol
CO:	Carbon monoxide
CO_2:	Carbon dioxide
CoA:	Coenzyme A
COD:	Chemical oxygen demand (g/L)
CODH:	Carbon monoxide dehydrogenase
CoM:	Coenzyme M
16S rDNA:	Genomic deoxyribonucleic acid
d:	Day
DGGE:	Denaturing Gradient Gel Electrophoresis
DNA:	Deoxyribonucleic acid
Ech-hydrogenase:	Escherichia coli hydrogenase
EDTA:	Ethylenedinitrolotetraacetic acid
FID:	Flame ionization detector
FT :	Fischer-Tropsch reaction

g:	Gram
GJ:	Gigajoule
H:	Henry's constant (L atm mol^{-1})
h :	Hour
H_2:	Hydrogen
H_2O:	Water
H_2S :	Hydrogen sulphide
Kb:	Kilobase
KJ:	Kilojoule
KPa :	Kilopascal
L:	Litre
LPS:	Lipopolysaccharides
µL:	Microlitre
µM:	Micromolar
mg:	Milligram
min:	Minute
mL:	Millilitre
mM:	Millimolar
mm:	Millimetre
mmol:	Millimole
ms:	Millisecond
MSW:	Municipal solid waste
Mtr :	Enzyme N-methyl-H_4SPT:CoM methyltransferase
N_2:	Nitrogen
Na_2EDTA:	Disodium ethylenediamine tetraacetate
ng:	Nanogram
Ni:	Niquel
O_2:	Oxygen

P_{CO}:	CO partial pressure
PCR:	Polymerase chain reaction
RDP:	Ribosomal Database Project
RNG :	Renewable natural gas
$\Delta G^{\circ\prime}$:	Standard Gibbs free energy
SD:	Standard Deviation
SDS :	Sodium dodecyl sulfate
sec:	Second
SO_4^{2-}:	Sulfate
TAE :	Tris-acetate-EDTA
TCD:	Thermal conductivity detector
T_d :	Doubling time
TEN:	Tris-EDTA-NaCl
TJ:	Terajoule
T_{opt} :	Temperature optima
t/year:	Tonne per year
UASB:	upflow anaerobic sludge blanket reactor
V:	Voltage
VFA:	Volatile Fatty Acids (mg/L)
VS:	Volatile solids (g/L)
VSS:	Volatile suspended solids (g/L)
WGS :	Water gas shift reaction
wt/vol:	Weight per volume
WW :	Wastewater

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CHAPTER 1

Introduction

Energy needs are increasing worldwide due to humanity's population growth and the accelerated development of industry leading to today's goal of replacing current non-renewable and scarce fossil fuel sources. A recent study estimated fossil fuel depletion at the current consumption rate for oil, coal and gas at approximately 35, 107 and 37 years, respectively[1]. Therefore it is necessary to find new alternatives for the production of sustainable energy to mitigate these energy needs. Nowadays, the use of renewable energy sources like biomass or solid waste has become one of the most promising sources for energy production, which at the same time supports the reduction of harmful fossil fuel gas emissions that largely contribute to global warming[2–5].

There are several well established processes for the conversion of different types of biomass into renewable energy sources like biodiesel or synthetic natural gas. However, some of the most established techniques for cleaner fuel production such as anaerobic digestion, direct fermentation of easily degradable substrates, still have issues regarding their efficiency since a large proportion of organic material cannot be degraded by the microorganisms[6,7]. One solution to overcome the limitation of poor biomass conversion with this kind of fermentation could be to gasify the biomass and further use the converted gas components (called synthetic gas or syngas) as a building block for the production of desired renewable fuels such as methane[7,8].

A recent report from the Canadian Gas Association together with the Alberta Research Council stated that the use of gasification of biomass versus anaerobic digestion has the potential to produce most of the renewable natural gas in Canada in the near future[9]. However, despite the advantages of using syngas fermentation for

clean fuel production, much more research in this field needs to be done. Deeper understanding of the microbiological aspects implicated in syngas fermentation will allow further improvement of the bioreactor setup, and consequently the advancement of syngas derived fuels at large scale.

One approach to lower the cost of this process is the use of already existing anaerobic consortiums, which in syntrophy will be able to convert syngas components (i.e. CO, H_2) into useful fuels such as methane. Anaerobic wastewater-treating sludge has been reported as a good source of carboxidotrophic microorganisms which can be exploited for methane production at large scale[10,11].

This study was therefore planned to assess the carboxidotrophic methanogenic potential present in a natural anaerobic consortium of microorganisms from an upflow anaerobic sludge blanket reactor (UASB). A second objective was to better understand the metabolic routes implicated in methane production from carbon monoxide (CO), the main component of syngas, under mesophilic temperatures.

1.1 Biomass as an Energy Source

Renewable energy sources like biomass or solid waste have become a promising sources for energy production[4]. This new trend of using organic waste for green energy production could be beneficial in two ways: it supports the reduction of fossil fuel gas emissions, such as the release of carbon dioxide into the atmosphere that largely contributes to the global warming, and at the same time contributes to the reduction of municipal and industrial solid wastes, whose accumulation has increased worldwide[12].

In a recent study in 2006, Levin et al. speculated that the residual biomass generated annually in Canada, approximately 1.45 x 10^8 t with an estimated energy value of 2.28 x 10^9 GJ, could account for about 22% of Canada's current annual energy use[13].

The most commonly used bioenergy conversion processes can be summarized as follows: the refining of oil from crops (i.e. sunflowers oil) or algae, anaerobic fermentation of sugar and starch feedstock (i.e. beet, cereals), anaerobic fermentation of wet biomass (i.e. organic waste, manure), and the pyrolysis, combustion or gasification of solid biomass[9,14,15]. The use of different technologies for biomass conversion depends on the type of material present in the biomass and the desired final use of the product (Figure 1).

These energy production techniques can be divided in three groups clearly defined depending on the mechanism used to transform the biomass into useful fuels. In biochemical conversion processes, the organic material is degraded directly by microorganisms for the production of energy sources. Examples of this group are the anaerobic digestion of organic biomass, which leads to the production of methane, and the carbohydrate fermentation, which leads to the production of bioethanol[15]. The second group of techniques for biomass conversion is the use of chemicals extracted from biomass for the production of improved fuels. An example of this group is the vegetable or algal oil refining process for liquid fuels production, such as

biodiesel[14]. The last group is the use of thermochemical processes for energy production, where biomass is converted into chemicals and heat at high temperatures and pressure, eventually followed by catalytic conversion into more valuable products. Gasification of biomass and Fischer-Tropsch reactions are good examples of this group[8,10].

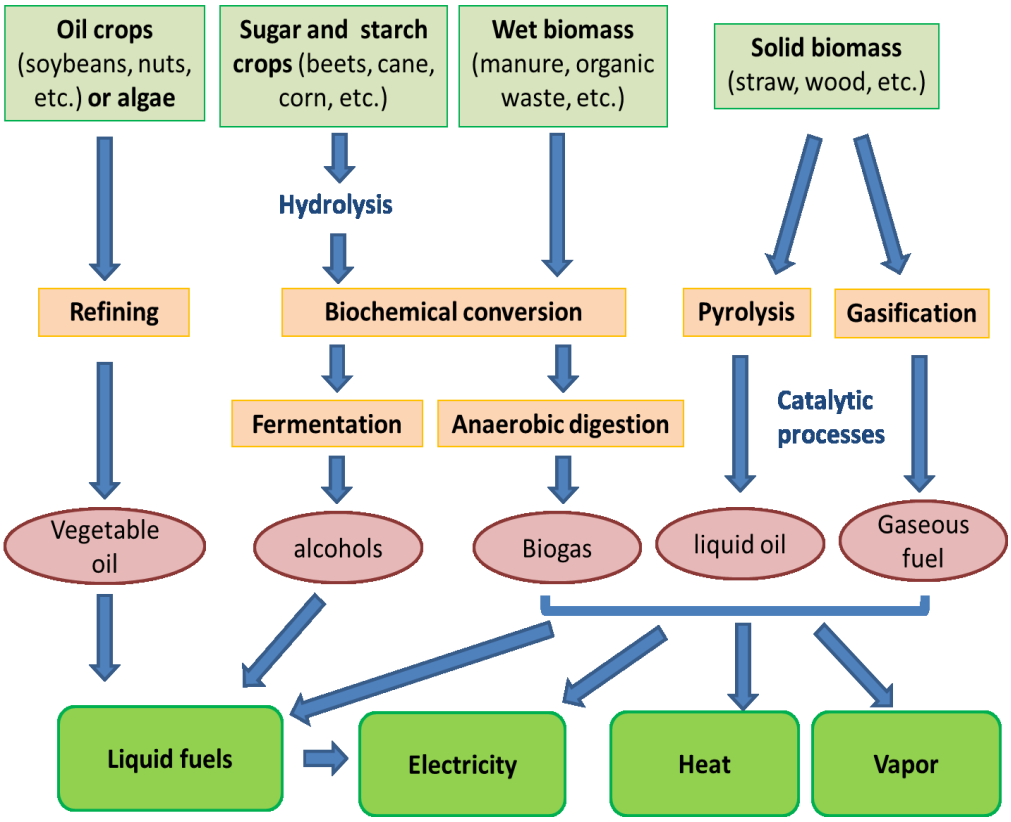

Figure 1. Bioenergy conversion processes from biomass[16].

Anaerobic digestion (AD) is one of the most cost-effective and widely studied processes for the production of biomass derived fuels and chemicals such as methane[17]. However, the main drawback of this method is the small percentage of organic material that can be degraded by microorganisms due to biomass' polymeric nature. Thus, it is necessary to perform a chemical hydrolysis of the poorly

degradable materials, increasing the cost of the process. An interesting alternative to overcome this issue is the gasification of biomass into syngas[7]. The main components of syngas, carbon monoxide (CO) and hydrogen (H_2), can then be used as a feedstock for a wide variety of microorganisms for fuel production, namely methane, to produce renewable natural gas (RNG)[18–20].

Another possible alternative is the direct use of hydrogen for energy production since it would provide higher energetic advantages than methane and would increase the overall efficiency of the process. However, storage options for hydrogen are limited due to high costs, security challenges (more flammable and buoyant than methane), missing infrastructure and short lifetimes of fuel cells[21–23]. In contrast, renewable natural gas (RNG) can easily be stored and distributed due to its higher energy density and boiling point. For example, liquid methane has three times the energy density of hydrogen and requires less storage space. Additionally, opposite to hydrogen, methane is compatible with the current natural gas network and gas devices (i.e. gas pipelines, engines, natural gas powered vehicles, etc.)[21].

Natural Gas Vehicles for America (NGVA) states that waste biomass could supply enough natural gas for about 11 million natural gas vehicles, which represents approximately 5 percent of the America's vehicles[24].

Moreover, in a recent study Alberta Innovates-Technology Future formerly Alberta Research Council claimed that methane production from Canadian waste in the next 5 to 10 years through anaerobic digestion processes will be the main source of RNG, with gasification contributing afterwards[9]. This conclusion was based on current technology, the level of industry acceptance, and the need for further technology development in the gasification industry[9].

In fact, the gasification of industrial and municipal solid waste is believed to be among the most efficient technologies for energy recovery nowadays, and at the same time contributes to the reduction of the continuously increasing municipal solid waste (MSW)[12].

1.1.1 Potential Methane Production from Organic Wastes Overview

Thanks to the continual technological advances in renewable energy production, most of today's waste has the potential to be converted into energy sources such as methane. The more organic materials are present in the waste, the more efficient the methane production process becomes. This type of biomass is mostly generated by the agricultural, forestry and municipal sector[9,25].

The knowledge of waste composition and its production rate in contemporary society can serve as a tool for the estimation of the methane production potential that can be achieved with different bioenergy processes. This will help improve economical waste management planning in order to reach higher energy yields.

Agricultural wastes mostly come from crop residues (i.e. wheat. corn, soybeans, etc.) and animal manures (i.e. cattle, chicken). Forestry residues include mainly wood and waste produced from wood processes. Besides, waste from the municipal sector is composed of solid waste from residential and industrial facilities (MSW), landfills, municipal wastewater (WW), and municipal biosolids collected from wastewater treatments[25,26].

In a recent report concerning methane's production potential from Canadian waste sources, prepared by Alberta Innovates Inc. in collaboration with the Canadian Gas Association, a potential production of 24.9 Mt/year of renewable natural gas (RNG) was estimated from the total Canadian waste produced by the agricultural, forestry and municipal sectors[9]. The authors calculated that the forestry sector has the potential of producing 12.9 Mt/year of RNG, in addition to 8.8 Mt/year from agricultural waste and 3.2 Mt/year from municipal waste. Moreover, statistical comparisons between anaerobic digestion (AD) and gasification processes for total methane production potential from Canadian wastes showed that the use of biomass gasification has the potential to produce 84% (21 Mt/year) of the total Canadian RNG output, whereas it was estimated that only 16% (3.9 Mt/year) of the total RNG can be

produced from anaerobic digestion processes since AD processes are limited by the polymeric nature of poorly degradable materials present in potential biomass sources. However, the latter is the most commonly used method for bioenergy production due to its technological availability and lower cost[9].

Moreover, the authors stated that the potential RNG estimated per year which corresponds to an energy value of 1.4 x 10^3 TJ, which could theoretically replace a significant amount of the current residential and commercial natural gas use[9].

Furthermore, many studies have documented that the production and capture of methane from organic waste contributes to the reduction of greenhouse gas emissions[13,14,27].

1.2. Gasification of Biomass

Gasification basically consists of the partial oxidation of organic material such as fossil fuels or biomass at temperatures between 500 and 1500˚C, without total combustion, in the presence of oxygen or steam as an oxidizer[10]. This process takes place in a gasifier, and the result is a mixture of combustible and non-combustible gases called synthesis gas. The syngas, following clean-up to remove impurities, can be directly used in gas turbines and internal combustion engines as well as fuel cells for generating heat and/or electricity[10,28]. Moreover, syngas can be used as a raw material for the production of synthetic and/or natural chemicals, as well as liquid and gaseous fuels such as methane which can be employed to replace natural gas derived from fossil fuel sources[8,9].

Gasification is a very efficient process in terms of energy production compared to other thermal bioenergy conversion techniques such as the combustion of biomass. This is mainly because compared to direct combustion, where part of the energy is lost in the combustion process, with syngas most of the energy contained in the organic material can be extracted with the further use of microorganisms[9,29]. Moreover, since any kind of material can undergo gasification, it is very useful when the organic matter is difficult or slow for microorganisms to degrade, such as relatively dry materials like straw or wood, or even when the organic matter is entirely non-biodegradable (i.e. plastic, rubber, etc.)[10].

There are 3 main steps in synthesis gas production. First, the organic material needs to be conditioned before its use depending on the feedstock (drying, sized, etc.). Then, the material is pyrolized at temperatures between 300-500˚C to produce gases, tars, bio-oils, and solid char, and is lastly gasified, where the products are transformed into syngas in the presence of an oxidizer[8,29] (Figure 2).

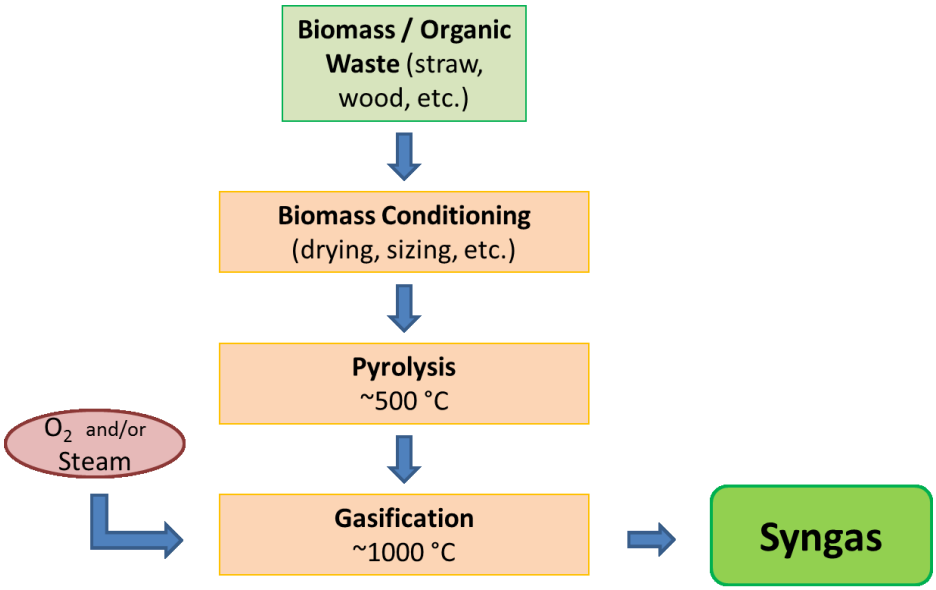

Figure 2. Main Steps in a Biomass Gasification System[8].

There are two kinds of gasification depending on the type of oxidant employed: direct gasification, where the organic material is partially oxidized using air and/or oxygen, and indirect gasification, which utilizes steam as the oxidizing agent. The latter being the more efficient as it is thermodynamically more favourable[8,30]. The use of the different methods depends mainly on the organic source used. For biomass gasification it is preferable to use air or oxygen as the oxidant, while indirect gasification is employed in the case of fossil fuels[6].

1.2.1 Synthesis Gas

Synthesis gas is derived from the gasification of a wide variety of organic sources such as coal, petroleum coke, oil, catalytic reforming of natural gas[31,32], and biomass including industrial and municipal solid wastes[32,33].

Syngas is mainly composed of carbon monoxide (CO), hydrogen (H_2), and carbon dioxide (CO_2), but also has minor amounts of other gases such as methane (CH_4), nitrogen (N_2), and hydrogen sulphide (H_2S). However, the gas composition of synthesis gas varies depending on the type of organic material used for gasification and its properties (moisture, ash, dust, tar content, etc.)[34], the gasification process employed (type of oxidant), the type of gasifier (fixed bed, fluidized bed, etc.), and the reactor's operational conditions (temperature, pressure, etc.)[8,35].

Thus the composition of synthesis gas can be modified through the optimization of the gasification process. It has been reported that gasification at high temperatures between 1500-1800˚C for coal, and 1100˚C for biomass produces higher CO and hydrogen concentrations within the syngas, which in turn are the main precursors for the production of different fuels such as methane[8,36].

Moreover, it has been proven that using pure oxygen as the oxidant agent can reduce N_2 content in the syngas to increase the concentration of targeted syngas components[37].

1.3. CO-Rich Syngas Conversion to Methane

Synthesis gas obtained from gasification of fossil fuels, biomass, and/or solid wastes can be further converted to methane, as previously commented, through the application of chemical or biochemical processes.

The main components of syngas, CO and hydrogen, can be used for methane production via the methanation catalytic reaction according to the following equation:

$$CO + 3H_2 \rightarrow CH_4 + H_2O \qquad \text{(Eq. 1)}$$

This reaction is carried out at elevated temperatures and pressure in the presence of chemical catalysts, making it a faster method than with bioconversion processes[32,38]. In order to increase the H_2/CO ratio necessary for the completion of the reaction, this process produces pure hydrogen through the catalytic water gas sift reaction (WGS), converting the CO present in the syngas into hydrogen and CO_2, (Eq. 2):

$$CO + H_2O \rightarrow H_2 + CO_2 \qquad \text{(Eq. 2)}$$

Catalytic reactions can also be applied to the production of a wide variety of fuels and chemicals such as ethanol, methanol, acetate, etc. (Fischer-Tropsch sysnthesis [39].

The main drawback with the use of chemical catalytic processes is the high sensitivity to the impurities present in synthesis gas. Hence, the process can be easily inactivated due to the presence of tar, oil and other gas contaminants such as sulfur[8]. In order to avoid catalyst poisoning these impurities need to be totally removed from

the syngas, or at a minimum reduced to a certain tolerated level[40]. However, the purification step to eliminate all of the impurities prior to methane production requires the use of advanced cleaning systems, making this method complex and costly. A solution to solve all these drawbacks with chemical catalysts processes is the use of microorganisms as a biocatalysts for syngas conversion to methane[7,10,32]. Anaerobic microorganisms can be exploited for the production of a variety of interesting metabolites from the syngas components. These products include gaseous fuels such as methane and H_2, organic acids (i.e. acetate, propionate, formate, butyrate, and lactate), as well as many alcohols such as methanol, ethanol and butanol[8,41].

Despite the slower syngas methane conversion rate achieved with the use of microorganisms as a catalyst, it still presents several advantages over the catalytic process. The higher specificity of the enzymes implicated in the biochemical reaction improves the product yield, which simplifies recovery, and also reduces the formation of toxic by-products[8,42]. Furthermore, the microorganisms act as a cheap catalytic source for methane production and possess higher tolerance to sulfur and other impurities present in syngas when compared to chemical catalysts[43,44]. Hence cleansing synthesis gas in order to remove impurities prior to its utilization can be avoided, decreasing the overall cost of the process.

Moreover, most of the microorganisms employed as catalysts are able to grow well on CO alone, permitting a low H_2/CO ratio in the syngas [45,46]. Lastly, the use of methanogenic microorganisms as biocatalysts for methane production can result in methane production solely from CO, according to Equation 3:

$$4CO + 2H_2O \rightarrow CH_4 + 3CO_2 \quad \text{(Eq. 3)}$$

However, there are a few drawbacks in using microorganisms as catalysts as the continuous supply of nutrients is necessary to maintain the efficiency of the process, in addition to maintaining total anaerobic conditions in the reactor since the

methanogenic microorganisms cannot usually survive in the presence of oxygen. Previous studies on methane production in soil showed that the presence of oxygen or high concentrations of oxidative inorganic compounds retarded and inhibited methane formation[47].

Besides, the maximum CO conversion rate achieved in the process will depend on the microorganism's capability to perform the reaction at the given environmental conditions, as well as the cell concentration in the reactor[48].

Moreover, one of the limiting steps in CO bioconversion processes is the gas-liquid mass transfer due to the low aqueous solubility of CO. Thus low CO mass transfer limits the microorganism's ability to convert CO into methane due to the low amount of substrate available[10,49].

Further steps might be needed once methane has been produced depending on its final use, such as a further separation of the methane and the carbon dioxide produced, or the need for compression processes for synthetic natural gas production[9,50,51].

1.3.1. Parameters that can Affect the CO Bioconversion to Methane

There are several operational parameters involved in syngas bioconversion processes that can seriously affect the methane production yield and growth of methanogens in the population. These parameters include but are not limited to pH, temperature, media composition, substrate pressure and gas-liquid mass transfer. Therefore, to achieve higher methane yields it is essential to optimize these parameters according to the needs of the targeted microorganisms and control these conditions during the bioconversion of CO-rich syngas.

1.3.1.1. Effect of pH

pH is an important parameter for the optimal activity and performance of the different microorganisms implicated in methane production from CO-rich syngas. Many studies working with anaerobic microbial populations have noted the relationship between the pH present in the medium and metabolite formation[8,52]. Therefore, when working with a mixed anaerobic consortium the production of one metabolite (i.e. acetate) in high quantities may decrease the pH in the medium and thus inhibit the activity of other microorganisms, such as the production of methane by methanogens[47,52]. This has already been demonstrated in many studies with methanogenic mixed cultures where the accumulation of fatty acids formed during the degradation of the organic matter in the soil decreased the pH in the media and methane production was inhibited[47,53,54].

Moreover, the pH might also affect some physiological aspects in the cell such as internal pH, membrane transport potential and the proton-motive force, which in fact might provoke the formation of metabolic by-products[8,52].

Due to the small pH range where the microorganisms are metabolically active, any change in pH in the medium can seriously affect cell growth and even cause the loss of biological activity by cell damage or death. Thus any change in pH in the medium affects the overall CO-rich syngas bioconversion process, as has been previously reported[47,55].

The optimum pH range observed for CO converting microorganisms varies between 5.5 and 7.5 depending on the different microbes' physiological group and species. For example *Citrobacter sp*, a hydrogen producer, has an optimal pH range of 5-5-7.5, and the acetogenic bacteria *Clostridium carboxidivorans* has an optimal pH of 6.2[7,32]. On the other hand, most of the methanogenic archaea reported until now also grow at an optimal pH between 6 and 8.5 near neutral conditions[17]. However, a few studies have observed some strains of *Methanosarcina barkeri* that

are able to grow at a pH as low as 4.3, however growing best under neutral conditions[56].

1.3.1.2. Effect of Temperature

The change in temperature during the CO-rich syngas bioconversion process has a similar effect as pH on the population. The temperature operational conditions of the process affect microbial growth and substrate utilization. However, a difference from the optimum pH tolerated is that optimal temperatures differ greatly between different species. While most of the CO-converting microorganisms' activities are better around neutral pH, the change of ±10 degrees Celsius will favour one type of CO-converting microbes with respect to another in the population. This is important when working with a mixed culture, since the change of a specific temperature range will lead to a shift in the population and thus a shift in metabolite formation from CO. The most favourable temperatures achieved for the growth of CO-consuming mesophilic and thermophilic anaerobic microorganisms range from 30-40˚C and 55-83˚C, respectively[7,32].

Moreover, all of the mesophilic methanogens currently known, such as *Methanosarcina barkeri* or *Methanobacterium formicicum*, present an optimal growth at temperatures between 30 and 45˚C, while thermophilic methanogens range between 55 and 70˚C[17].

It must be noted that temperature also affects the solubility of gaseous substrates, namely CO, in liquid media. Hence increasing the temperature of the process leads to the reduction of gas solubility in the culture while the rate of gas-liquid mass transfer may increase due to lower viscosity[57].

1.3.1.3. Effect of Media Composition

The components of syngas, namely CO, serve as a source of carbon and energy for the growth of a variety of microorganisms used as biocatalysts in the CO conversion to methane process. However, all bacteria need elements such as nitrogen, sulfur and phosphorus for the synthesis of cell material[52,58]. Moreover, it has been observed in previous studies that the addition of various minerals and vitamins in the media results in higher metabolic activities[8,58].

Experimental evidence with different methanogenic microorganisms indicate that sodium and potassium play important roles for ATP synthesis and nutrient transport in the cell[58]. Moreover, sulfur, nickel and vitamin B12 are involved in CO dehydrogenase (CODH) activity, the enzyme responsible for the conversion of CO[58]. A significant boost in methane production from acetate has been reported through the addition of iron, nickel, and cobalt[59]. Moreover, magnesium is required for the activity of many enzymes, including methyl-CoM reductase, the enzyme that catalyzes the final metabolic reaction in methanogenesis[58].

Therefore, nutrient limitations can cause limitations in the maintenance of cell metabolism, intracellular enzyme production and cofactor formation[52] .

1.3.1.4. Effect of Substrate Partial Pressure

The partial pressure of the syngas components is a key factor in the metabolism of the microorganisms forming part of the consortium. The partial pressure of CO (P_{CO}) and/or the P_{CO} to P_{CO2} ratio can greatly affect the microbial growth and the metabolite production since some enzymes involved in the metabolic processes can be entirely or partly inhibited by substrate exposure[60].

Many microorganisms are reported to use CO as a carbon and energy source since CO can act as an electron donor via CODH for the production of reducing equivalents, namely methane[8,61,62]. In fact it has been recently reported that electron production from CO is always thermodynamically more favourable than electron production from H_2, totally independent of pH, ionic strength, electron carrier pairs, and gas partial pressure[63].

However, the lower aqueous solubility of CO compared to the other components of syngas might lead to a limitation of the gas-liquid mass transfer rate to the media, thus decreasing the metabolic activity of the microorganisms[10,52]. When the mass transfer becomes a limiting factor the amount of gaseous substrate uptake is proportional to the partial pressure of that component in the gas phase[52,64].

Hence, a method to overcome this mass transfer limitation is by increasing the initial partial pressure of CO which improves the net electron production with CODH. Furthermore, it has been proven that the volume of the reactor can be reduced by increasing the partial pressure of the gaseous components present in the syngas[52].

Many studies have reported a high tolerance to CO by a variety of microorganisms, such as *Clostridium aceticum* observed to grow at high partial pressure of CO up to 2 atm without cell growth inhibition[65]. Or in another study the authors reported that *R. rubrum* was able to grow under partial pressures of CO up to 1.4 atm without any effect in the CO consumption rate, cell growth and H_2 yield[66].

However, some microorganisms are less tolerant to high CO partial pressures, and the increase in P_{CO} can lead to a longer growth doubling time or metabolite inhibition[52]. This is the case of methanogenesis inhibition when increasing the CO partial pressure in the media, as has been reported in many studies[18,62,67]. A couple of studies have shown that exposure to higher CO leads to the apparent down-regulation of the mtr operon, which encodes for the enzyme N-methyl-H_4SPT:CoM methyltransferase (Mtr) involved in both the hydrogenotrophic pathway and the acetoclastic pathway for methane formation in methanogens, thus decreasing the methane production yield[68,69].

Moreover, many studies report that a change in P_{CO} in the gas phase can result in a shift of metabolite formation. O'Brian et al. reported that *M. barkeri* produced H_2 at P_{CO} higher than 0.2 atm in the gas phase with methane as the main metabolite at CO concentrations below this value, suggesting that *M. barkeri* CODH produces hydrogen as a by-product from the CO transformation, and that the hydrogenase production activity is not inhibited at high CO concentrations[70]. In another study with *M. acetivorans*, the authors discussed that the methane production rate is not inhibited at high CO concentrations, but the increase in CO partial pressure leads to the rate increase of acetate and formate production from CO which could cause a decrease of the final amount of CO converted to methane[67].

However, in a later study it was reported that an *M. acetivorans* strain isolated from prolonged incubation at a high partial pressure of CO was capable of producing methane at a high rate[71].

1.3.1.5. Mass Transfer Effect

Another important condition that can affect the CO bioconversion processes is the gas-liquid mass transfer rate due to the low aqueous solubility of CO and H_2. This diffusion limitation results in a low availability of substrates for the microorganisms, which decreases the overall productivity of the process[10,49]. The mass transfer limitation might originate from the transport of the gaseous substrates into the liquid interface, into the fermentation media, into the liquid layer around the microbes, and finally by the diffusion of the substrates across the cell membrane into the microbial cytoplasm[52,57]. However, the major mass transfer resistance observed during syngas fermentation processes is the mass transfer across the gas-liquid interface[52,72].

Moreover, it is also known that the yield of the process is affected by the cell concentration in the media and the CO consumption rate, parameters which might vary during the course of the process[8].

Based on the theoretical equations for CO-rich syngas conversion to methane, for the production of one mol of methane, one mol of CO and 3 mols of H_2 (Eq. 1) have to be transferred into the media, or in the case of direct CH_4 production from CO (Eq. 3), four moles of CO are necessary per mol of CH_4 produced. However, since at mesophilic temperatures the solubility of CO and H_2 is low, more moles of the gaseous substrates need to be transferred to the media per carbon equivalent consumed to achieve higher yield and productivity during the process[8,73].

To have a better understanding of the mass transfer rate in the media, it is important to know the volumetric mass transfer coefficient, K_La (s^{-1}), which can be determined using the following equation:

$$\text{Overall mass transfer rate} = K_La / H (P^g - P^l) \quad \text{(Eq. 4)}$$

where H is Henry's constant (L atm mol^{-1}), and P^g and P^l (atm) are the partial pressures of the gaseous substrate in gas and liquid phase, respectively[8].

Therefore, to improve gas solubility in the liquid phase and thus achieve high product yields and efficiency it is necessary to increase the operational pressure conditions during the process. However, it should be noted that the increase in CO concentration can lead to the inhibition of the microorganisms' metabolism, and thus the inhibition of the CO conversion to methane or other targeted chemicals[18,69]. As previously commented it has also been observed that adaptation of the microbial culture to high CO pressures can be achieved by gradually increasing the pressure in the system[48].

It is therefore important to evaluate the kinetics of the reaction and have a good correlation between the substrate diffusion into the medium and the specific substrate consumption rate[57].

Several studies have pointed to an increase in agitation speed for improving mass transfer, since the speed increases the break up of the gaseous compounds bubbles formed in the medium, thus increasing the gas-liquid interfacial area. However, this solution consumes a lot more power and becomes unfeasible in large scale methane production processes due to the higher costs associated with this method [8,52].

Moreover, many studies have examined mass transfer using different bioreactors, and the volumetric mass transfer coefficient depends mainly on reactor geometry, configuration, process operational conditions and liquid phase properties[72,74]. For example, Klasson et al.[72] compared the performance of a packed bubble column and a trickle-bed reactor for the conversion efficiency of syngas components CO, H_2 and CO_2 to methane in a tri-culture of *R. rubrum*, *M. formicicum* and *M. barkeri*. The authors concluded that the trickle-bed reactor has a higher mass transfer rate and considerably higher productivity due to the longer gas residency time in the media and improved mass transfer properties.

Furthermore, many chemicals such as surfactants, bio-polymers, organic compounds, catalysts and small particles can be added to the media to increase the gas-liquid mass transfer rates[8,66].

1.4. CO-Consuming Anaerobic Microorganisms

A wide variety of microorganisms within different trophic groups are able to metabolize carbon monoxide (CO). Microbes which use CO as their carbon and/or energy source are known as carboxidotrophic microorganisms. This nomenclature is usually used in literature concerning CO-consuming microbes with aerobic respiratory systems[32,75], however in this work the term "carboxidotrophic" will be used for all of the microorganisms which utilize CO. Since there is a clear distinction between aerobic and anaerobic CO metabolisms due to their different enzyme systems and oxidants employed, aerobic CO-metabolism won't be discussed here.

Carbon monoxide dehydrogenase (CODH) is the key enzyme involved in the conversion of carbon monoxide (CO), which oxidizes CO according to the following reversible reaction:

$$CO + H_2O \leftrightarrow CO_2 + 2H^+ + 2e^- \qquad \text{(Eq. 5)}$$

This enzyme is widely distributed among different anaerobic bacteria and archaea, and is characterized by the presence of nickel as a cofactor[32,76]. Ni-containing CODH could be classified according to its catalytic activity as a monofunctional CODH, which only catalyzes the oxidation of CO coupled to anaerobic respiration (eq. 3), and bifunctional CODH/acetyl-coenzyme A (CoA) synthase, which also catalyzes the cleavage (or the synthesis) of acetyl-CoA to form the methyl-group coenzyme A (CoA), and CO[7,20,77]. The reducing equivalents produced from CO oxidation are then funneled along a hydrophobic channel into a respiratory chain for the final reduction of the terminal electron acceptor, driving the synthesis of ATP by the translocation of ions across the cytoplasmic membrane[62,77,78].

The thermodynamically favorable electron production with CO makes this substrate an excellent source of energy, able to reduce most redox-active cofactors[62,63]. However, a limited number of anaerobes are capable of using CO as their only source of carbon and energy. It is argued that this is likely due to the sensitivity of metal containing enzymes to CO exposure, which results in cell growth inhibition[20,62].

The known anaerobic respiratory processes which can coupled to CO oxidation are shown in figure 3: carbonate respiration (methanogenesis and acetogenesis), proton respiration (hydrogenogenesis), and sulfate or sulfur respiration (sulfate or sulfur reduction)[19,20,32,62].

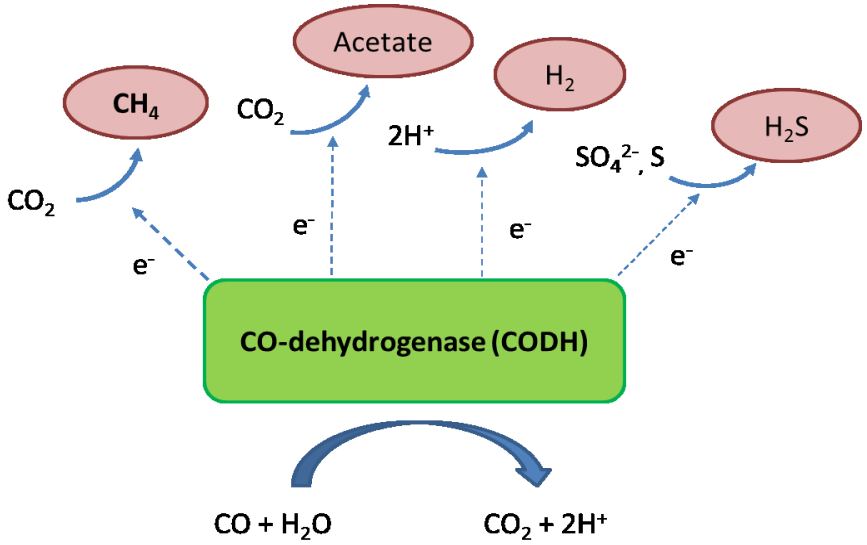

Figure 3. Anaerobic respiration coupled to CO oxidation (adapted from Oelgeschläger et al.) [62].

1.4.1. Hydrogenogens

Hydrogenogenic carboxidotrophs are a group of microorganisms capable of using CO as their only energy and carbon source to produce H_2 in the absence of an electron acceptor. These bacteria can grow by oxidizing CO and reducing the protons derived from H_2O in order to produce equimolar amounts of H_2 and CO_2, analogous to the water-gas-shift reaction (WGS) previously described (Eq. 2)[62]. This reaction is coupled to the translocation of ions across the cytoplasmic membrane of the cell which drives the formation of ATP by an ATP synthase[62,79].

The carboxydotrophic hydrogenogenic metabolism has been shown in both mesophilic Gram-negative bacteria such as *Rhodospirillum rubrum*[80], and thermophilic Gram-positive bacteria such as *Carboxydothermus hydrogenoformans*[7,81]. Table I present some characteristics of several CO-oxidizing hydrogenogenic bacteria.

Generally, growth rates of mesophilic hydrogenogens on CO are low, and high CO concentrations in the medium might lead to growth inhibition of the bacteria. However, Kerby et al. reported that *R. rubrum* was able to achieve rapid anaerobic growth in darkness, converting CO into H_2 and CO_2 by increasing the nickel content in the medium[32,80].

On the contrary, thermophilic gram positive bacteria can achieve higher growth rates with CO alone, and are able to grow at high CO concentrations without growth inhibition[32]. Despite that, *C. hydrogenoformans*, one of the most frequently studied bacteria in this group, has been shown to use a similar carboxydotrophic hydrogenogenic pathway as *R. rubrum*, a mesophilic hydrogenogen with a much slower growth rate. Therefore, it has been proposed that its ability to grow much more rapidly with CO than other species might lie in the fact that this bacterium possesses various genes encoded for the enzyme CODH and CODH/ACS, and thus probably regulates the synthesis of both hydrogenases differently depending on the metabolic needs of the bacteria[62]. Recent work with *C. hydrogenoformans* supports

this hypothesis, as in the study it is described how both hydrogenase-linked CODH and CODH/ACS operons are regulated for efficient consumption of CO across a wide range of concentrations[82]. The authors demonstrated that under high partial pressures of CO the bacteria is able to bypass more CO into energy production by the overexpression of hydrogenase, while at low CO concentrations the CO is mainly used towards carbon fixation by the enzyme CODH/ACS.

Table I. Characteristics of some anaerobic carboxydotrophic microorganisms [7,32].

CO Oxidizing Microorganisms	T_{opt} (˚C)	pH	T_d (h)	CO-tolorance (KPa)*	Products Formed	Ref.
Hydrogenogenic Bacteria						
Rubrivivax gelatinous	34	6.7-6.9	6.7	101	H_2	83,84
Rhodospirillum rubrum	30	6.8	8.4	101	H_2	80
Citrobacter sp Y19	30-40	5.5-7.5	8.3	50	H_2	85,86
Carboxydothermus hydrogenoformans	70-72	6.8-7.0	2	101	H_2	81
Acetogenic Bacteria						
Clostridium ljungdahlii	37	6	3.8	105	Acetate, CO_2	87
Clostridium carboxidivorans	38	6.2	6.25	160	Acetate, Ethanol, Butyrate, Butanol	88
Moorella thermoautrophica	58	6.1	7	214	Acetate, CO_2	89
Acetobacterium woodii	30	6.8	13	30	Acetate, CO_2	90
Eubacterium limosum	38-39	7.0-7.2	7	152	Acetate, CO_2	90,91
Butyribacterium methylotrophicum	37	6.0	12-20	120	Acetate, Ethanol, Butyrate, Butanol	92,93
Methanogens						
Methanosarcina barkeri	37	7.4	65	101	CH_4, CO_2	94
Methanosarcina acetivorans strain C2A	37	7.0	24	100	Acetate, Formate, CH_4	95
Methanothermobacter thermoautotrophicus	65	7.4	140	45	CH_4, CO_2	96
Sulfate Reducing Bacteria						
Desulfovibrio desulfuricans	37	n.r	n.r	<20	H_2, CO_2, H_2S	97
Desulfovibrio vulgaris	37	n.r	n.r	<4.5	H_2, CO_2, H_2S	98
Desulfotomaculum carboxydivorans	55	7.0	1.7	180	H_2, CO_2, H_2S	99

*maximal CO concentration tested; n.r, not reported.

1.4.2. Acetogenic Carboxidotrophs

Acetogens are a diverse group of anaerobic microorganisms characterized by their production of acetate from CO_2 via the reductive acetyl-CoA pathway[32,100]. In this metabolic pathway two molecules of CO_2 are reduced to a methyl and carbonyl group, which are further combined with Coenzyme A by the enzyme CODH/ACS to form acetyl-CoA[32,62]. This acetyl-CoA will be then converted into acetate for energy production. Figure 4 presents an overview of carbon flow in the different metabolisms that employ the aceyl-CoA pathway.

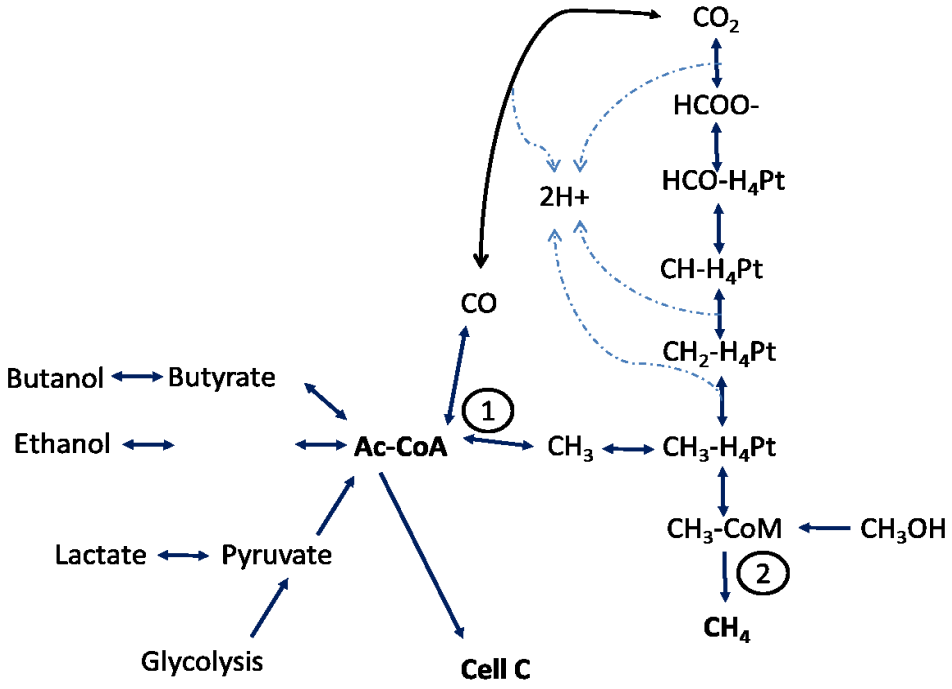

Figure 4. Carbon flow in the metabolisms that employ the acetyl-CoA pathway, (adapted from Sipma et al.)[24]. 1, step catalyzed by the bifunctional CODH/acetyl-coenzyme A (CoA) synthase. 2, final step in methane production catalyzed by the enzyme methyl-CoM reductase, shared by the three metabolic methanogenic pathways. Abreviations: Pt,pterin carrier; HCO-, formyl; CH-, methenyl; CH_2-, methylene; CH_3-, methyl; CoA, coenzyme A; CoM, coenzyme M.

It has been demonstrated in several studies that acetogens can also use CO through the acetyl-CoA pathway for energy production according to equation 10:

$$4CO + 2H_2O \rightarrow CH_3COO^- + 2CO_2 + H^+ \quad (\Delta G^{\circ\prime} = -176 KJ/reaction) \quad (Eq.10)$$

The production of acetate from CO has been also shown to be coupled to the formation of an ion motive force across the cytoplasmic membrane, which is used for energy production in the cell (ATP)[62,101].

Many acetogens are reported to grow with CO as their sole carbon and energy source at high CO concentrations (Table I). Moreover, some acetogenic bacteria (i.e. *Clostridium sp*.) are able to produce certain amounts of ethanol, butyrate and butanol from CO in addition to acetate[7].

Although the conversion of CO acetate is mainly managed by the enzyme CODH/ACS, the presence of this enzyme doesn't imply the bacteria's ability to use CO as its sole energy and carbon source[32]. Nonetheless, some acetogens are able to metabolize CO if grown together with other substrates such as hydrogen and carbon dioxide[102].

1.4.3. Carboxidotrophic Methanogens

Many biochemical studies with methanogens propose 3 main but overlapping methanogenic pathways for energy production with these microbes, although most methanogens have been known to use only one[17,62]. Most known methanogenic archaea reduce CO_2 to methane via coenzyme-bound intermediates, using electrons derived from the oxidation of hydrogen, the hydrogenotrophic pathway[17,62] (Eq. 6). The methylotrophic pathway instead reduces methylated compounds, such as methanol and methylamines, to carbon dioxide and methane. In this pathway the oxidation of 1 mol of the substrate is necessary to provide the electrons needed for the reduction of 3 mol of methanol to methane[62,103] (Eq. 7). In the third pathway, acetate is activated to acetyl-CoA, and is then split into enzyme-bound CO, a methyl group, and a coenzyme A by the CODH/ACS acetoclastic pathway. The carbonyl group is then oxidized to CO_2 which generates the electrons required for further reduction of the methyl group to methane[103,104] (Eq. 8). Table II shows general overview characteristics of some methanogenic archaea.

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad (\Delta G^{\circ\prime} = -130\text{KJ/reaction}) \qquad \text{(Eq. 6)}$$

$$4CH_3OH + H_2 \rightarrow 3CH_4 + CO_2 + 2H_2O \quad (\Delta G^{\circ\prime} = -318\text{KJ/reaction}) \quad \text{(Eq. 7)}$$

$$CH_3COOH + H_2O \rightarrow CH_4 + CO_2 \quad (\Delta G^{\circ\prime} = -31\text{KJ/reaction}) \qquad \text{(Eq. 8)}$$

It has been argued that ferredoxin might be the last electron acceptor in methanogenesis, however it is still not known how exactly the electrons are funnelled[103,105]. The key step in the three metabolic pathways is the final reduction of methyl-CoM to methane catalyzed by the enzyme methyl-CoM reductase. This reaction generates the terminal electron acceptor, which is finally reduced by electrons derived from H_2 oxidation or a reduced coenzyme[62].

Table II. General characteristics of some methanogenic archaea, (adapted from Demirel et al. 2008)[37].

Methanogenic Archaea	T_{opt} (°C)	pH	Substrate
Methanobacterium bryantii	37	6.9-7.2	H_2/CO_2
Methanobacterium formicicum	37-45	6.6-7.8	H_2/CO_2, Formate
Methanothermobacter thermoautrophicum	65-70	7.0-8.0	H_2/CO_2, CO
Methanobrevibacter smithii	37-39	-	H_2/CO_2, Formate
Methanococcus vannielii	65	7.0-9.0	H_2/CO_2, Formate
Methanomicrobium mobile	40	6.1-6.9	H_2/CO_2, Formate
Methanospirillum hungatei	30-40	-	H_2/CO_2, Formate
Methanosarcina acetivorans	35-40	6.5	Acetate, Methanol, CO
Methanosarcina barkeri	35-40	5-7	H_2/CO_2, Methanol, Methylamines, Acetate, CO
Methanosarcina mazeii	30-40	6-7	Methanol, Methylamines, Acetate, H_2/CO_2
Methanococcoides methylutens	42	7.0-7.5	Methanol
Methanosaeta concilii	35-40	7.0-7.5	Acetate
Methanosaeta thermophila	55-60	7	Acetate

The enzyme CODH/ACS used in the acetyl-CoA pathway has also been shown to participate in the carbon fixation, thus making this enzyme essential in methanogens. Briefly, carbon fixation in methanogens involves the CO_2 reduction pathway and the reverse acetoclastic pathway previously discussed[62,106].

Therefore, CO could be considered as an important substrate for methane production since it is involved as an intermediate in acetoclastic energy metabolism and carbon fixation by the enzyme CODH/ACS. However, so far only three methanogenic archaea have been found capable of growing with CO as the sole

energy source, *Methanothermobacter thermoautotrophicus*, *Methanosarcina barkeri*, and *Methanosarcina acetivorans*[7,32,69] (Table I). Methane production from CO by methanogenic archaea has been studied extensively by many authors[7,19,67,69,96].

During growth with CO alone *M. thermoautotrophicus* and *M. barkeri* oxidize four mols of CO to CO_2 for every mol reduced to methane according to equation 9.

$$4CO + 2H_2O \rightarrow CH_4 + 3CO_2 \ (\Delta G^{\circ\prime} = -211KJ/reaction) \qquad \text{(Eq. 9)}$$

Both microorganisms have been shown to use the hydrogenotrophic pathway for methane production from CO, thus they could be classified as a hydrogenophilic methanogens. Several studies regarding CO metabolism in archaea discuss the production of H_2 by these two methanogens when growing on CO, where afterwards this H_2 produced is further metabolized for the production of methane[32,70]. Hence carboxidotrophic growth in these two microbes is considered as hydrogenotrophic combined with CO-dependent H_2 formation[62].

This observation is consistent with many studies working with *M. barkeri* on CO alone, such as the one reported by O'Brian et al. as previously discussed, which stated that *M. barkeri* CODH produces hydrogen as a by-product from the CO transformation due to the substantial amounts of H_2 observed when methanogenesis was blocked by high CO partial pressures[70]. In another study with *M. barkeri,* also concerning the production of hydrogen when growing with CO, showed that the deletion of the genes encoding Ech-hydrogenase, and thus eliminating hydrogen production, blocked the archaea's growth with H_2, CO_2 and CO, demonstrating that hydrogen is an intermediary of methane production[107]. However, despite the recent discoveries that electron production from CO is thermodynamically more favourable compared to electron production from hydrogen[63], *M. Thermoautotrophicus* and *M. barkeri* have been shown to grow slowly with CO alone compared to growth with H_2 as the electron donor .

In contrast, *M. acetivorans* exhibits higher growth rates with CO as the sole energy source, thus this archaea is recognized as growing well on CO alone, although hydrogen is not an intermediate metabolite in the conversion of CO to methane in *M. acetivorans* due to the lack of a functional hydrogenase[62,108]. Hence *M. acetivorans* is considered a strict acetoclastic methanogen.

It is argued that the fact that this archaea lacks a hydrogenase might be the cause of the apparent adaptation to growth with CO alone at higher concentrations, thus achieving higher growth rates than other methanogens under these conditions[62,69] since hydrogenases generally have been shown to be inhibited by small amounts of CO in the medium[62]. However, Rother et al. reported that an increase in CO concentration in the medium leads to a decrease in methane production by *M. acetivorans*, and acetate and formate become the main metabolites produced from carbon monoxide[69].

High sensitivity of methanogens to high levels of CO has been observed, and thus both growth and methane production ceases by increasing the CO partial pressure in the gas phase[32,67]. However, resistance to high CO concentrations with *M. barkeri* and *M. acetivorans* has been demonstrated after an adaptation period by slowly increasing the CO concentration[70,71].

1.4.4. Carboxidotrophic Sulfate-reducing Microorganisms

Most sulfate reducing bacteria which can use CO as an energy source convert CO to CO_2 and H_2 and further use this hydrogen for the reduction of sulfate[32,62] according to equation 11.

$$4CO + SO_4^{2-} + H^+ \rightarrow 4CO_2 + HS^- \quad (\Delta G^{\circ\prime} = -231 KJ/reaction) \quad (Eq.\ 11)$$

Nearly all of the known sulfate reducing bacteria are sensitive to high CO concentrations in the medium, thus it is suggested that the production of H_2 as an intermediate in sulfate reduction on CO might serve as a CO-detoxification pathway in these microorganisms[32,62]. However, *Desulfotomaculum carboxydivorans* isolated from a full-scale anaerobic wastewater-treatment plant not only grows under an atmosphere of 100% CO in the gas phase but is also able to grow on CO as a hydrogenogen in the absence of sulfate[109]. This ability of growing without sulfate as an electron acceptor has also been observed in sulfate reducing archaea (i.e. *A. fulgidus*), which can grow as an acetogen with CO[110].

The acetyl-CoA pathway also has been shown to participate in the carbon assimilation in sulfate reducers, thus it is probable that the enzyme CODH/ACS participates in the oxidation of CO in these organisms[32,62,111].

1.5. Syntrophic Methane Production from CO in a Natural Anaerobic Consortium.

As discussed in previous sections, anaerobic conversion of carbon monoxide (CO) can sustain a variety of microorganisms from different trophic groups within a microbial community. Therefore, in a mixed anaerobic consortium methane production from CO can also be coupled to other metabolic pathways in syntrophy with different groups of microorganisms able to oxidize CO into the main methanogenic precursors[7,10,42].

Therefore, in a microbial community methane may be produced directly from CO as previously reported with *M. acetivorans* and *M. barkeri*[94,95], and/or indirectly via acetate, methanol, H_2/CO_2 or formate, all of which can be produced from CO by several anaerobic bacteria[7,32,112,113] (Table I).

Some carboxydotrophic acetogenic bacteria such as *Butyribacterium methylotrophicum* or *Clostridium carboxidivorans* which are able to grow on CO alone as an energy and carbon source, have been shown to produce acetate, ethanol, butyrate and butanol from carbon monoxide[88,114]. Several studies have reported methane production from ethanol, butyrate, propionate and butanol in methanogenic co-cultures with ethanol, butyrate, propionate and butanol oxidizing bacteria, respectively[92,115,116]. Moreover, several methanogenic co-cultures have been described as capable of using long chain fatty acids for methane production at mesophilic conditions[112,115,117].

Thus the production of methane from CO in a mixed culture could be considered as a two-step process: formation of the methane precursor from CO (i.e. H_2, acetate) directly from CO or indirectly by oxidation of other CO products (i.e. ethanol); and the biomethanation of the precursors[42]. Table III summarize some of the reported reactions with CO by several carboxydotrophic microorganisms.

Table III. Reported reactions from CO and CO/H_2. (adapted from Sipma et al.)[24].

Product formed	Reaction	ΔG°'KJ/mol CO*
From CO		
Hydrogen	$CO + H_2O \rightarrow H_2 + CO_2$	-20
Formate	$CO + H_2O \rightarrow HCOO^- + H^+$	-16
Acetate	$4\ CO + 2\ H_2O \rightarrow CH_3COO^- + H^+ + 2\ CO_2$	-44
Butyrate	$10\ CO + 4\ H_2O \rightarrow CH_3(CH_2)_2COO^- + H^+ + 6\ CO_2$	-44
Ethanol	$6\ CO + 3\ H_2O \rightarrow CH_3CH_2OH + 4\ CO_2$	-37
n-Butanol	$12\ CO + 5\ H_2O \rightarrow CH_3(CH_2)_3OH + 8\ CO_2$	-40
Methane	$4\ CO + 2\ H_2O \rightarrow CH_4 + 3\ CO_2$	-53
From CO/H_2		
Acetate	$2\ CO + 2\ H_2 \rightarrow CH_3COO^- + H^+$	-67
Butyrate	$4\ CO + 6\ H_2 \rightarrow CH_3(CH_2)_2COO^- + H^+ + 2\ H_2O$	-80
Methanol	$CO + 2\ H_2 \rightarrow CH_3OH$	-39
Ethanol	$2\ CO + 4\ H_2 \rightarrow CH_3CH_2OH + H_2O$	-72
n-Butanol	$4\ CO + 8\ H_2 \rightarrow CH_3(CH_2)_3OH + 3\ H_2O$	-81
Methane	$CO + 3\ H_2 \rightarrow CH_4 + H_2O$	-151

* Standard Gibbs free energy changes (273.15 k; 101.325 kPa) at pH 7.

Many anaerobic bacteria such as *Peptostreptococcus productus,* a carboxydotrophic acetate producer able to grow rapidly under 90% of CO in the gas phase[118], are known to produce acetate from H_2 and CO_2 [7,42] according to equation 12:

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O \quad (\Delta G^{\circ\prime} = -104 KJ/reaction) \qquad \text{(Eq. 12)}$$

These bacteria also known as homoacetogens, are capable of acetate oxidation (reverse reaction) when the hydrogen partial pressure in the gas phase is low enough for the reaction to became thermodynamically favourable[100]. Many studies have

reported acetate oxidation by homoacetogenic bacteria when growing syntrophically with hydrogen-utilizing bacteria or archaea[119,120]. Further studies reported that syntrophic acetate oxidation is the main mechanism for acetate degradation in the presence of inhibitors such as high concentration of volatile fatty acids (VFA) or ammonia[121,122] due to the higher sensitivity of acetoclastic methanogens versus hydrogenophilic methanogens[123].

Therefore, the compatibility of microorganisms present in the culture with substrates and products is essential for efficient methane production in a mixed culture at large scale. Hence the use of already existing anaerobic consortiums is one interesting approach to lower the cost of this process. Anaerobic wastewater-treating sludge from UASB reactors has been reported as a good source of carboxidotrophic microorganisms which can be exploited for methane production at large scale[10,11].

1.6. Advantages and Disadvantages of CO Bioconversion to Methane by Natural Anaerobic Biofilms from a Wastewater-Treatment UASB reactor.

The use of anaerobic biofilms such as natural anaerobic granules from wastewater-treating upflow anaerobic sludge bed (UASB) reactors in the conversion of syngas components, namely CO, to different desirable compounds presents several advantages in achieving high productivity at a large scale. Some of the advantages are the following:

- Source of microbes adapted to harsh conditions that prevail with crude syngas
- Higher toxicity tolerance
- Higher process productivity
- Industrial wastewater-treating anaerobic granules have the potential to consume CO
- Possibility to enrich carboxydotrophic function
- Low operating costs

A UASB reactor mainly consists of a square or cylindrical tower surmounted by a three-phase separator, with upward feeding of the wastewater. The three-phase separator allows for gas-liquid separation and retention of the granular biomass. It is widely used as wastewater treatment technology. The long solid retention time achieved with the reactor leads to the formation of microbial microenvironments by gradually converting the suspended biomass into biogranules of about 1-3mm in

size[124]. These anaerobic biogranules which are capable of converting complex pollutants into methane have been extensively studied[125,126]. The microorganisms composing the biogranule can be roughly divided into 3 trophic groups, acidogens, acetogens, and methanogens which contribute to the final CH_4 production (Figure 5). Hence the configuration of the observed granular morphology allows the microbes to work in syntrophy which improves the flux of metabolites and the electron transfer between them, resulting in higher methane yields compared to suspended biomass. Many studies have reported the advantage of granules over suspended biomass in a bioreactor[124,126,127]. Moreover, according to these studies the methanogens will be situated in the interior of the granule, thus well protected from inhibition by high CO concentrations in the medium allowing higher methane yields. A few studies have already shown the potential of wastewater-treatment anaerobic granules for higher productivity in CO conversion processes to methane[10,18].

Furthermore, these anaerobic wastewater-treating sludges are available in large quantities for free or at a low cost, thus using them as biocatalysts for syngas conversion to methane decreases the total cost of processes at large scale.

However, a few disadvantages need to be noted when working with anaerobic wastewater-treating sludge from a UASB reactor:

- Unexpected reactions when working with a mixed culture
- Difficult optimization of the different metabolic pathways to achieve higher product yields
- Intragranular substrate diffusion limitation

One of the major limitations when working with a natural mixed culture is the difficulty in reaching optimal operational conditions to achieve high productivity of the process. This is mainly due to the great diversity of microorganisms present in the consortium, and consequently the multiple metabolic pathways potentially implicated in the conversion of CO-rich syngas to methane. Moreover, the different microbes in

the consortium working in syntrophy probably have different optimal growth conditions, which render the operational control of the overall process even more difficult.

Furthermore, the morphology of the granular biofilm systems may limit the intragranular diffusion of CO-rich syngas, thus decreasing the availability of the carbon source, namely CO, to all of the potentially carboxidotrophic microorganisms present in the granular consortium. Hence this might limit the biofilm thickness and the CO turnover rate in the overall process[128].

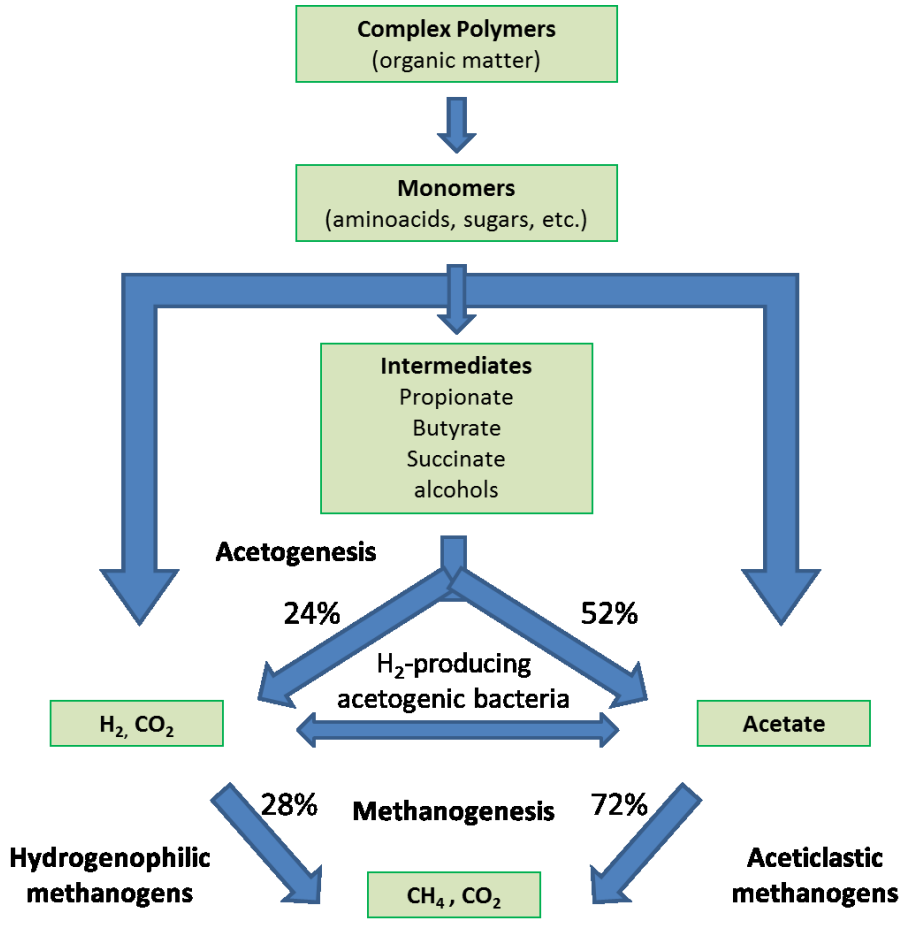

Figure 5. Anaerobic microbial conversion of biomass to methane adapted from Demirel et al. (2008)[37]. The organic substrates, such as proteins, lipids and carbohydrates are first hydrolyzed to soluble aminoacids, monosaccharides, long-chain fatty acids and alcohols.

These components are then degraded by acidogenic bacteria to reduced intermediate compounds such as volatile or short chain fatty acids (VFAs), alcohols, lactate, etc., which serve as substrates for the production of acetate, formate, CO_2 and H_2 by acetogenic bacteria. Those intermediates (acetate, formate, CO_2 and H_2) are then converted to methane by two different metabolic groups, acetoclastic and hydrogenophilic methanogens.

CHAPTER 2

Objectives and hypothesis

Previous efforts to evaluate syngas bioconversion to methane from a wastewater-treatment anaerobic granular sludge in a 30L gas-lift reactor in our lab suggested an interesting carboxidotrophic methanogenic potential at a partial pressure of 0.2 atm CO. We concluded that the conversion of CO was likely hydrogenotrophic combined with CO-dependent hydrogen formation, due to the detection of H_2 in the reactor, either under mesophilic or thermophilic conditions. Nonetheless, based on the limited batch experiments performed we could not accurately distinguish between the possible routes of CO conversion to methane. Moreover, many studies working with anaerobic bioreactor sludge state that at mesophilic temperatures the conversion of CO is only via acetate as an intermediary, followed by acetoclastic methanogenesis[18,20]. Thus a deeper understanding of the microbiological aspects implicated in CO-rich syngas fermentation was necessary to allow further improvement of the bioreactor setup.

Hypothesis

The hypothesis of this work is that anaerobic granular sludge possesses significant carboxidotrophic methanogenic potential, that this potential might be sensitive to CO levels, and that organisms present in the community might use either the hydrogenotrophic or acetogenic pathway, or a combination of these, to produce methane from CO.

Objectives

The major objective of this study was to assess the carboxidotrophic methanogenic potential present in an anaerobic microbial population from an upflow anaerobic sludge blanket reactor (UASB) under different CO concentrations at mesophilic temperatures. In order to further characterize this system, it was necessary to assess CO toxicity. Given the preliminary evidence cited above for a primarily hydrogenotrophic route of methane production, in contradiction to published reports, a second objective was to better elucidate the metabolic routes involved in methane production from CO with the help of specific inhibitors of methanogenesis and gram positive bacteria (i.e acetogens), 2-bromoethanesulfonic acid (BES) and vancomycin, respectively. Moreover, the effect of adaptation to high CO concentrations over time was also examined. An important carboxidotrophic methanogenic potential under CO partial pressure higher than 0.2 atm was expected, as well as determining the impact of the input of hydrogenophilic methanogens in the conversion of CO to methane at 35˚C.

CHAPTER 3

Preliminary Work: Effectiveness of the Selected Inhibitors and Characterization of the Methanogenic Potential Pathways of the Anaerobic Sludge

Use of Specific Inhibitors for Characterization of Methanogenic Potential Pathways from CO in a Natural Consortium from an Anaerobic Digestion Reactor

As discussed earlier, anaerobic conversion of carbon monoxide (CO) can sustain a variety of microorganisms from different trophic groups within a microbial community, including methanogens. Carbon monoxide dehydrogenase (CODH) is the enzyme involved in the oxidation of CO, which is present in all of the known carboxidotrophic microorganisms, and provides the energy necessary for the production of methane and/or methanogenic precursors (i.e. acetate, H_2, formate)[61]. Therefore, in a microbial community methane may be produced directly from CO as previously reported with *M. acetivorans* and *M. barkeri*[94,95], and/or indirectly via acetate, methanol, H_2/CO_2 or formate, all of which can be produced from CO by various anaerobic bacteria[7,32,112,113].

Several studies have also demonstrated the conversion of CO into ethanol, butyrate and butanol by carboxidotrophic bacteria[7,88]. These substrates can then be further converted by acetogenic bacteria into assimilable metabolites for methanogens such as formate, acetate or H_2. Previous work with mixed cultures have reported methane production from ethanol, butyrate, propionate and butanol in methanogenic co-cultures with ethanol, butyrate, propionate and butanol oxidizing bacteria, respectively[92,115,116].

Hence, when working with a mixed anaerobic consortium it is important to consider all of the possible reactions involved in the conversion of CO to methane, and thus inhibitory activity tests were necessary. 2- bromoethanesulfonate (BES), an analog of coenzyme M, is commonly used as an inhibitor of methane production by methanogenic archaea. Another widely used inhibitor, vancomycin, is presented as an inhibitor of general bacteria in many metabolic studies[11,18,129,130]. However,

vancomycin specificity is questioned since general literature sources in microbiology refer to vancomycin as an inhibitor of gram positive bacteria only by blocking the proper synthesis of their cell wall[131]. Furthermore, there are additional questions regarding the stability of vancomycin under 35˚C since there is no registered data about it in literature and several studies state that it degrades and loses its effectiveness over time[132,133].

Therefore, a first series of tests were performed in our lab to specify the inhibitory targets of the inhibitors used in the study, as well as to determine the efficiency and stability of vancomycin over time. Afterwards, a preliminary study was performed to characterize the microbial composition of the anaerobic sludge, as well as the potential methanogenic pathways present in the consortium.

Methodology

Specific Activity Tests

Firstly, to test the inhibitory effect of both inhibitors used in the identification of the metabolic routes involved in methane production from CO in this study, 2-bromoethanesulfonic acid (BES) and vancomycin, a series of activity tests with CO_2/H_2, glucose, acetate and CO (0.2 atm partial pressure in N_2) as a substrate were performed in duplicate with and without the presence of the inhibitors. Afterwards, to characterize the microbial composition of the wastewater-treating sludge used and determine the potential metabolic pathways implicated in methane production, specific activity tests were arranged in triplicate and duplicate on the suspended anaerobic inoculum in the absence and presence of inhibitors, respectively. The substrates used for that purpose were the following: formate, hydrogen, acetate,

propionate, butyrate, methanol, ethanol and butanol. The substrates and inhibitor concentrations used are shown in Table V.

The tests were performed in 120 mL and 60 mL serum bottles for the liquid and gaseous substrates, respectively. The bottles were filled with 20 mL of inoculum diluted with 0.05 M phosphate buffer at pH 7.5 to an initial concentration of 5 gVSS/L for the tests fed with liquid as a substrate (i.e. acetate), and at a concentration of 2 gVSS/L for the hydrogenotrophic and carboxidotrophic tests. To establish anaerobic conditions in the tests with liquid substrates the bottles were capped, sealed and flushed with N_2/CO_2 gas (80/20%, v/v) to obtain 1 atm of total pressure in the headspace. Then, the bottles were injected with the substrate solution to obtain the initial concentration required, except for the endogenous controls. In the case of the carboxidotrophic tests, once the bottles were capped and sealed they were flushed with N_2 gas (100%) for 3 minutes. Afterwards, CO was injected into the bottles under anaerobic conditions using a gas tight syringe to obtain the required CO concentration in the headspace (20% CO, N_2 balance). The hydrogenotrophic activity tests were carried out likewise, but using H_2/CO_2 (80/20%, v/v) pressurized at 2.5 atm in the headspace, and shaking the bottles at 400 rpm instead of 100 rpm to maximize the gas-liquid mass transfer. All the bottles were incubated at 35 ± 3 ºC in the presence of inhibitors at concentrations of 50 mM BES (Sodium salt, 98% purity, Sigma-Aldrich, Netherlands), and 0.07 mM vancomycin (hydrochloride hydrate, Sigma-Aldrich, USA). These concentrations of the inhibitors were chosen based on other metabolic studies on pure cultures and environmental samples where high inhibitory effects were evidenced for the desired activity[11,134–136].

The bottles were sampled at regular intervals of time according to the different consumption rates observed for each substrate used, and the tests were ended before the total depletion of the substrate except for the carboxidotrophic test where the CO was totally consumed. The activities were determined and calculated as in previous studies with anaerobic sludge[10,137] by measuring the rate of methane production and substrate depletion at their inflexion point (expressed in mmols of CH_4 and/or substrate per unit of volatile suspended solids (VSS) per day). VFAs and

alcohols were analyzed at the end of the experiment, with the exception of the carboxidotrophic test where VFAs and alcohols were analyzed every two days. An endogenic test (without substrate) was also performed in parallel and used as a control.

Table IV. Substrate and specific inhibitors concentrations applied in the bottles.

Substrate	Substrate Initial Concentration (mg/L)	Inhibitor	Inhibitor Concentration (mM)
Formate	1000	-	-
		Vancomycin	0.07
		Methyl viologen	7.5
Acetate	3000	-	-
		BES	50
		Vancomycin	0.07
Propionate	500	-	-
Butyrate	1000	-	-
H_2/CO_2[a]	na	-	-
		Vancomycin	0.07
		BES	50
Methanol	1000	-	-
		Vancomycin	0.07
		BES	50
Ethanol	2000	-	-
		Vancomycin	0.07
		BES	50
Butanol	2000	-	-

[a] 80%/20% vol./vol.; 2.5 atm total pressure. na, not applicable.

Vancomycin Inhibitory Stability

To validate the efficiency and stability of vancomycin over time, activity tests with 0.2 atm CO partial pressure (0.3mM) in the presence of vancomycin were performed at 35 ºC. The tests were carried out over 32 days at vancomycin concentrations of 0.07, 0.14 and 0.21 mM per triplicate. The amount of CO, methane, and H_2 produced was checked every four days. The concentration of VFAs and alcohols was estimated at the beginning, middle, and end of the experiment. Moreover, to avoid the possibility of vancomycin degradation over time, three other bottles with 0.07 mM of vancomycin were re-injected with an additional 0.07 mM of vancomycin every 10 days. No addition of CO was needed during the incubation period. An endogenous activity test (without substrate) was performed in parallel and used as a control.

Additionally, one-way ANOVA was performed in order to compare the variances between the four treatments used (0.07, 0.14, and 0.21 mM of vancomycin, and 0.07 mM vancomycin with re-addition of the inhibitor over time) for the CO conversion and methane production activity of each sampled day. The level of significance used in the statistical tests was 0.05.

Results

Inhibitory Specificity of BES and Vancomycin

The activity tests performed to define the role of each inhibitor are presented in Table IV. In the presence of vancomycin the glucose started to degrade from the very beginning of the test, and no lag phase was observed for the methane production (data not shown). Moreover, its specific depletion activity or conversion rate was

almost 70% of the rate of the test without inhibitor (3.9 versus 5.9 mmol/VSS· d) with slightly higher methanogenic activity (1.9 versus 1.6 mmol/VSS· d). No intermediate metabolites such as VFAs, alcohols or H_2 were found at the end of the experiment.

Table V. Fermentative and methanogenic specific activities of the anaerobic sludge under different substrate conditions and effecting presence of vancomycin (0.07 mM), and BES (50 mM), at 35 ˚C. Average ± SD of duplicates.

Substrate	Specific Activity				
	Type and Unit	Without Inhibitor	With Vancomycin	With BES	With Vancomycin and BES
Glucose	mmol CH_4/gVSS·d	1.6 ± 0.0	1.9 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
	mmol Gluc/gVSS·d	5.9 ± 0.3	3.9 ± 0.0	5.0 ± 0.7	2.8 ± 0.1
H_2/CO_2[a]	mmol CH_4/gVSS·d	12.6 ± 1.4	14.6 ± na	0.0 ± 0.0	0.0 ± 0.0
	mmol H_2/gVSS·d	80.1 ± 18	70.7 ± na	23.8 ± 9	0.7 ± 1.0
Acetate	mmol CH_4/gVSS·d	3.7 ± 0.1	2.8 ± 0.3	0.0 + 0.0	-
	mmol Ac/gVSS·d	2.9 ± 0.4	2.2 ± 0.2	0.6 ± 0.0	-
CO/N_2[b]	mmol CH_4/gVSS·d	0.9 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	mmol CO/gVSS·d	5.4 ± 0.26	0.4 ± 0.0	2.4 ± 0.5	0.0 ± 0.0

[a] 80%/20% vol./vol.; 2.5 atm total pressure. [b] 20%/80% vol./vol.; 1 atm total pressure. (-) not performed.

Both the H_2/CO_2 and acetate tests in the presence of vancomycin presented similar values for substrate conversion and methane production rate as in the absence of an inhibitor. Moreover, methane production from these substrates started from the beginning of the test. Together these results with Glucose, acetate and H_2/CO_2 demonstrate that vancomycin does not inhibit all of the fermenters and archaea in the sludge.

On the other hand, the glucose conversion rates in both the presence and absence of BES were similar (5 and 5.9 mmol/VSS· d, respectively), although no methane was produced as expected. Acetate and propionate were the major glucose metabolites in the presence of BES. Moreover, when the sludge was incubated with H_2/CO_2 or acetate in the presence of BES the substrate conversion rate dropped drastically from 80.1 to 23.8 for the hydrogenotrophic test, and from 2.9 to 0.6 for the acetate test. No methane was found, as expected. These tests confirmed that only methanogenic archaea were inhibited by BES.

The experiments performed with glucose in the presence of both inhibitors, vancomycin and BES, showed a much pronounced decrease in its conversion rate (from 5.9 to 2.8 mmol/VSS· d). We assume that this is probably due to a feedback inhibition of the products formed, since acetate and propionate accumulated to a large extent under those conditions. Moreover, no substrate consumption was observed in the hydrogenotrophic or acetate tests in the presence of both inhibitors.

As previously mentioned, these inhibitors were suggested to decipher the metabolic pathways preferentially used in the conversion of CO to methane in the sludge. Hence it was necessary to confirm the inhibitory effects observed with vancomycin and BES when CO is the only substrate. Vancomycin decreased the CO conversion rate to methane by a factor of 10 (from 5.4 to 0.4 mmol/VSS· d), however the methane yield at the end of the experiment was higher than in the tests without vancomycin. On the contrary, BES completely inhibited the methane production, while the CO conversion rate was only half of that in the test without inhibitor and accumulation of acetate, propionate and H_2 was observed (discussed in Chapter 4). However, when both inhibitors vancomycin and BES were added, the carboxidotrophic activity was almost negligible and the only product formed was H_2 (data not shown). The last observation suggested that hydrogen producing bacteria were probably not inhibited under these conditions. However, the drastic decrease in both carboxidotrophic and methanogenic activities likely indicates their limited presence in the microbial population.

Vancomycin Inhibitory Stability at 35 ˚C

Since the effectiveness of vancomycin over time is uncertain due to the lack of literature regarding its stability at 35˚C with environmental samples, and that clinical studies recognize the decrease of its inhibitory effect after 7 days at 25˚C[133], a series of activity test were performed to clarify this issue. Figure 6 shows the CO consumption and methane production over time at different vancomycin concentrations.

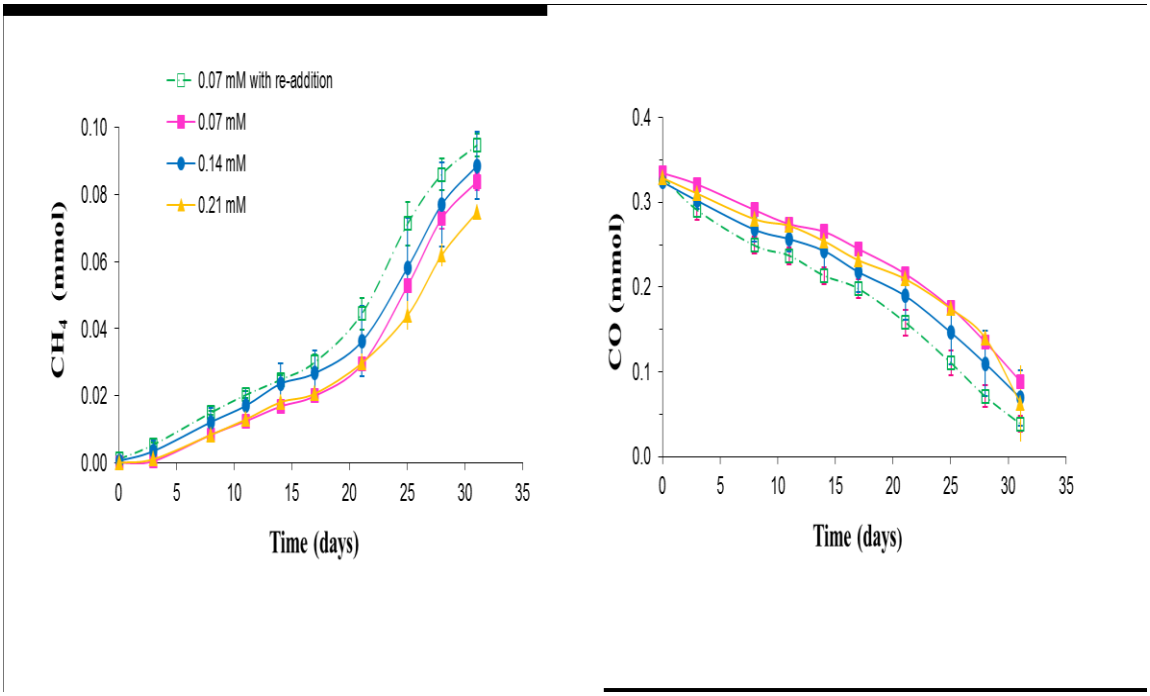

Figure 6. Comparison of the CH_4 and CO time course under different vancomycin concentrations, and vancomycin (0.07 mM) re-addition every 10 days. The tests are performed at 0.2 atm CO partial pressure, and 200 rpm agitation at 35˚C. Mean ± SD of triplicates.

There was no significant difference between the bottles incubated with different vancomycin concentrations, and neither when vancomycin was added over

time. The carboxidotrophic and methanogenic activities were very similar in the four treatments, with overlapping standard deviations (ANOVA, $p > 0.05$).

Potential Methanogenic Pathways of a Typical Anaerobic Sludge

The methanogenic activities achieved and the substrates consumption rate to indirectly establish the presence of different bacterial trophic groups and methanogens in the sludge are shown in Table VI. All the substrates used started to be consumed from the beginning of the test except for butyrate, which had a lag phase of 2 days (data not shown).

In the acetate tests, the similar activity rates observed in both the control (without inhibitors) and in the presence of vancomycin (when most acetate oxidizers are inhibited), together with the drastic decrease in acetate conversion rate when methanogenesis was inhibited (from 2.9 to 0.6 mmol/VSS· d) suggest an important acetoclastic methanogenic activity in the sludge for acetate conversion. These results were similar in the hydrogenotrophic test, as in the presence of BES the conversion of H_2/CO_2 dropped drastically (from 80.1 to 23.8 mmol/VSS· d), and in the presence of vancomycin the activities were similar to the ones without an inhibitor, suggesting potential methane production by hydrogenophilic methanogens in the sludge. Nonetheless, in the presence of vancomycin a slight increase in methanogenic activity and methane yield was observed. This result might be due to competition for H_2 between hydrogenophilic methanogens and homoacetogenic bacteria in the sludge.

Moreover, the methanogenic activity and conversion rate of methanol were the same in both the presence and absence of vancomycin, and when methanogenesis was blocked by BES methanol consumption rate decreased approximately 60% (from 1.0 to 0.4 mmol/VSS· d). Thus it is possible that methanol was also directly converted to methane by methanogens[129]. On the other hand, ethanol was probably transformed to methane via acetate as an intermediary in syntrophy with ethanol

oxidizing bacteria. Stoichiometric amounts of acetate from ethanol were found when methanogenesis was blocked (data not shown).

Table VI. Anaerobic digestion sludge methanogenic activity from different substrates in the absence and presence of different specific inhibitors at 35˚C. The methane yield is also calculated based on previous studies.

Substrate	Inhibitor	Specific Activity (mmol CH_4/gVSS·d)	Specific Activity (mmol Substrate/gVSS·d)	CH_4 Yield* (% of the Stoichiometric)
Formate[1]	-	7 ± 0.1	22.9 ± 0.0	124 ± 28
	Vancomycin	5 ± na	23.4 ± na	78 ± na
	Methyl viologen	0.0 ± 0.0	6.8 ± 0.4	0.0 ± 0.0
Acetate[2]	-	3.7 ± 0.1	2.9 ± 0.4	122 ± 18
	Vancomycin	2.8 ± 0.3	2.2 ± 0.2	116 ± 13
	BES	0.0 ± 0.0	0.6 ± 0.0	0.0 ± 0.0
Propionate[3]	-	0.9 ± 0.0	0.4 ± 0.0	132 ± 36
Butyrate[4]	-	1.0 ± 0.2	0.4 ± 0.0	109 ± 16
Hydrogen[5]	-	12.6 ± 1.4	80.1 ± 18	69 ± 18
	Vancomycin	14.6 ± na	70.7 ± na	81 ± na
	BES	0.0 ± 0.0	23.8 ± 9	0.0 ± 0.0
Methanol[6]	-	0.8 ± 0.0	1.0 ± 0.1	132 ± 38
	Vancomycin	0.7 ± 0.0	1.0 ± 0.2	132 ± 25
	BES	0.2 ± 0.0	0.4 ± 0.3	43 ± 42
Ethanol[7]	-	5.6 ± 0.2	9.3 ± 2.4	104 ± 20
	Vancomycin	5.2 ± 0.2	7.9 ± 0.6	113 ± 4
	BES	2.7 ± 0.2	3.7 ± 0.0	52 ± 3
Butanol[8]	-	2.7 ± 0.1	3.5 ± 0.4	34 ± 7

na, no applicable

*Stoichiometric methane yields as reported in the literature:

1. ¼ mol CH_4 per mol of formate[61]
2. 1 mol CH_4 per mol of acetate[61]
3. 1.75 mol CH_4 per mol of propionate[138]
4. 2.5 mol CH_4 per mol of butyrate[138]
5. ¼ mol CH_4 per mol of hydrogen[61]
6. ¾ mol CH_4 per mol of methanol[61]
7. 1.5 mol CH_4 per mol of ethanol[116]
8. 2 mol CH_4 per mol of butanol[139]

Discussion

Inhibitory Specificity of BES and Vancomycin, and Vancomycin Stability at 35°C

In previous studies[11,18] with anaerobic wastewater-treating sludge, vancomycin and BES are presented as inhibitors of general bacteria and methanogens, respectively. However, in many studies vancomycin is referred to as an inhibitor of gram positive bacteria blocking the polymerization of N-acetylmuramic acid and acetylglucoseamine units to peptidoglycan, and thus inhibiting proper cell wall synthesis[131,140]. Moreover, Quintiliani[141] highlights the intrinsic resistance of most gram-negative bacteria to vancomycin due to their outer membrane which is impermeable to large glycopeptide molecules such as vancomycin. Hence it was necessary to determine the specificity of each inhibitor in the microbial population.

The activity tests performed with glucose as a substrate showed that vancomycin does not inhibit all of the fermentative bacteria present in the sludge. This was confirmed in the presence of both inhibitors, vancomycin and BES, where the glucose turnover was half of that in the control test (without inhibitors). Moreover, in the tests performed with CO in the presence of both inhibitors, CO conversion to hydrogen was detected which highlights that hydrogenogens (mostly gram negative bacteria) are not inhibited by vancomycin. Besides, the tests carried out with CO_2/H_2 or acetate as a substrate substantiate the non-inhibitory effect of vancomycin on methanogens, as has been discussed in many studies[142–144].

Hence, the use of vancomycin to identify direct carboxidotrophic methanogenic activity versus indirect methane production via acetate or hydrogen as intermediates, as previously reported[11,18], could be misinterpreted since most known mesophilic hydrogenogens are gram negative bacteria, and thus probably not inhibited by vancomycin[7,62].

The non-inhibitory effect of vancomycin in gram negative was corroborated with the molecular analyses performed in this study (Appendix III). The comparison of the DGGE profiles in the presence and absence of vancomycin confirmed its inhibitory potency on gram positive bacteria.

A clear difference in the eubacterial community composition was observed between both conditions (the presence and the absence of vancomycin). In the presence of vancomycin, the inhibition of gram positive bacteria such as *A. wieringae* and *C. propionicum* leads to the emergence of different gram negative species related to the phylum *Proteobacteria*, such as *Magnetospirillum gryphiswaldense*[145], *Brevundimonas* sp.[146], and *Syntrophobacter fumaroxidans*.

Furthermore, we determined that BES effectively blocks methanogenesis, as previously described by many authors[11,18,116,136], since nearly no methane was produced in any of the tests performed in the presence of the inhibitor.

Finally, since there are several studies concerning the resistance of some gram positive genera to vancomycin[140,147,148], the presence of vancomycin resistant bacteria strains in the sludge was investigated (Appendix III). None of the vancomycin resistant genes tested were found to be present in our samples, indicating the possible absence of vancomycin resistant bacteria in the sludge.

The stability of vancomycin at 35˚C was also under question as it degrades and loses its effectiveness over time[132,133]. However, the experiments performed to confirm the stability of vancomycin in this study showed no difference in the microbial activity between the four treatments during the 32 day assay. Therefore, despite the fact that some studies have indicated that vancomycin stability at room temperature decreases by approximately 10% every 7 days[133], these analyses indicate that 0.07 mM of vancomycin in the bottles is enough to maintain an inhibitory effect for at least 32 days at 35˚C with the wastewater-treating sludge.

This outcome is supported by a few clinical studies for the evaluation of the vancomycin stability storage[132,149], which showed that vancomycin solutions stored at

25˚C could maintain at least 90% of its initial concentration for up to 30 days. In addition, another study carried out to establish vancomycin's antimicrobial potency and stability concluded that there were no differences in its antimicrobial potency over 1 month when at room temperature[150].

Hence, in this study an initial concentration of vancomycin of 0.07 mM will be used to achieve the desired inhibitory effect in the microbial population.

Potential Methanogenic Pathways of a Typical Anaerobic Sludge

The methanogenic activities achieved with the substrates applied in this study are quite consistent with other studies working with different anaerobic digestion sludge from UASB reactors under mesophilic temperatures[126,151]. Although, surprisingly, the acetoclastic activity was relatively low in the sludge used as opposed to other anaerobic digestion studies[151–153]. Similarly, methane production from formate, butyrate and propionate was also low in the conditions tested. The methanogenic activity achieved with the different volatile fatty acids (VFAs) used was in decreasing order: formate > acetate > butyrate and propionate, which is in accordance with the level of standard free energy released per reaction according to previous studies[61,154].

The experiments set with acetate indicated that acetoclastic methanogenic activity was the dominant pathway for acetate conversion in the microbial population, as reported in many studies with anaerobic digestion sludge[122,152,155].

Moreover, the lower activity of hydrogen-oxidizing bacteria observed compared to hydrogenophilic methanogens when the sludge was incubated with H_2/CO_2 might be related to the lower kinetics of their growth and the less favourable free energy balance of the homoacetogenic reaction as compared to hydrogenophilic methanogenesis[156], thus making the latter a better competitor for hydrogen in the sludge.

Furthermore, the tests performed with methanol as a substrate suggest possible methane production directly from methanol by methylotrophic methanogens[129], while methane from ethanol was probably in synthrophy with ethanol oxidizing bacteria as has been discussed in earlier studies[116,157]. However, direct utilization of ethanol by methanogenic archaea has been reported with *Methanogenium organophilum*, which oxidizes 2 mol of ethanol to acetate for every mol of methane produced[158]. Nonetheless, direct utilization of ethanol by methanogens is quite unusual and growth is less efficient[159].

Therefore, we proved that both direct H_2/CO_2 and acetate conversion to CH_4 at mesophilic temperatures exists in the sludge used and methanogens were able to grow with primary alcohols as hydrogen donors. Hence the consortium used contains, aside from acetoclastic methanogens, other methanogenic populations such as methanol, hydrogen and formate utilizing methanogens for the conversion of a variety of compounds into methane[17,103,160].

CHAPTER 4

Article

This article, entitled "Biomethanation of CO: identification and classification of metabolic pathways in a natural consortium from an anaerobic digestion reactor" was written following the experiments I performed from January 2010 to February 2012. The article was completely written by me, and was revised by my mentor, Ruxandra Cimpoia and my research director, Dr. Serge Guiot. The paper will be submitted shortly to the journal “Applied Microbiology and Biotechnology”. The authors of the article are Silvia Sancho Navarro, Ruxandra Cimpoia and Serge R. Guiot.

Biomethanation of CO: Identification and Classification of Metabolic Pathways in a Natural Consortium from an Anaerobic Digestion Reactor

ABSTRACT

The gasification of biomass produces a mixture of gas (mainly carbon monoxide (CO), carbon dioxide (CO_2) and hydrogen (H_2)) called synthesis gas, or syngas, through thermal degradation without combustion. The components of syngas can serve as substrates for a wide range of microorganisms. This study evaluates the carboxidotrophic methanogenic potential present in anaerobic sludge from a UASB reactor treating wastewater and elucidates the CO conversion routes to methane at 35±3˚C.

Kinetic activity tests under CO at partial pressures varying from 0.2 to 1.6 atm (0.3-2.6 mmol/L) showed an interesting carboxidotrophic activity potential for growth on CO alone. However, the maximum methanogenic activity of 0.99 mmolCH_4/gVSSd was achieved at 0.2 atm of CO (0.3 mmol/L), with the rate decreasing with the amount of additional CO supplied. Thus the intermediary metabolites acetate, H_2 and propionate started to accumulate at higher CO concentrations. Inhibition experiments with 2-bromoethanesulfonic acid (BES), and vancomycin showed that in a mixed culture CO was converted mainly to acetate by acetogenic bacteria, which was further transformed to methane by acetoclastic methanogens. Methanogenesis was totally blocked at a high CO partial pressure (P_{CO}) in the bottles (>1 atm). However, it is possible to achieve higher methanogenic potential under an atmosphere of 100% CO after acclimation of the sludge to CO.

Moreover, it seems that this adaptation to high CO concentrations leads to a shift in the archaeal population dominated by hydrogen-utilizing methanogens. These

results suggest a possible enrichment potential with anaerobic biofilms for large scale methane production from CO-rich syngas, and further advances the knowledge base for anaerobic reactor development.

1. INTRODUCTION

Energy needs are increasing worldwide due to humanity's population growth and the accelerated development of industry leading to today's goal of replacing non-renewable and scarce fossil fuels sources. Therefore, it has become necessary to find new alternatives for the production of sustainable energy to mitigate these energy needs. Synthesis gas, or "syngas", produced by the thermal gasification of biomass, has received increased attention for energy recovery in the past decades due to its higher efficiency compared to other bioenergy processes[7,34,161].

The principal components of syngas, CO, CO_2 and H_2, can serve as substrates for conversion into higher-value fuels, namely methane, through a wide range of microorganisms[8,10,18]. Biomethane can therefore be used to replace natural gas extracted from fossil fuel sources and can be re-injected into the natural gas grid. Moreover, the use of methane as a green energy source is advantageous compared with other gaseous fuels due to its higher boiling point and higher energy density, making it easier to manipulate and thus lowering its storage costs[13]. However, only a small number of microorganisms able to reduce syngas' CO into methane have been discovered so far[19,62,69,96]. On the other hand, anaerobic wastewater-treated sludge has been reported as a good source of carboxidotrophic microorganisms which can be exploited for methane production at large scale[10,11].

The anaerobic conversion of carbon monoxide (CO) can sustain a variety of microorganisms from different trophic groups within a microbial community. Therefore the pathways involved in methane production from CO become more complex when working with a mixed anaerobic consortium. Carbon monoxide dehydrogenase (CODH) is the enzyme involved in the following CO oxidation reaction:

$$CO + H_2O \rightarrow CO_2 + 2H^+ + 2e^-$$

This enzyme is present in all of the known carboxidotrophic microorganisms, including methanogens. The oxidation of CO by CODH provides the energy required to reduce the different substrates in order to produce H_2, acetate, and methane[61]. Recent studies have pointed out that the electron production from CO is thermodynamically more favorable as compared to H_2. Thus CO can theoretically replace H_2 as electron donor in all of the microorganisms that contain CODH[62,63].

CO can be metabolized by the four main trophic groups of microorganisms: methanogenic archaea, hydrogen producing bacteria (hydrogenogens), acetogenic bacteria, and sulfate reducers[62,162]. Thus, when working with a mixed methanogenic consortium it is important to consider all of the possible reactions involved in the conversion of CO to methane. Carboxidotrophic methanogenic archaea are able to convert CO directly to methane through the following reaction:

$$4CO + 2H_2O \rightarrow CH_4 + 3CO_2 \ (\Delta G^{\circ\prime} = -210 \text{ kJ/ reaction})$$

However, methane can also be produced from CO indirectly via other metabolites such as H_2 and CO_2 produced by hydrogenogens followed by hydrogenophilic methanogenesis, or acetate produced from CO by acetogenic bacteria with subsequent acetoclastic methanogenesis. The main indirect carboxidotrophic methanogenic reactions can be summarized as follows:

$$CO + H_2O \rightarrow H_2 + CO_2 \ (\Delta G^{\circ\prime} = -20 \text{ kJ/ reaction}) \ (1a)$$

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \ (\Delta G^{\circ\prime} = -135 \text{ kJ/ reaction}) \ (1b)$$

$$CO + 3H_2 \rightarrow CH_4 + H_2O \ (\Delta G^{\circ\prime} = -150 \text{ kJ/ reaction}) \ (1c)$$

$$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2 \ (\Delta G^{\circ\prime} = -176 \text{ kJ/ reaction}). \ (2a)$$

$$CH_3COOH + H_2O \rightarrow CH_4 + CO_2 \ (\Delta G^{\circ\prime} = -31 \text{ kJ/ reaction}) \ (2b)$$

In addition, homoacetogenic bacteria might participate in the conversion of H_2 and CO_2 to acetate, a thermodynamically favorable reaction ($\Delta G^{\circ\prime}$ = -134 kJ/ reaction), or acetate oxidation when the conditions are favorable. Moreover, it has been shown that some carboxidotrophic bacteria are able to convert CO into other metabolites such as formate, ethanol, butyrate, and butanol, all of which can then be converted into methane by methanogens directly or indirectly via acetate and H_2/CO_2[7,88,163].

Therefore a deeper understanding of the microorganisms concerning the production of methane from CO and the biochemical pathways involved in a natural methanogenic consortium under different environmental conditions (temperature, pressure, CO, H_2, and CO_2 content, etc.) is necessary. This will provide the possibility of enhancing carboxidotrophic methanogenic potential which would facilitate further development of reactor design and operation optimization, in order to enable a subsequent scaling-up.

To address this issue this study is primarily focused on the assessment of the carboxidotrophic methanogenic potential present in an anaerobic wastewater-treating sludge from an upflow anaerobic sludge blanket (UASB) reactor as well as the identification of CO conversion routes to methane under mesophilic conditions (35ºC) with the use of specific metabolic inhibitors for bacteria and archaea (methanogens).

2. MATERIAL AND METHODS

SLUDGE

The tests carried out for this study were performed under mesophilic conditions (35˚C), using anaerobic granular sludge from a full scale UASB plant treating fruit processing wastewater (Lassonde Inc., Rougemont, QC, Canada). In order to minimize the effect of the granular structure of AD sludge on metabolic pathways as well as to evaluate its CO toxicity, the tests were performed with a disaggregated inoculum. The granular structure was disrupted by sieving with a 0.25 mm diameter grid pore sieve, and crushed with a mortar under a N_2 atmosphere. The biomass was re-suspended in 0.05 M phosphate buffer at pH 7.5.

EXPERIMENTAL DESIGN

Identification of methanogenic carboxydotrophic potential and toxicity

Carboxydotrophic specific activity tests were performed in triplicate on the suspended anaerobic inoculum. The tests were carried out with CO as a sole substrate in 60mL serum bottles, and the carboxidotrophic and methanogenic activities were determined by measuring the rate of CO consumption and methane production, respectively, at their inflection point (expressed in mmols of CO or CH_4 per unit of volatile suspended solids (VSS) per day), calculated as in previous studies with anaerobic sludge[10,137]. The bottles were filled with 20 mL of the inoculum diluted with 0.05 M phosphate buffer at pH 7.5 to an initial concentration of 2gVSS/L. To establish anaerobic conditions the bottles were capped, sealed with butyl rubber stoppers and flushed with N_2 gas (100%) for 3 minutes. Afterwards CO was injected

into the bottles under anaerobic conditions using a gas tight syringe to obtain the required CO concentrations in the headspace. The CO partial pressure ranged between 0.2 and 1.6 atm (20-100% CO, N_2 balance), and corresponded to liquid CO concentrations varying from 0.33 to 1.65 mM. The bottles were immediately placed in dark environmental conditions in a rotary shaker (New Brunswick, Edison, NJ) controlled thermostatically at 35 ± 3 ºC and operated at 200 rpm to maximize the liquid-gas mass transfer. During the incubation period the bottles were sampled for CH_4, H_2 and CO at regular time intervals depending on the initial CO concentration until the CO was totally depleted. At the end of each assay liquid samples from each bottle were analyzed for the presence of volatile fatty acids (VFA) and alcohols. Four control tests were also performed: an endogenic test (without substrate), an endogenic inhibited test (with cyanide), a negative control (with CO and cyanide) and lastly an abiotic test (with a basal medium without sludge).

Identification of possible routes to methane

To identify the actual routes for CO conversion to methane, inhibitory studies were necessary. The tests were performed in the same manner as described above, however the bottles were injected at the start of the test, prior to incubation, with the following metabolic inhibitors: 50 mM 2-bromoethanesulfonic acid (BES) (Sodium salt, 98% purity, Sigma-Aldrich, Netherlands), used as a methanogenic inhibitor[136], and 0.07 mM vancomycin (hydrochloride hydrate, Sigma-Aldrich, USA) used as an inhibitor of gram-positive bacteria[131], which are generally acetogenic bacteria. The concentrations of the inhibitors of the desired activity were chosen based on the results of metabolic studies[11,134,135]. All of the inhibitory tests were carried out in duplicate.

Effect of long-term exposure to CO on the consortium

To evaluate the effect of long-term exposure to high CO concentrations on the carboxydotrophic and methanogenic microbial populations, further activity tests were carried out similarly as described above. For this purpose the sludge was incubated during 63 days with continuous CO injections in the headspace, creating an atmosphere of 100% CO. In addition, a molecular approach (DGGE experiments) was performed in parallel to examine changes in the microbial community structure over time. Samples for DGGE analyses were taken from the bottles every two weeks over the 63 day incubation period.

MOLECULAR ANALYSES

Total genomic DNA was extracted from 2 mL homogenized sludge samples as previously described[164,165], and then purified and concentrated using a QIAEX gel extraction kit (Hoffman-La Roche AG, USA) according to the manufacturer's instructions. DGGE experiments were performed as previously described by Tresse et al.[166]. In summary, 16S rDNA sequences were amplified using the primers 341f (5'-CCTACGGGAGGCAGCAG-3')[167] and 758r (5'-CTACCAGGGTATCTAATCC-3')[168] for Eubacteria, and the primers 931f (5'-AGGAATTGGCGGGGGAGCA- 3')[169] and 1392r (5'- ACGGGCGGTGTGTAC -3')[170] for Archaea. After electrophoresis, bands of interest were excised from the gel, reamplified and submitted for sequencing (Université Laval, Québec, QC, Canada). The sequences were analyzed and then compared to those in the GenBank database using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) to determine the phylogenetic affiliations.

ANALYTICAL METHODS

The gas components (O_2, H_2, CH_4, N_2, CO, CO_2) were determined by gas chromatography. 250 μL of gas sample (model 1750 gas-tight syringe, Hamilton, Reno, NV) was injected on an Agilent 6890 gas chromatograph (Wilmington, DE) equipped with a TCD and a 5 m x 2.1 mm Carboxen-1000 column (Supelco, Bellafonte, PA) with argon as carrier gas. The column temperature was held at 60ºC for 7 min and increased to 225 ºC at a rate of 60 ºC per min. Volatile fatty acids (VFA) (acetate, propionate, and butyrate) and alcohols (methanol, ethanol, acetone, 2-propanol, tert-butanol, n-propanol, sec-butanol, and n-butanol) were measured on an Agilent 6890 gas chromatograph (Wilmington, DE) equipped with a flame ionization detector (FID) as described by Guiot[10]. The volatile solids (VS), volatile suspended solids (VSS) and chemical oxygen demand (COD) analyses were performed according to standard methods[171].

3. RESULTS

CARBOXIDOTROPHIC METHANOGENIC POTENTIAL

First, the anaerobic sludge was characterized for its carboxidotrophic and methanogenic potential at different CO partial pressures (P_{CO}) in the gas phase. Typical time courses for substrate consumption and methane production are shown in Figure 7. The non-adapted anaerobic sludge presented an interesting carboxidotrophic potential. No lag time was observed; however the activity was fully expressed only after a certain time interval, depending on substrate concentration and the presence of inhibitors (i.e. availability of biochemical pathways). At higher CO concentrations, there is an increase in the time interval required for the full expression of the carboxidotrophic activity (figure 7B). Some correlation is also observed when inhibitors were applied to the media: the time interval needed to achieve full carboxydotrophic activity increased when methane production was blocked with BES, and this delay in the activity was even higher in presence of vancomycin. Generally, accumulation of H_2 was detected in the bottles and achieving maximum H_2 concentrations after the carboxydotrophic activity was fully expressed. Moreover, it should be noted that methane production appears to begin when hydrogen starts to be consumed.

Since CO is known to act as an inhibitor of methanogenesis, an activity test-based kinetic study was assessed to define the optimal CO concentration required to achieve maximum methanogenic activity (Table VII). The carboxidotrophic activities observed ranged between 5.4-8.6 mmolCO consumed/g VSS·d. The CO activity increased with the amount of CO supplied, and reached its maximum at 0.5 atm P_{CO} in the gas phase (0.84 mM). On the contrary, the optimal CO partial pressure for methanogenesis was observed around 0.2-0.3 atm (0.3-0.4 mmol CO/L), and after the methane production rate decreased with the increase in CO concentration until it was

totally blocked at a P_{CO} of 1 atm (1.8 mM). The maximum methanogenic activity rate was 0.99 ± 0.02 mmol methane produced/ g VSS·d.

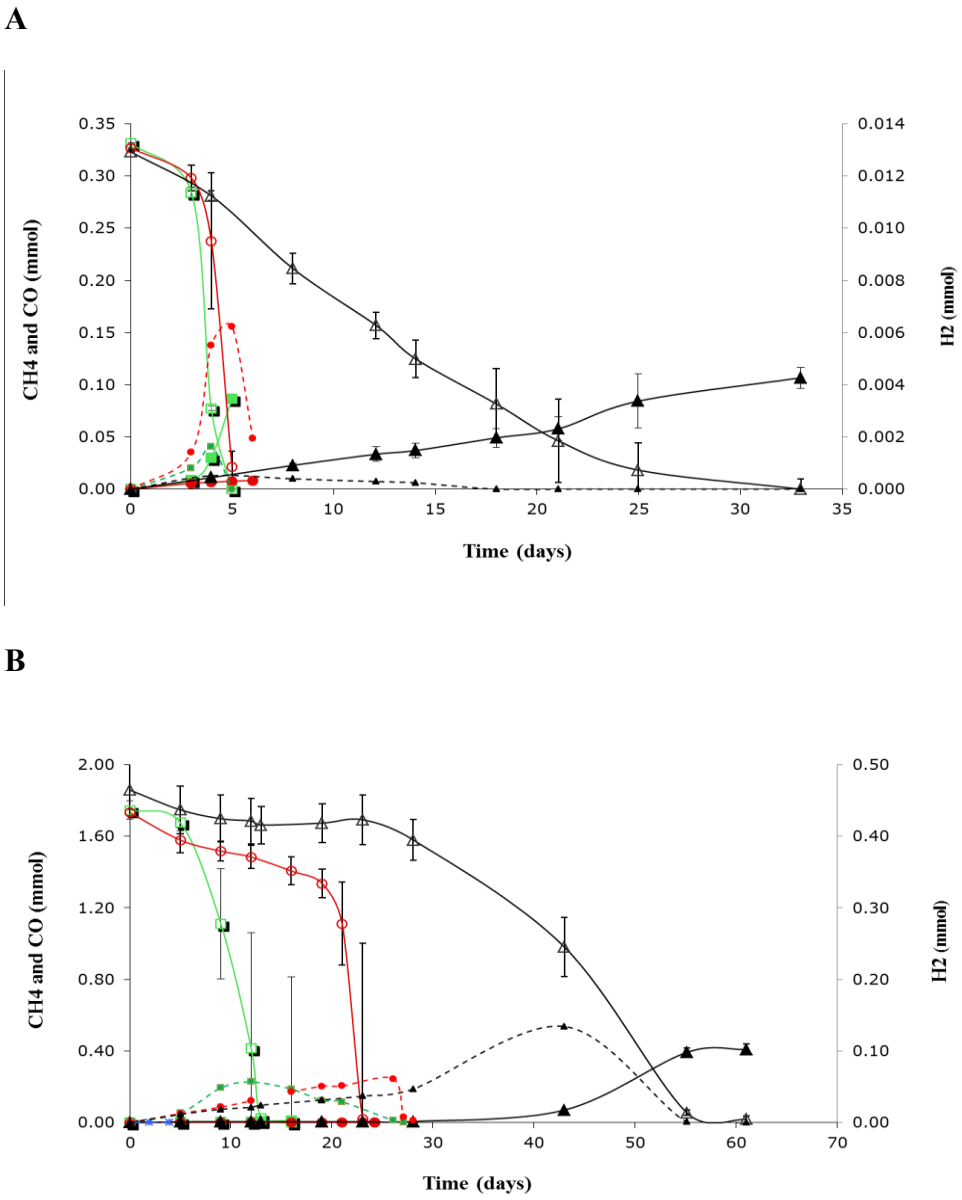

Figure 7. CO consumption (open symbols) and CH_4 and H_2 production (closed symbols) without inhibitors (square), in presence of BES (circle), and vancomycin (triangle), at 0.2 atm of CO (A) and 1.1 atm of CO (B) in the headspace. Production of hydrogen is represented with dotted lines. Average ± standard deviation of triplicates for the test without inhibitors, and duplicates in presence of inhibitors.

Methane, acetate, propionate and H_2 were the main products of CO conversion, and their yield varied depending on the initial CO concentration. High concentrations of CO clearly affected the CH_4 yield as has been reported in previous studies using anaerobic sludge and pure cultures[18,62,67]. At the point when methanogenesis started to decrease, methane precursors began to accumulate. We then observed an increase of acetate, propionate and H_2 proportionally to the increase of dissolved CO concentration (P_{CO}). Both acetogenic and hydrogenogenic bacteria were still active at 1.6 atm P_{CO} in the gas phase (2.59 mmol CO/L).

Table VII. Carboxidotrophic and methanogenic activities of anaerobic sludge and product formed under different initial CO concentrations in absence of inhibitors at 35˚C.

CO Initial Concentra-tion mM (atm)	**Time to Reach Maximum activity* (d)**	**CO Specific Activity (mmol CO/g VSS·d)**	**CH_4 Specific Activity (mmol CH_4/g VSS·d)**	**CH_4 Yield[1] (% of the Stoichio-metric Yield)**	**Other Products Formed (% of the Stoichiometric Yield)**
0.33 (0.22)	3	5.37 ± 0.26	0.99 ± 0.02	104 ± 4.75	Propionate[2] (7)
0.45 (0.29)	5	7.12 ± 1.27	0.82 ± 0.02	61.88 ± 5.01	Acetate[3] (22) Propionate (9) H_2[4] (3)
0.84 (0.53)	6	8.62 ± 0.89	0.45 ± 0.08	23.85 ± 3.01	Acetate (38) Propionate (9) H_2 (4)
1.78 (1.12)	14	8.62 ± 1.93	0.04 ± 0.00	2.31 ± 0.49	Acetate (45) Propionate (21) H_2 (14)
2.59 (1.65)	37	7.09 ± 0.57	0.00 ± 0.00	na	Acetate (45) Propionate (15) H_2 (8)

1. ¼ mol of CH_4 per mol CO.
2. ⅐ mol of propionate per mol CO.
3. ¼ mol of acetate per mol CO.
4. 1 mol H_2 per mol CO.

* Average

CO CONVERSION ROUTES

The major CO conversion metabolites observed in the study were CH_4 and acetate, although the presence of hydrogen and propionate was also noted in the microcosms. This data suggests that acetate is the main methane precursor. However, to determine the main routes from CO to methane, it is essential to determine the different metabolic pathways present in the consortium. In order to accomplish this, specific inhibitors for bacteria and archaea (methanogens) were used (Figure 8). The use of vancomycin as acetogenic inhibitor (mostly gram positive bacteria) allowed the evaluation of methane formation directly and/or indirectly via H_2 and CO_2. BES in contrast permitted the identification of the different methane precursors which were accumulated when methanogenesis was blocked. The activity tests performed in the presence of inhibitors at different P_{CO} in the gas phase are presented in Table VIII.

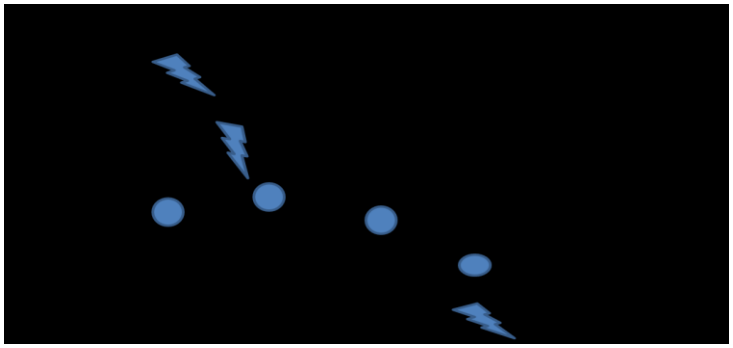

Figure 8. Blocking of potential pathways involved in CO conversion to methane by inhibitors.

In general, the carboxidotrophic activity in the presence of BES was reduced to approximately 50% in all of the tests performed in this study, and led to the accumulation of acetate, propionate and hydrogen as the final products from CO as previously observed. This pronounced decrease in activity could be due to the input of direct carboxidotrophic methanogenesis in the sludge. Nonetheless, based on the

data obtained with vancomycin tests a better explanation for the decrease in the CO conversion rate could be the feedback inhibitory effect by the accumulated products from CO (i.e. acetate and/or H_2) when methanogenesis is blocked. This scenario has been described earlier by various authors working with anaerobic biofilms[172,173]. In the absence of methanogenesis acetate was the metabolite with the highest accumulation of all the CO concentrations tested, which suggests that the partial pressure of CO didn't have any effect on the metabolic pathways involved in methane production, and that acetate was the main intermediate.

Table VIII. Carboxidotrophic and methanogenic activities of anaerobic sludge and product yields under different CO concentrations in the presence of BES (50 mM), and Vancomycin (0.07 mM) at 35˚C.

CO Initial Conc. mM (atm)	Time to Reach Maximum Activity(d)	Relative CO Conversion Activity rate (% control)*	Relative CH_4 Production rate (% control)*	CH_4 Yield[1] (% of the Stoichio-metric Yield)	Other Products Formed (% of the Stoichiometric Yield)
BES					
0.33 (0.20)	4	57 ± 18.0	1 ± 0.1	9 ± 0.0	Acetate[2] (32) Propionate[3] (7) H_2[4] (4)
0.51 (0.32)	5	42 ± 7.0	4 ± 2.6	7 ± 5.6	Acetate (64) Propionate (17) H_2 (12)
0.85 (0.54)	8	54 ± 6.5	0 ± 0.1	2 ± 0.0	Acetate (45) Propionate (10) H_2 (6)
1.72 (1.12)	21	74 ± 23.9	3 ± 0.3	0.0	Acetate (51) Propionate (16) H_2 (9)
2.37 (1.56)	31	51 ± 4.7	0 ± 1.4	0.0	Acetate (42) H_2 (15) Propionate (11)

Vancomycin					
0.32 (0.20)	na**	8 ± 2.7	14 ± 1.4	129.6 ± 3.5	Propionate (14) Acetate (4)
0.48 (0.30)	20	9 ± 1.79	21 ± 12.4	112.5 ± 2.7	Propionate (12)
0.84 (0.55)	44	12 ± 1.4	59 ± 14.5	96.7 ± 2.3	Propionate (7)
1.86 (1.13)	40	22 ± 7.1	2240 ± 29	88.8 ± 0.2	H_2 (15) Propionate (3)
2.53 (1.62)	55	17 ± 2.9	12061 ± 3746	65.6 ± 21.7	H_2 (14) Propionate (5) Acetate (3)

* The relative activity rates are calculated as the ratio in percentage of the actual rate (with inhibitors) to the corresponding rate in the control assays (without inhibitor). The following formula was used to determine the variance of the rate's ratios of the actual rates:

$$\Delta z/z = \mathbf{SQRT}\ [(\Delta x/x)^2 + (\Delta y/y)^2]$$

Where Δz is the error for the sum, Δx is the error for the first variable, and Δy is the error for the second variable.

** The activity rate did not change for the duration of the experiment

[1] ¼ mol of CH_4 per mol CO
[2] ¼ mol of acetate per mol CO.
[3] ⅐ mol of propionate per mol CO
[4] 1 mol H_2 per mol CO.

In the presence of vancomycin, the observed CO conversion rate for all of the CO concentrations tested was very low compared to the control tests (without vancomycin). Generally, in the absence of active acetogens only 10% of carboxidotrophic activity was expressed. The maximum rate of 1.89 mmol CO/g VSS·d was achieved at 1 atm CO partial pressure (1.86 mM), which represented only 22% of the CO conversion rate in the absence of the inhibitor. This data suggests that direct CO conversion to methane or via H_2/CO_2 (or formate) as intermediates are not important pathways in the anaerobic sludge used. Moreover, when acetogenic bacteria were inhibited by vancomycin, the methanogenic activity of the sludge increased with the amount of CO applied, and almost all of the CO was converted to

methane at the end of the tests. The maximum methanogenic activity achieved in the presence of vancomycin of 0.74 ± 0.06 mmol CH_4/gVSS·d was obtained at 1.1 atm P_{CO} in the gas phase, and is comparable with the maximum methanogenic activity observed among all of the tests performed in this study. Although the methane production rate increased with the amount of CO supplied, the methane yield from CO decreased, and the accumulation of H_2 was observed at higher CO concentrations.

These outcomes suggest that under test conditions both main methanogenic pathways, via H_2/CO_2 and via acetate, appear to be accessible in the sludge. However, acetoclastic methanogenesis seems to be the dominant pathway when the conditions are favourable for methanogenesis to happen.

EFFECT OF LONG TERM EXPOSURE TO CO

Since a drastic increase in methane production was observed in the vancomycin assays at higher CO concentrations, as well as an increase in the time required to fully achieve carboxidotrophic activity, a possible selection and/or adaptation of the microbial population (methanogens) present in the sludge by exposure to high CO concentrations over the time was suggested. To evaluate this hypothesis, activity tests under a fixed atmosphere of 100% CO in the gas phase were performed over 63 days.

The carboxidotrophic activity and methane potential achieved over the time are presented in Table IX. There was a clear correlation between the time exposure to CO and the methanogenic potential in the consortium. Both the carboxidotrophic and methanogenic activity increased drastically between days 30 and 40 of incubation, achieving a maximum methane production rate of 5.48 ± 1.18 mmol CH_4/gVSS·d at day 40.

Under previous acclimation of the sludge it was possible to reach 90% CO conversion to CH_4 at 1 atm P_{CO}, even though only 2% of the CO was transformed to methane in normal conditions. However, the accumulation of acetate in the bottles over time due to the continuous addition of CO probably affected the microorganism's activities, since both CO conversion and methane production activity, as well as acetate and hydrogen accumulation, decreased during the last two weeks of the experiment.

To examine the possible variation in the microbial population over the time due to an adaptation to CO, DGGE experiments were performed in parallel to the activity tests. DGGE analyses for eubacterial and archaeal 16S rDNA sequences of interest are presented in Table X of the supplementary information. The results confirmed a shift in both eubacterial and archaeal populations, corresponding to the increased methanogenic potential observed at 40 days of incubation.

Table IX. Comparison of the sludge CO conversion rate and methanogenic potential under 100% CO in the gas phase over time.

Time (days)	**CO Specific Activity (mmol CO/gVSS·d)**	**CH_4 Specific Activity (mmol CH_4/gVSS·d)**	**CH_4 Yield*** **(% of the Stoichio-metric Yield)**	**Cumulative Acetogenic Yield % (mol/mol) and Conc. (mM)**	**Max H_2 Conc. (mmol/L)**
Without Inhibitor					
0	8.52 ± 2.73	0.04 ± 0	1.96 ± 0.63	10.3 ± 4.1 (0.18 mM) pH 7.46	2.2 ± 0.5
30 ± 3	11.73 ± 1.55	0.67 ± 1.30	23.0 ± 44.48	3.2 ± 0.4 (17 mM) pH 6.92	6 ± 1
40 ± 1	24.23 ± 5.95	5.48 ± 1.18	90.40 ± 29.56		1.7 ± 0.6
63 ± 7	2.15 ± 0.97	0.19 ± 0.05	34.88 ± 18.64		0.8 ± 0.2

* ¼ mol of CH4 per mol CO

Clostridium propionicum, a propionate producing bacterium[174], and *Acetobacterium wieringae*, an acetate producing bacterium[175], which were not detected at the beginning of the test, appeared to be dominant in the eubacterial population after a month of incubation at high CO concentrations, suggesting that long-term exposure to CO had stimulated their growth. This increase in the abundance of these two species in the microbial community corresponded to the previously observed acetate and propionate accumulation at high CO concentrations. In addition to these two bacteria, *Petrimonas sulfuriphila*, a fermentative acetate and H_2/CO_2 producing bacterium[176], and *Geobacter uraniireducens* sp., an acetate oxidizing bacterium[177], were detected after one or two months of CO exposure. Variations in the archaeal population were also observed, with a notable shift over time towards a dominance of hydrogen-utilizing methanogens. Microorganisms belonging to the orders *Methanomicrobiales* and *Methanobacteriales* were found to be present in the consortium after one or two month of CO exposure, suggesting a better adaptation of those populations to CO conditions.

4. DISCUSSION

Based on the data obtained with the specific inhibitory assays we determined that methane production from CO was mainly via acetate as an intermediate metabolite, as was observed in many studies at mesophilic temperatures[18,172,178]. This was further confirmed by the dominance of *Methanosaeta* species in the microbial population. However, it is important to note the presence of hydrogenophilic methanogenesis in the sludge.

When methanogens were inhibited in the presence of BES, acetate was the major metabolite accumulated in all of the CO concentrations tested, although H_2 and propionate were also present but to a lesser extent.

In the absence of an inhibitor these metabolites were completely converted to methane under optimal methanogenic conditions (0.2 atm P_{CO}), but started to accumulate at higher CO concentrations, probably due to the inhibitory effect of CO to methanogenesis, as reported in previous work[62,67]. Rother and Metcalf [66] reported that higher exposure to CO leads to the apparent down-regulation of the operon mtr, which encodes for the enzyme N-methyl-H_4SPT:CoM methyltransferase (Mtr) involved in both the hydrogenotrophic and the acetoclastic methanogenic pathways in methanogens, thus decreasing the methane production yield at high P_{CO}. Hence, at higher CO concentrations when the conditions are unfavourable for methanogens, CO-utilizing hydrogenogenic and acetogenic bacteria may take over in the population. These results are consistent with literature, which reports many acetogens and hydrogenogens which can grow at high CO concentrations[32,62,179].

Methane production from CO via acetate as the main intermediate was further supported in the presence of vancomycin when acetogenic bacteria were inhibited. The pronounced decrease in carboxidotrophic activity observed under these conditions clearly indicated that direct methane production from CO or indirectly via H_2/CO_2 was secondary in the sludge studied, as has been reported in previous studies

working with anaerobic sludge[10,62,180]. This might be explained by the better energy balance of the CO-utilizing acetogenic reaction versus the hydrogenogenic one (i.e. $\Delta G^{\circ\prime}$= -176 kJ/reaction, vs. $\Delta G^{\circ\prime}$= -20 kJ/reaction), as well as the slightly smaller doubling time of acetogens as compared to hydrogenogens[7]. Hence this makes acetogenic bacteria a better competitor for CO than hydrogenogens, the former thus becoming dominant in the population under high CO concentration, as was shown in the molecular analyses performed over a long term exposure to CO.

On the other hand, the minimal direct CO to methane conversion observed in the consortium might be due to the poor kinetic properties of methanogens compared to CO-utilizing hydrogenogenic and acetogenic bacteria. This is consistent with previous work where the authors reported higher CO affinity of the carbon monoxide dehydrogenase (CODH) enzyme in carboxidotrophic hydrogenogens and acetogens than in methanogens[62].

An interesting phenomenon in the vancomycin assays was the increase in methanogenic activity with the amount of CO supplied, contrary to the tests without an inhibitor. Thus, since under these inhibitory conditions the achievement of a fully carboxidotrophic activity took much longer than when all of the metabolic pathways were available, the adaptation of methanogens by long-term exposure to CO was proposed as an explanation. This hypothesis was confirmed through the tests performed under 100% CO over 63 days, where the sludge achieved the highest methanogenic activity after 40 days of exposure to CO, with 90% CO conversion to methane.

Previous studies with *M. acetivorans*[69] and *M. barkeri*[70] demonstrated the microorganisms' ability to grow at 100% CO in the headspace after an adaptation period by the stepwise increase of CO concentration, although the methane production achieved at high CO levels in the gas phase was very low. Nonetheless, a recent study reported that a *M. acetivorans* strain isolated from prolonged incubations with CO was capable of producing methane directly from CO at a high rate[71].

Moreover, recent work with *Carboxydothermus hydrogenoformans* describes the regulation of both hydrogenase-linked CODH and CODH/ACS operons for

efficient consumption of CO across a wide range of concentrations[82]. In the study the authors presented that under high P_{CO} the bacteria was able to catabolize more CO into energy by overexpression of the hydrogenase, while at low CO concentrations the CO was mainly used for carbon fixation. It seems that methanogens needed a longer adaptation time to achieve methane production at high CO concentrations.

However, the abrupt decrease in activity observed after day 55 remains unexplained. This decrease could be related to the accumulation of acetate in the bottles over time, the slightly decrease of pH in the media from 7.5 to 6.9, the accumulation of other by-products that are inhibitory, or another unknown factor[172,173].

The molecular analyses performed in this study showed an adaptation of the sludge's microbial population to high P_{CO}, with an evolution towards the dominance of acetate producers and acetate oxidizers. The archaeal population first dominated by acetoclastic methanogens, namely *Methanosaetaceae* species, evolved into a mix of acetate and hydrogen-utilizing methanogens dominated by hydrogenophilic methanogens (*Methanobacteriales* and *Methanomicrobiales*).

The decrease of *Methanosaetaceae* species in co-cultures has already been related to the presence of high concentration of VFA or high ammonia levels in many studies[122,123,181]. Besides, the decrease of *Methanosaetaceae* in the population might lead to the dominance of acetate oxidizers in syntrophic cooperation with hydrogen-utilizing methanogens, as already reported elsewhere[122]. In addition, other studies highlight the noticeable sensitivity of acetoclastic methanogens to different products in the media[122,182,183], including CO[184].

Therefore, based on the data obtained in this study we conclude that at low CO concentrations CO is converted mainly to acetate by acetogenic bacteria which is subsequently transformed into methane by acetoclastic methanogens (*Methanosaetaceae*), while at high P_{CO} the methanogenic activity seems to be

generally inhibited by the amount of CO applied, as previously discussed[62,67,103]. However, it was demonstrated that methanogens are able to adapt to high CO concentrations over time, possibly through the regulation of CODH/hydrogenase activity at the molecular level. The sludge's adaptation to high P_{CO} over time lead to a shift in the microbial population, to be dominated by acetate oxidizers and hydrogen-utilizing methanogens (*Methanobacteriales* and *Methanomicrobiales*). The proposed CO conversion routes at low and high P_{CO} prior to and after adaptation to high CO concentrations in the sludge are illustrated in figure 9.

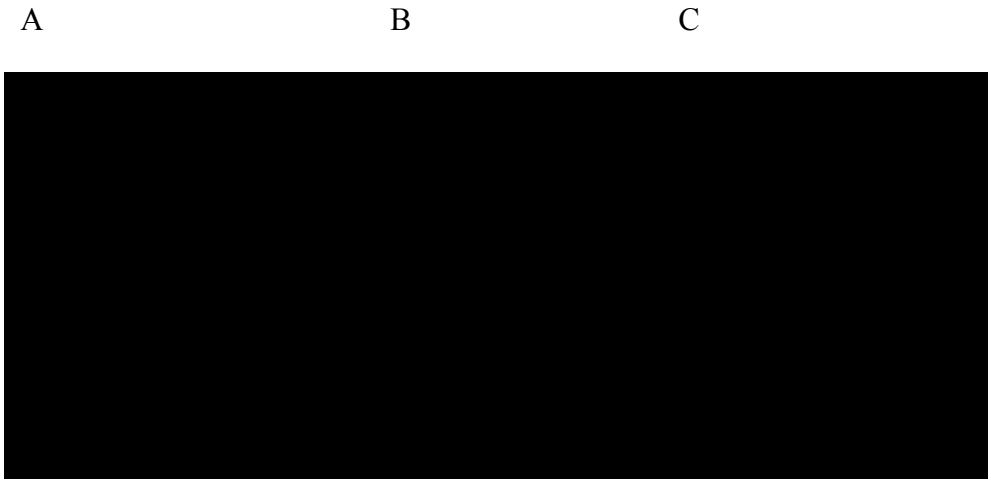

Figure 9. Suggested pathways for conversion of CO into CH_4 present in the anaerobic consortium. Under low P_{CO} (< 0.5 atm) (A), under high P_{CO} (> 0.5 atm) (B), and after acclimation of the sludge to high CO concentrations (100% CO in the gas phase) (C). The width of the arrow is indicative of the predominance of the pathway, corresponding to a level of 60-70% (thick), 20-40% (intermediate) and 5-20% (thin). The dotted lines indicate the blocking of methane production by CO.

5. CONCLUSION

In this study increasing CO partial pressures lead to an increase in carboxidotrophic activity. Therefore, the non-adapted anaerobic sludge presents an interesting carboxidotrophic potential for growing conditions with CO alone.

In general the results obtained for CO conversion to methane are very similar to previous studies with anaerobic digester sludge under mesophilic conditions[5,6,33]. Direct methane production from CO appears to be negligible, and acetate seems to be an important intermediary metabolite for methane production in the sludge used. However, it is important to take into account the effect of hydrogenotrophic methanogenesis on the total CO conversion to methane, based on the presence of hydrogen-utilizing methanogens in the consortia, and the accumulation of H_2 at high CO concentrations when methanogenesis is blocked by CO.

The optimal methanogenic activity achieved under mesophilic conditions was observed at P_{CO} in the gas phase lower than 0.3 atm, and further increase in the amount of CO supplied lead to the inhibition of methanogenesis. However, it was possible to achieve methane production at high P_{CO} through the sludge's previous acclimation to CO, contrary to what is reported in many studies[18,62,67].

After long-term exposure to high CO concentrations, acetogenic bacteria were found to play an important role in the population, and acetate oxidation became the main pathway for methane production followed by hydrogenotrophic methanogenesis. Nevertheless, more targeted techniques will be required to better understand the microbial population shifts under different environmental conditions, as well as the change in their metabolic pathways and the adaptation or regulation of CO conversion to methane by some methanogenic microorganisms.

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7. Supplementary information

Table X. Evolution of the eubacterial and archaeal population in the sludge over time, in the absence of inhibitors under 100% CO.

Identified Microorganism (GenBank Accession Number)	% Similarity (Sequence Length)	Day 0*	Day 30*	Day 45*	RDP Classifier Classification
Eubacteria					
Clostridium propionicum strain JCM 1430 (AB649276.1)	100% (400/400)	-	+	+	*Clostridium* XlVb (100%)
Acetobacterium wieringae strain DP9 (HQ384240.1)	99% (394/396)	-	+	+	*Acetobacterium* (100%)
Uncultured *Bacteroidetes* bacterium clone D1 16S (HQ003602.1)	99% (357/391)	-	-	+	*Bacteroidetes* (99%)
Petrimonas sulfuriphila strain BN3 (NR042987.1)	94% (391/415)	-	-	+	*Bacteroidales* (100%), *Petrimonas* (96%)
Geobacter uraniireducens Rf4 (CP000698.1)	97% (399/411)	-	+	+	*Desulfuromonadales* (100%), *Geobacteraceae* (97%), *Geobacter* (94%)
Archaea					
Methanosaeta concilii strain NBRC 103675 (AB679168.1)	99% (429/434)	+	+	+	*Methanosaeta concilii* (98%)
Uncultured *Methanolinea* sp. clone SMS-T-Pro-2 (AB479406.1)	99% (431/433)	-	+	+	*Methanolinea tarda (89%)*

Uncultured *Methanobacteriaceae* clone:AR-H2-B (AB236069.1)	100% (429/429)	-	-	+	*Methanobacterium congolense (98%)*

* As determined by DGGE; (+) presence of the microorganism; (-) absence of the microorganism.

CHAPTER 5

Discussion

The characterization of CO conversion by anaerobic digestion sludge at different CO partial pressures clearly indicates that the non-adapted anaerobic sludge presents a significant carboxidotrophic growth potential with CO alone.

The carboxidotrophic activity of the microbial population increased with the amount of CO supplied in the assays performed, reaching its maximum at 0.5-1 atm of CO. However, the optimal methanogenic activity was observed at CO partial pressures in the gas phase lower than 0.3 atm and a further increase in the amount of CO supplied lead to the inhibition of methanogenesis as reported in previous work[62,67]. Hence, when methanogenesis starts to decrease due to the increase of the amount of CO supplied, the methane precursors start to accumulate in the medium.

Acetate, propionate, and H_2 were the main metabolites accumulated in absence of methanogenesis at high CO partial pressure, but when the concentration of CO was low enough for methanogenesis to resume, all of the CO was converted to methane.

As previously discussed, CO can be metabolized by different trophic groups of microorganisms, such as hydrogen producing bacteria (hydrogenogens), acetogenic bacteria, and methanogenic archaea[62,162]. Therefore, a deeper understanding of the metabolic pathways implicated in the CO conversion to methane, and thus the interaction between the microbial communities in the sludge, was necessary to further improve the optimal conditions and achieve higher methanogenic potential from CO at large scale.

The specific inhibitory tests performed for this purpose in the presence of BES, an inhibitor of methanogenesis, and vancomycin, an inhibitor of gram positive

bacteria (most acetogens) which blocks the murein biosynthesis, suggests that methane production from CO in the sludge was mainly via acetate as an intermediate metabolite, as was observed in many studies with anaerobic sludge at mesophilic temperatures[18,172,178].

When methanogenesis was blocked in the presence of BES, acetate was the main metabolite accumulated in all of the CO concentrations tested (0.2-1.6 atm P_{CO}). Moreover, the pronounced decrease in carboxidotrophic activity observed in the presence of vancomycin which inhibited acetogenic bacteria, and therefore the production of acetate from CO, indicates that direct methane production from CO or indirectly via H_2/CO_2 is a secondary pathway in the sludge used, as has been reported in previous work[10,62,180]. However within the scope of this study, it was impossible to distinguish between direct CO conversion to methane or via H_2/CO_2.

The dominance of CO-utilizing acetogenic bacteria in the sludge might be explained by the more favourable free energy balance of the carboxidotrophic acetogenenic reaction than that of the carboxidotrophic hydrogenogenesis (i.e. $\Delta G^{\circ\prime}$= -176 KJ/reaction, vs. $\Delta G^{\circ\prime}$= -20 KJ/reaction), as well as the slower growth rate reported for hydrogenogens[7]. Hence this makes acetogenic bacteria a better competitor for CO becoming a dominant group of bacteria in the population under CO conditions. On the other hand the low input of direct methanogenesis observed in the population could be explained by the lower CO affinity of the enzyme carbon monoxide dehydrogenase (CODH) in methanogens compared to that of CO-hydrogenogens or CO-acetogenic bacteria, as has been discussed in previous work[62].

The molecular analyses performed in this study further confirmed the indirect production of methane via acetate by the predominance of *Methanosaeta* species in the microbial population (Appendix III). However, it is important to note that methane was also produced in part via H_2/CO_2 since presence of hydrogen-utilizing methanogens was also observed in the population.

Many studies have reported the adaptation of microorganisms to high CO concentrations[82,94,95], and thus it was interesting to identify whether adaptation to high CO partial pressure was possible in the microbial population used in order to achieve higher methane potential. Performing carboxidotrophic activity tests under 100% CO over two months confirmed this hypothesis. After 40 days of incubation the sludge achieved the highest methanogenic activity observed in all of the tests performed in the study, with a nearly 90% conversion of CO into methane.

Previous studies with *M. acetivoran*s and *M. barkeri* demonstrated the microorganisms' ability to grow at 100% CO in the headspace after an adaptation period with progressive increase of the CO concentration[71,94]. Moreover, Techtmann et al. highlights the regulation mechanisms of both hydrogenase-linked CODH and CODH/ACS operons for efficient consumption of CO across a wide range of concentrations with *Carboxydothermus hydrogenoformans*[82]. In the study the authors conclude that at high CO concentrations the bacteria catabolize more CO into energy by the overexpression of hydrogenase, while at low CO concentrations the CO is mainly used for carbon fixation. Therefore, it is possible that methanogens need longer adaptation time to achieve methane production at high CO concentrations.

Nonetheless, after 55 days of incubation at higher CO partial pressure the carboxidotrophic and methanogenic activities in our study dropped drastically. It was suggested that the decrease of activity in the sludge was related to the accumulation over time of acetate or other by-products in the bottles, which may have affected the microbial performance[172,173].

The DGGE analyses performed after long term exposure to high CO concentrations further confirmed a variation in the microbial population over time (appendix III). These results reflect a selection of the microorganisms better adapted to high CO concentrations.

A notable difference in the eubacterial community was observed after one month of incubation. In the absence of inhibitors, *Clostridium propionicum*, a propionate producing bacterium[174], and *Acetobacterium wieringae*, an acetate producing bacterium[175], were detected after a month of incubation at high CO

concentrations and possibly became a dominant group in the eubacterial population. This increase in the abundance of these two species may explain the accumulation of acetate and propionate observed in our study at high CO partial pressures in the gas phase. In addition, other fermentative bacteria were detected in the sludge after one or two months under high CO conditions such as *Petrimonas sulfuriphila*[176], a H_2 and acetate producing fermentative bacterium, and *Geobacter uraniireducens* sp., an acetate oxidizing bacterium[177] (homoacetogen). Thus, the eubacterial population shifted towards an increased carboxidotrophic activity, probably due to the predominance of these species in the consortium after a month of exposure to high CO concentrations.

In parallel, the DGGE analyses also showed an evolution in the archaeal population after the two months of incubation (Appendix III). Both acetoclastic and hydrogenophilic methanogenic species were present at the beginning in the sludge. The initial archaeal population was primarily composed of *Methanosaetaceae* species such as *M. concilii*, a strict acetoclastic methanogen[185], and *Methanomicrobiales* species like *Methanolinea tarda*, which is a new species isolated from a propionate-degradation enrichment culture from municipal sewage sludge and which utilizes H_2 and formate for growth and methane production[186].

However, after 63 days of incubation under 100% of CO in the gas phase a shift was observed. Hydrogen-utilizing methanogens became predominant in the archaeal population. An abundance of *Methanobacterium* species, such as *M. congolense*, which grows only on H_2/CO_2 as a substrate[187], was detected.

These results concerning the archaeal population in the sludge are in agreement with previous studies reporting that *Methanosaetaceae* is a very important group of acetoclastic methanogens in anaerobic bioreactors due to its higher affinity for acetate, and thus have a competitive advantage when acetate concentration is low. However, the decrease of *Methanosaetaceae* species in co-cultures has been related to the presence of high concentration of VFA in the medium (namely acetate)[122,123,181]. In addition, many studies report the inhibition of acetoclastic methanogens to high VFA concentrations and state that acetoclastic methanogens are

much more sensitive to high VFA concentrations than hydrogenophilic methanogens[122,123]. Besides, Karakashev et al. underline that the decrease of *Methanosaetaceae* in the population might lead to the predominance of acetate oxidizers in syntrophic cooperation with hydrogen-utilizing methanogens as observed in our study[122].

Therefore, when the acetate removal rate is low and acetate starts to accumulate in the medium at high CO concentrations, as previously discussed, the production of methane from CO must be performed mainly in syntrophy between acetate-oxidizing bacteria (i.e. *Clostridium* sp., or *Geobacter* sp.*)* and hydrogen-utilizing methanogens (*Methanobacteriales*, *Methanomicrobiales* and *Methanosarcinaceae)*. This shift of the microbial population lead to the observation of higher methanogenic potential in our study, probably due to the higher free energy release of the methanogenic reaction from H_2 compared to the acetoclastic methanogenic reaction[156].

This adaptation to high CO concentrations of the sludge opens a new perspective regarding the potential to achieve higher carboxidotrophic methanogenic potential at large scale through the sludge's previous acclimation to high CO concentrations.

Perspective

The work performed in this study constitutes only a part of an effort to develop an integrated conversion of residual biomass into bioenergy production, namely methane-enriched bio/syngas at high yield. Therefore, the concluding remarks of this project provide the possibility of enhancing the conversion potential of the carbon monoxide produced as a result of biomass gasification into methane, which would facilitate further development of reactor design and operation optimization in order to enable a subsequent scaling-up.

Nevertheless, more targeted techniques are required to better understand the microbial population shifts under different environmental conditions, as well as the change in their metabolic pathways and the adaptation or regulation of CO conversion to methane by some methanogenic microorganisms.

Moreover, an interesting follow up study of the results obtained would be to focus on its reproducibility with the use of all of the components of syngas (i.e. CO, H_2), and the effect of impurities on the conversion process for the advancement of syngas derived fuels at large scale.

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SUPPLEMENTARY WORK

During the 2.5 years I spent in the lab for my master, I performed many different techniques, analyzed and interpreted a large quantity of data and obtained many results that have not been included for the scientific publication. However, all of these results obtained helped us to understand the whole picture of our work and to build the final conclusion of our main paper. Furthermore, these experiments gave me the chance to learn new methodologies and acquire a deeper understanding of the general anaerobic microbial population structure and dynamics. This gave me the technical skills to become a better and more objective scientist, a prerequisite for writing a strong scientific paper.

Appendix I, II, and III contain different experiments I carried out during my master program but without the objective of publishing, at least at the present time. The methodologies and results obtained are discussed therein.

APPENDIX I

Pressure effect

One of the limiting steps in CO conversion processes at a large scale is the gas-liquid mass transfer rate, due to the low aqueous solubility of CO. Therefore, one way to achieve higher gas-liquid mass transfer rates and increase process productivity is to increase the pressure in the gas phase of the process. However, the increase in the CO pressure can lead to the metabolism inhibition of the microorganisms present in the bioreactor, and thus the inhibition of the CO conversion to methane or other desirable chemicals. Hence to further understand the effect of CO partial pressure (P_{CO}) and gas total pressure in the system on the carboxidotrophic methanogenic potential in the sludge, an additional series of tests were performed at different P_{CO} and total pressure.

Methodology

The anaerobic digestion sludge was tested under a P_{CO} of 10% CO (N_2 balance) at a total pressure in the gas phase of 1 and 3 atm, and under 100% CO at 1 and 1.6 atm total pressure in the headspace of the bottles. The specific activity tests were carried out as previously described in chapter 4, as was the calculation of carboxidotrophic and methanogenic activities. The tests were performed in both the absence and presence of inhibitors, namely BES (50mM) and vancomycin (0.07mM).

Results

The pressure effect on carboxidotrophic and methanogenic activities in the anaerobic sludge at 10% and 100% CO partial pressure in the bottles is presented in Table XI.

Under low CO concentration (0.09 mM), in the absence of inhibitors, the increase in total pressure decreased both the CO consumption rate and the methane production by approximately 40 and 60%, respectively. This negative effect of total pressure applied on CO bioconversion becomes even more pronounced when methanogenesis is blocked by BES. However, in the presence of vancomycin the increase in total pressure raised the carboxidotrophic specific activity and the CH_4 production rate and yield to similar levels of that achieved in the test without inhibitors.

On the other hand, under high CO concentrations the increase in total pressure in the bottles decreased both activities, even when vancomycin was added to the media.

Table XI. Pressure effects on carboxidotrophic and methanogenic activities at low and high dissolved CO concentrations in absence and presence of inhibitors at 35˚C.

Inhibitor	Total Pressure in the Bottle (atm)	Specific Activity (mmol CO/gVSS·d)	Specific Activity (mmol CH_4/gVSS·d)
CO Concentration: 0.09 mM			
-	1.01	*1.2*	*0.7*
	3.04	0.7 ± 0.3	0.3 ± 0.0
BES	1.01	*1.3*	*0*
	3.04	0.3 ± 0.0	0.0 ± 0.0
Vancomycin	1.01	*0.2*	*0.1*
	3.04	0.6 ± 0.1	0.4 ± 0.0
CO Concentration: 1.6 mM			
-	1.01	8.6 ± 1.94	0.04 ± 0.00
	1.52	7.09 ± 0.57	0.00 ± 0.00
BES	1.01	6.63 ± 0.28	0.00 ± 0.00
	1.52	3.59 ± 0.17	0.00 ± 0.00
Vancomycin	1.01	1.89	0.7
	1.52	1.22 ± 0.18	0.31 ± 0.06

Discussion

The effect of the CO partial pressure and consequently the amount of substrate available for the cells is already extensively discussed in chapter 4. In

parallel to previous observations, the results obtained with the tests where the total pressures in the bottles were varied, suggest that total pressure has an effect on the microbial activity.

The resistance to high pressures varies greatly between microorganisms. However the main mechanisms leading to the cell inactivation under these conditions are mainly related to intrinsic factors affecting the cell structure, such as the cell membrane, which plays an important role in cell transport, permeability and respiration[188,189]. Hence it is expected that increasing the total pressure in the system has a negative effect on the activity and productivity of the cells. This negative effect matches the results obtained in our study. At low CO concentrations we observed a decline in the microorganisms' metabolic activities by increasing the total pressure in the bottles, which was even more visible when methanogenesis was blocked by BES. However, the relative activity increase when gram positive bacteria were inhibited in the presence of vancomycin was not expected, since many studies reported that there is a higher resistance in gram positive bacteria compared to gram negative bacteria due to the thicker peptidoglycan layer of the cell wall in the former[189,190].

A possible explanation for the higher carboxidotrophic and methanogenic activity observed in the presence of vancomycin might be due to the increase of permeability in the cell membrane of gram negative bacteria by the high pressure applied, thus allowing higher absorption of CO increasing the rate of substrate metabolization in the cell. It has been shown that the outer membrane of gram-negative bacteria can act as a permeability barrier against many substances and antimicrobial compounds[113,191,192]. The structure and the presence of lipopolysaccharides (LPS) in the cell wall of gram negative bacteria lead to the exclusion or selection in the penetration of many compounds, providing them protection against environmental stress conditions[193].

Therefore, since the concentration of dissolved CO was very low in the study (0.09 mM), it was necessary to increase the total pressure in the bottles to allow better diffusion of the substrate from the media to the cell and thus achieve higher metabolic activity.

On the tests conducted at high dissolved CO concentration (1.6 mM) methanogenesis was already partly inhibited by the amount of CO provided. Hence the increase of total pressure in the gas phase increased the CO availability and the inhibitory effect of CO, as has been previously observed[18,62,67]. In that case both CO and CH_4 specific activities, in addition to the CH_4 yield, were reduced.

Therefore, high pressure in the system may affect the osmosis, metabolites transport, enzymes activity, proteins, as well as the thermodynamics in the cell. Many findings concerning metabolic barotolerant mechanisms in gram negative bacteria have been previously reported[194,195]. Even so, very few archaea barotolerance mechanisms have been studied in depth, although tolerance to high pressure has been observed[195]. Moreover, it is possible that some microorganisms may decouple growth and methane production to survive at higher pressures, as has been extensively discussed in previous work with *Carboxydothermus hydrogenoformans*[82].

Hence, in the present study the substrate concentration available for the microorganisms is the determining factor for achieving higher productivity.

APPENDIX II

Granules vs. Suspended Biomass

The disruption of the granular structure of the sludge used in this study was mainly done in order to have complete exposure of the microbial community to CO and to estimate the CO toxicity without bias due to the biofilm's structure. However, the increase of carboxidotrophic activity observed with the amount of CO applied significantly differed from previous results obtained with integral granular sludge[10,69]. Guiot et al. (2011) reported a maximum CO consumption rate of 8.1 mmol CO/g VSS·d at 0.2 atm CO initial partial pressure (0.3 mM), and when increasing the CO concentration the activity dropped drastically to 2-3 mmol CO/g VSS·d. This discrepancy in results led us to question the impact of the biomass configuration on carboxidotrophic activity and metabolic pathways.

Therefore, a new set of kinetic activity tests with entire granules were assessed in the same manner and under the same conditions as with the crushed sludge to define more precisely the impact of the biomass configuration as a biofilm on CO conversion to methane.

Methodology

The Kinetic activity tests were performed as previously described in chapter 4. The tests were performed in duplicate with and without inhibitors (BES and vancomycin), and the carboxidotrophic and methanogenic activities were determined by measuring the rate of CO consumption and methane production at their inflection point (expressed in mmol CO or CH_4 per unit volatile suspended solids (VSS) per day). The CO partial pressure ranged between 0.1 and 1.2 atm (10-100% CO, N_2 balance), and corresponded to CO concentrations in liquid varying from 0.17 to 1.82 mM.

Results

The experimental results obtained followed a similar pattern in both granules and suspended biomass (Figure 10).

Despite the fact that the carboxidotrophic activity was lower in general in the granules than in the suspension, the activity of granules also increased with the amount of CO supplied. The maximum activity reached with granules was 7.1 mmol CO/g VSS·d, for a P_{CO} of 1.2 atm in the gas phase (1.8 mM in the liquid). On the other hand, the methane production rate with granules stayed more or less constant at 1.2 mmol CH_4/g VSS·d, and then decreased at P_{CO} higher than 0.5 atm (0.8 mM in the liquid), in contrast to the suspended sludge, for which the methane production rate started to decrease at a P_{CO} higher than 0.2 atm (data not shown).

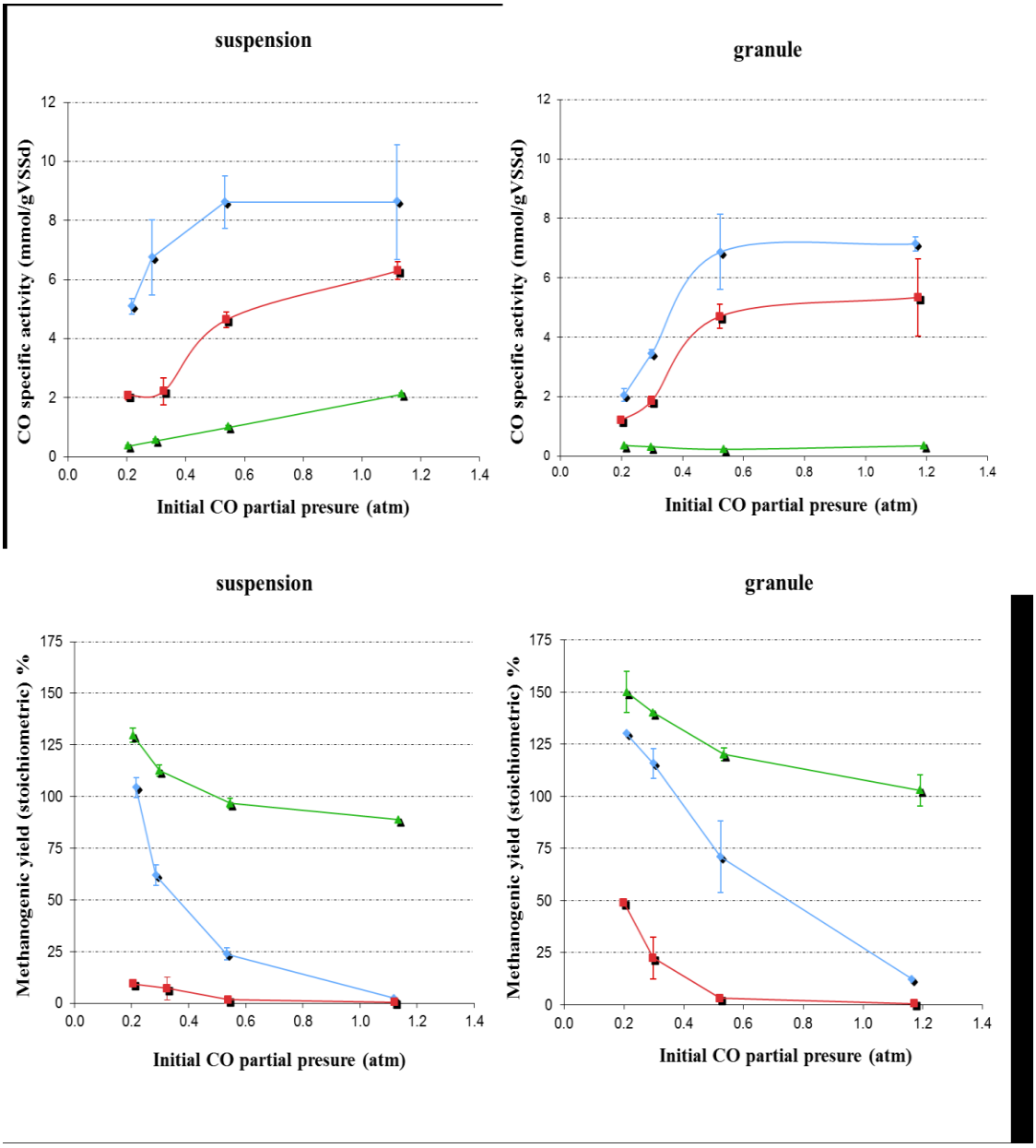

Figure 10. Comparison of the carboxidotrophic specific activity and methanogenic yield under different P_{CO} for granules and suspended biomass. In the absence of inhibitor (○), and the presence of BES (□) and vancomycin (Δ).

Nonetheless, despite the differences in methane production expressed by both sludge structures and the higher methane potential achieved with granules (Figure 11), the methane yield decreased with the amount of CO supplied independently of the sludge morphology.

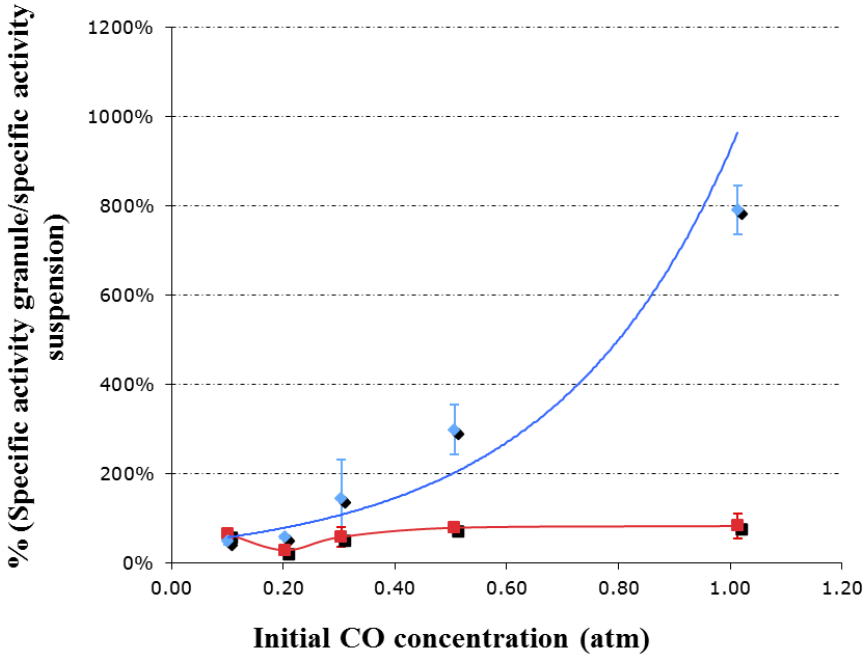

Figure 11. Carboxidotrophic (□) and methanogenic (○) specific activity ratio of granules versus suspended biomass.

The higher stoichiometric product yields observed at low CO concentrations under restrictive conditions are probably due to the degradation of organic matter present in the sludge. The fermentation of the organic material to H_2 and CO_2 and/or acetate might afterwards be transformed into CH_4 thus increasing the final yield. This was confirmed with endogenous tests (no substrate) and vancomycin (data not shown). However, these values were not subtracted from the results obtained in the CO tests because it is important to account for the inhibitory effect that CO brings to the consortia.

Discussion

The comparison of the specific bioactivity in both the disaggregated granules (the sludge mainly used in the study) and the original granules (integral granules) of the same origin, leads us to believe that the disruption of the granule has a negative impact on methanogenic activities. This is probably due to the lack of CO protection that the granule offers to the methanogens present in the consortia and the lack of synergy between the different trophic groups of microorganisms that participate in the CO conversion to CH_4. In the study the granules achieved approximately four times higher methane yields at high CO concentrations, and the methane production rate increased almost 8 times.

This advantage of granules over suspended biomass has been reported in many studies[124–127]. The authors of these studies discuss the benefits of the distribution of the different trophic groups in the consortium forming juxtaposed layers, which allows them to improve the flux of metabolites produced from CO between species, as well as the electron transfer between them for the final CH_4 production. According to these studies the outermost layer of the granule is composed of hydrolytic and fermenting bacteria, the middle layer mostly populated by acetogens and hydrogen-producing bacteria, while methanogens are mostly in the core of the granule. This location in the granule protects methanogens against toxic CO concentrations, allowing the maintenance of the methanogenic potential.

On the other hand, the lower carboxidotrophic activity observed in granules as compared to the biomass in suspension might be explained by the diffusion limitation that readily arises in a granular biofilm, which therefore would lower the CO availability to the carboxidotrophic microorganisms, and would thus limit the activity of the overall microbial community. This could be a disadvantage when working with granules, since to increase the mass transfer from the medium to the microbial population it is necessary the increase of the total pressure in the system or the amount of CO supplied to achieve higher methanogenic potential, increasing the costs of the process.

Furthermore, the comparison between both sludge morphologies suggested that even though there are a few differences in methanogenic activity, the general performance of the anaerobic sludge and the main pathways implicated in CO conversion to methane don't vary between granules and suspended biomass. Therefore the performance of the overall process depends on the microbial composition present in the sludge, which mainly depends on the operational conditions and type of substrates digested in the UASB reactor. Hence, different environmental conditions in the operational plant lead to different microbial populations in the sludge and thus affect the behaviour and the fate of the different metabolic groups present.

APPENDIX III

Molecular Analyses

The carboxidotrophic methanogenic potential increase observed in the kinetic activity tests after long-term exposure to CO led us to investigate the microbial ecology of the consortium over time and to evaluate the possible adaptation of the microbial population to high CO concentrations. We used a molecular approach (DGGE experiments) to examine the changes in the microbial community structure over time, in both the presence and absence of vancomycin. The presence of vancomycin resistant bacteria in the sludge was also analyzed by PCR experiments.

Methodology

DNA Preparation

Bottles were incubated for two months at 1 atm CO partial pressure with and without the addition of vancomycin. CO was continuously supplied to achieve an atmosphere of 100% CO in the gas phase during the incubation period. During this period, sludge samples were taken every two weeks for further analyses. Total genomic DNA was extracted from 2 mL homogenised sludge samples as previously described in population dynamic studies working with environmental samples[164,165]. Briefly, 0.7 mL of TEN buffer (Tris-EDTA-NaCl: 100 mM-100 mM-100 mM; pH 8) were added to the 2 mL samples, which were then incubated at 37˚ C for 15 min after the addition of 35 μL of SDS 20%. 250 mg of 0.5 and 0.1 mm glass beads were then added into the solution and shaken twice for 5 seconds between 4 and 6.5 ms^{-1} in a DNA FastPrep system bead-beater (Bio 101, Savant, Farmingdale,NY). The DNA was then precipitated with ethanol (95-100%), resuspended in Tris (10 mM, pH 8.5), and quantified using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Finally, the extracted DNA was visualized on a 0.8% agarose gel. The DNA was afterwards purified and concentrated using a QIAEX gel extraction kit (Hoffman-La Roche AG, U.S, 2001) according to the manufacturer's instructions.

DGGE Experiments

The purified DNA was used as template to amplify the 16s rDNA region of the eubacterial and archaeal populations of the sludge by PCR. DGGE experiments were performed as previously described by Tresse et al. (2004)[196]. The universal

eubacterial primers 341f (5'-CCTACGGGAGGCAGCAG-3')[167] and 758r (5'-CTACCAGGGTATCTAATCC-3')[168] were used for Eubacteria, and primers 931f (5'-AGGAATTGGCGGGGGAGCA- 3')[169] and 1392r (5'- ACGGGCGGTGTGTAC - 3')[170] were used for Archaea. A 40 bases GC clamp was added to the forward primers[167]. PCR amplifications were performed in a PCR thermal cycler (Eppendorf Mastercycler pro, NB, Canada), in a final volume of 25 µL containing 5 µL of TAQ green master mix (EconoTaq PLUS GREEN 2X Master Mix Lucigen), 0.5 µM of each primer, 2 ng of genomic DNA and sterile Millipure water. Amplification conditions were as follows: an initial denaturation step of 3 min at 94ºC followed by 35 cycles of 30 s at 94 ºC, 30 s at 55 ºC and 30 s at 72 ºC. The final extension step was 10 min at 72 ºC. PCR products were then verified by gel electrophoresis on 1.5% agarose gel followed by visualization with UV illumination after Sybr Safe staining (Invitrogen, Carlsbad, CA, USA).

DGGE analyses of PCR products were performed with a DCode GeneTM System (Bio-Rad, Hercules, CA, USA). PCR samples were concentrated and 400 ng were loaded onto a 40% to 60% urea-formamide denaturant gradient gel (8% (wt/vol) polyacrylamide in 1X TAE (40 mM Tris-acetate pH 7.4, 20 mM acetate, 1 mM Na_2EDTA). Electrophoresis was performed in 1X TAE buffer at a temperature of 60°C and at a constant voltage of 80 V for 16 hours. After electrophoresis, the gel was stained for 30 min with Vistra Green (Molecular Dynamics, CA, USA). Densitometric scanning of fluorescent DNA fragments was performed with the Molecular FluorImager (Molecular Dynamics, Sunnyvale, CA, USA) and results were analyzed using ImageQuaNT software (Molecular Dynamics).

Bands of interest were excised form the gel and the DNA was then eluted and re-amplified by PCR as described above except using forward primers without a GC clamp. PCR products were then purified using the QIAquick purification kit (Qiagen, Valencia, CA, USA), quantified by densitometry, and sequenced at the Université de Laval (Québec, QC, Canada). Sequences were compared to those in the GenBank database using the Basic Local Alignment Search Tool (BLAST)[197] at the National

Center for Biotechnology Information (NCBI) to determine the phylogenetic affiliations.

PCR Experiments

To search for vancomycin resistant strains in the sludge, PCR experiments were performed using specific primers targeting the vancomycin resistance encoding genes *vanA*, *vanB2*, *vanC*, *vanC3*, *vanE*, *vanH*, *vanS*, *vanY*, and *vanZ*. The sequences and properties of the primers are listed in Table XII. DNA preparation and PCR conditions were the same as described above for DGGE experiments. The control strains used as a reference to test the specificity of each primer set were *Enterococcus casseliflavus* MA-52407 (*vanA*, *vanC3*), *Enterococcus faecalis* MA-58123 (*vanB2*), *Enterococcus gallinarum* MA-52409 (*vanC*), *Enterococcus faecalis* MA-62440 (*vanE*), and *Enterococcus faecium* BM4147 (*vanH*, *vanS*, *vanY*, and *vanZ*).

Table XII. PCR primers used to determine the presence of vancomycin resistant genes.

Primers Set	Targeted Gene	Size of PCR Product (bp)	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	Ref.
F*van*A-R*van*A	*vanA*	1029	ATGAATAGAATAAAAGTTGCAATAC	CCCCTTTAACGCTAATACGAT	198
F*van*B2-R*van*B2	*vanB2*	536	AAGCTATGCAAGAAGCCATG	CCGACAATCAAATCATCCTC	199
F*van*C-R*van*C	*vanC*	822	GGTATCAAGGAAACCTC	CTTCCGCCATCATAGCT	200
F*van*C3-R*van*C3	*vanC3*	484	CGGGGAAGATGGCAGTAT	CGCAGGGACGGTGATTTT	200
F*van*E-R*van*E	*vanE*	513	TGTGGTATCGGAGCTGCAG	GTCGATTCTCGCTAATCC	201
F*van*H-R*van*H1	*vanH*	943	ATCGGCATTACTGTTTATGGAT	TCCTTTCAAAATCCAAACAGTTT	198
F*van*S-R*van*S	*vanS*	1094	AACGACTATTCCAAACTAGAAC	GCTGGAAGCTCTACCCTAAA	202
F*van*Y-R*van*Y	*vanY*	866	ACTTAGGTTATGACTACGTTAAT	CCTCCTTGAATTAGTATGTGTT	202
F*van*Z-R*van*Z	*vanZ*	454	TTATCTAGAGGATTGCTAGC	AATGGGTACGGTAAACGAGC	202

Results

DGGE Experiments

The evolution of the microbial population structure and dynamics under strictly CO conditions was studied through DGGE over the two month experimental set up (Figure 12).

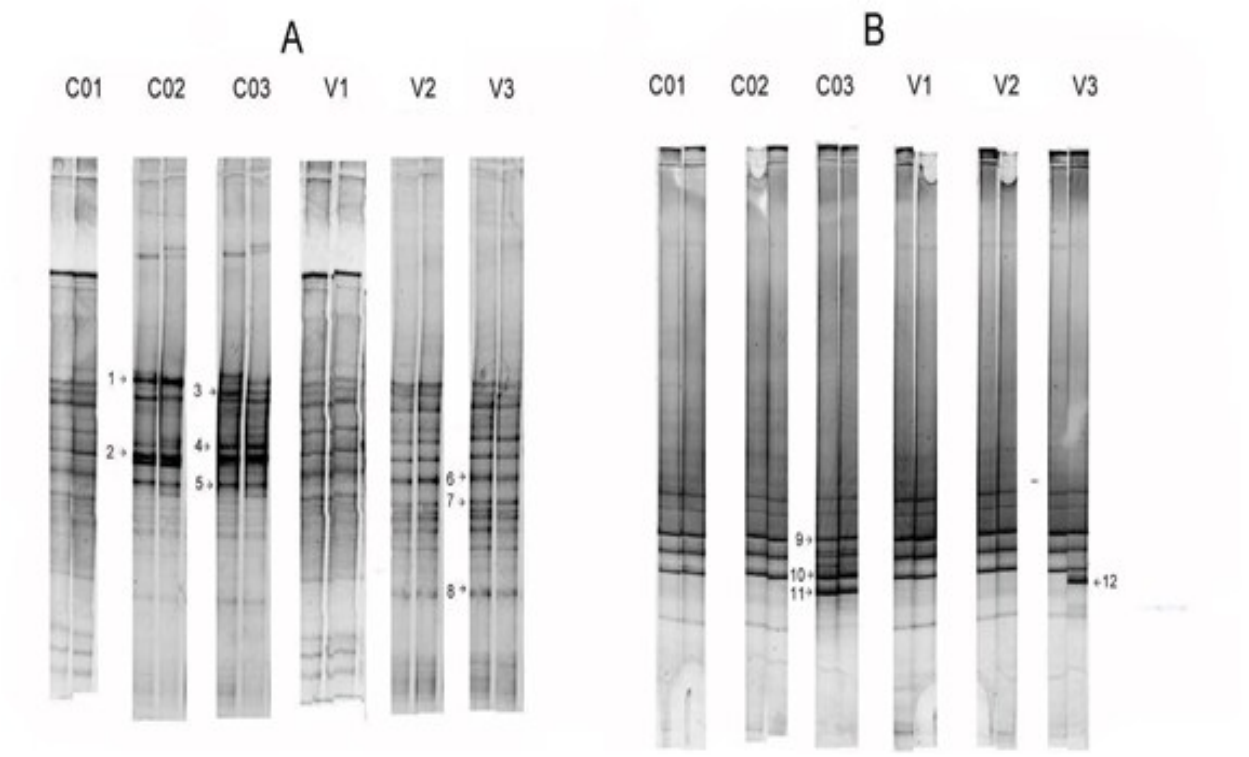

Figure 12. DGGE analysis of the microbial population diversity in the anaerobic digestion sludge over the 2 month incubation period. A and B correspond to the analysis of the eubacterial and archaeal population structure, respectively. Lanes CO1, CO2, and CO3 correspond to samples taken at day 0, 30, and 60 in the absence of vancomycin. Lanes V1, V2, and V3 correspond to samples taken at day 0, 30, and 60 in the presence of vancomycin. The bands selected for further analysis are indicated with an arrow and numbered from 1 to 12.

The comparison of the DGGE profiles of the sludge samples taken at three different times in the absence and presence of vancomycin, led to the selection of 39 bands of interest (Figure 12). These bands were extracted from the archaeal and eubacterial DGGE gels and submitted to sequencing. Due to the poor quality of some sequences (either high background or doubled sequences), only 12 bands were finally taken into consideration and compared either to the GenBank database by using BLAST or to the Ribosomal Database Project (RDP) 16S rDNA database by using the RDP classifier platform. Phylogenetic affiliations determined by BLAST and RDP classifier for the selected eubacterial and archaeal 16S rDNA sequences are presented in Table XIII and XIV, respectively.

A notable difference in the eubacterial community between the two tests (with and without vancomycin) was observed after one month of incubation (Figure 12A). When vancomycin was not present in the media *Clostridium propionicum* (Figure 12A. band 1), a propionate producing bacterium[174], and *Acetobacterium wieringae* (Figure 12A. band 2), an acetate producing bacterium[175], were detected after a month of incubation at high CO concentrations. In addition, other fermentative bacteria were detected in the sludge after one or two months under CO conditions. H_2 and acetate producers such as *Petrimonas sulfuriphila*[176], and *Geobacter uraniireducens* sp., an acetate oxidizer bacterium[177](homoacetogen), were detected after 40 days of incubation.

In parallel, the DGGE analyses also showed an evolution in the archaeal population after the two months of incubation in both conditions (presence and absence of vancomycin) (Figure 12B). The initial archaeal population was notably composed by *Methanosaetaceae* species such as *M. concilii* (Figure 12B. band 9), a strict acetoclastic methanogen[185], and *Methanomicrobiales* species like *Methanolinea tarda* (Figure 12B. band 10), which utilizes H_2 and formate for growth and methane production[186].

Table XIII. Evolution of the eubacterial population in the sludge over the time, in the presence and absence of vancomycin under an atmosphere of 100% CO as determined by DGGE experiments.

Band Number	Identified Microorganism (GenBank Accession Number)	% Similarity (Sequence Length)	Day 0	No Inhibition		Vancomycin Inhibition		RDP Classifier Classification
				Day 30	Day 45	Day 30	Day 45	
1	*Clostridium propionicum*, strain: JCM 1430 (AB649276.1)	100% (400/400)	-	+	+	-	-	*Clostridium* XlVb (100%)
2	*Acetobacterium wieringae* strain DP9 (HQ384240.1)	99% (394/396)	-	+	+	-	-	*Acetobacterium* (100%)
3	Uncultured *Bacteroidetes* bacterium clone D1 16S (HQ003602.1)	99% (357/391)	-	-	+	-	+	*Bacteroidetes* (99%)
4	*Petrimonas sulfuriphila* strain BN3 (NR042987.1)	94% (391/415)	-	-	+	-	+	*Bacteroidales* (100%), *Petrimonas* (96%)
5	Geobacter uraniireducens Rf4 (CP000698.1)	97% (399/411)	-	+	+	-	-	*Desulfuromonadales* (100%), *Geobacteraceae* (97%), *Geobacter* (94%)
6	Brevundimonas bullata, strain: NBRC 13290	98% (380/388)	-	+	-	+	+	*Alphaproteobacteria* (100%), *Caulobacteraceae* (99%), *Brevundimonas* (97%)
7	*Magnetospirillum gryphiswaldense*, strain: NBRC 15271 (AB680821.1)	100% (372/372)	-	-	-	-	+	*Magnetospirillum* (100%)
8	*Syntrophobacter fumaroxidans* MPOB (JQ346744.1)	99% (404/407)	-	-	-	+	+	*Syntrophobacteraceae* (100%), *Syntrophobacter* (100%)

(+) Presence of the microorganism; (-) Absence of the microorganism in the population.

After 63 days of incubation a shift was observed, and hydrogen-utilizing methanogens became dominant in the archaeal population. An abundance of *Methanobacterium* species (Figure 12B. band 11), namely *M. congolense*, that grows only on H_2/CO_2 as a substrate[187] was detected. In the presence of vancomycin, *Methanosarcina mazei* became a dominant species in the population after two months at high CO concentrations (Figure 12B. band 12). This archaea has previously been reported capable to survive with a wide variety of substrates such as H_2/CO_2, acetate, all methylamines and methanol[203], which likely makes it easier to survive when conditions are unfavorable.

Table XIV. Evolution of the archaeal population in the sludge over the time, in the presence and absence of vancomycin under an atmosphere of 100% CO as determined by DGGE experiments.

Band Number	Identified Microorganism (GenBank Accession Number)	% Similarity (Sequence Length)	Day 0	No Inhibition		Vancomycin Inhibition		RDP Classifier Classification
				Day 30	Day 45	Day 30	Day 45	
9	*Methanosaeta concilii* , strain: NBRC 103675 (AB679168.1)	99% (429/434)	+	+	+	+	+	*Methanosaeta concilii* (98%)
10	Uncultured *Methanolinea* sp. , clone: SMS-T-Pro-2 (AB479406.1)	99% (431/433)	-	+	+	-	-	*Methanolinea tarda (89%)*
11	Uncultured *Methanobacteriaceae* archaeon , clone:AR-H2-B (AB236069.1)	100% (429/429)	-	-	+	-	-	*Methanobacterium congolense (98%)*
12	*Methanosarcina mazei* Go1 (JQ346757.1)	100% (428/428)	-	-	-	-	+	*Methanosarcina mazei(99%)*

(+) Presence of the microorganism; (-) Absence of the microorganism in the population.

In parallel, the comparison of the DGGE profiles in the presence and absence of vancomycin, an inhibitor of gram positive bacteria[131], confirms its inhibitory potency on the bacterial population. A clear difference in the eubacterial community composition was observed between both conditions. In the presence of vancomycin, the inhibition of gram positive bacteria, such as *A. wieringae* and *C. propionicum*, led to the emergence of different gram negative species related to the phylum Proteobacteria, such as *Magnetospirillum gryphiswaldense* (Figure 12A. band 7), *Brevundimonas* sp. (Figure 12A. band 6), and *Syntrophobacter fumaroxidans* (Figure 12A. band 8)[204,205].

Vancomycin Resistance

The presence of vancomycin resistant bacteria strains in the sludge was investigated by PCR, using primers targeting 9 different well characterized vancomycin resistance encoding genes. None of the gene tested were found to be present in our samples, indicating the possible absence of vancomycin resistant bacteria in the sludge (Figure 13).

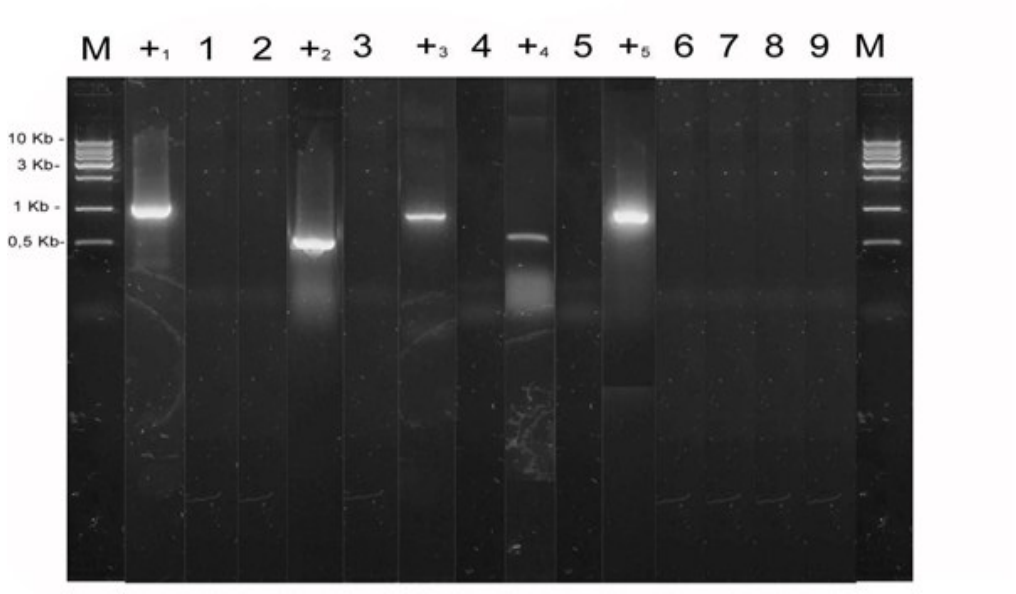

Figure 13. PCR experiments for the detection of vancomycin resistance encoding genes in the sludge. Lanes: M, 10-Kb DNA ladder (Bioshop Canada Inc.); 1, 2, 3, 4, 5, 6, 7, 8, and 9 correspond to the amplification of *vanA*, *vanC3*, *vanB2*, *vanC*, *vanE*, *vanH*, *vanS*, *vanY*, and *vanZ* genes, respectively. Positive controls; +1, *Enterococcus casseliflavus* strain MA-52407, +2, *Enterococcus faecalis* strain MA-58123, +3, *Enterococcus gallinarum* strain MA-52409, +4, *Enterococcus faecalis* strain MA-62440, +5, *Enterococcus faecium* strain BM4147.