Kinetics, multivariate statistical modelling, and physiology of CO2-based biological methane production

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HIGHLIGHTS

• Gas-to-gas conversion processes are analyzed with respect to bioenergy production.
• CO2-BMP modeling is performed and model validity is discussed.
• Multivariate data analysis and biological gas conversion mechanistic is integrated.
• Gas limitation and liquid limitation in pure culture biological CH4 production are highlighted.
• Continuous culture CH4 bioprocessing from H2/CO2 is discussed.

ABSTRACT

Conversion of surplus electricity to chemical energy is increasingly attracting attention. Therefor, biological energy conversion and storage technologies are one of several viable options. In this work, the inherent challenges faced in analyzing the CO2-based biological methane production (CO2-BMP) process for energy conversion and storage are discussed. A comprehensive assessment of key process parameters on several CO2-BMP process variables was conducted. It was found that literature data often misses important information and/or the required accuracy for resolution of the underlying mechanistic effects, especially when modelling reactor dependent variables. Multivariate dependencies inherently attributable to gas-to-gas conversion bioprocesses are particularly illustrated with respect to CO2-BMP. It is concluded that CO2-BMP process modelling requires the application of process analytical technology. The understanding of the CO2-BMP mechanistic process is discussed to assist with the analysis and modelling of other gas-to-gas conversion processes. The findings presented in this work could aid in establishing a biotechnology-based energy to gas conversion and storage landscape.

1. Introduction

Converting surplus electricity to chemical energy is increasingly attracting attention [1]. In this frame, chemical or biological energy conversion and storage technologies for the power-to-gas concept are one of several viable options [2,3]. Due to decreasing reserves of fossil fuels and growing awareness for global warming, carbon dioxide (CO2) utilization has become a topic of industrial relevance [4]. An effective reduction of CO2 emissions will be achieved in the long term if renewable energy production can be linked with power conversion and storage technologies. Furthermore, the production of renewable energy is significantly more carbon neutral when compared to fossil fuel-based energy production [5,6]. Therein, a renewable energy production scenario that consumes CO2 and produces biofuels could become an integral part of a biorefinery scenario for reducing CO2 emissions [7]. However, the environmental impact of biofuels production, utilization, and surplus (or excess) energy conversion systems still needs to be evaluated and re-assessed.

Production of 1st generation biofuels would currently be able to compete with fossil fuels in the case where certain energy crops (e.g. Saccharum officinalis) are employed in bioethanol production [5]. 2nd generation biofuel production from e.g. lignocellulose could also become competitive to fossil fuels and are already applied on industrial scale for energy production [5,6]. Biofuel production systems of the 3rd and 4th generations have only reached pilot and pre-industrial scales concerning biodiesel production from algae and photo-fermentation of
molecular hydrogen (H₂) respectively [7]. Recent advances in bioprocess technology [2,3,8,9] and the development of biorefinery concepts favored the development of 5th generation biofuels, which employ microorganisms to convert gaseous substrate(s) to gaseous end products. 5th generation biofuels encompass CO₂-based biological methane (CH₄) production (CO₂-BMP) and H₂ production from C₁ compounds [9,10]. CO₂-BMP and H₂ production from C₁ compounds are known to be the only gaseous biofuel production technologies that have no immediate requirement for photosynthesis. Thus, integrating surpass renewable power conversion with CO₂ capture and storage can be performed by applying the CO₂-BMP process.

The CO₂-BMP process is characterized by utilizing hydrogenotrophic methanogenic archaea (methanogens) for CH₄ production [9]. Because CO₂-BMP is a bioprocess, it encompasses distinct and emergent advantages compared to its chemical counterpart – the Sabattier process. One such advantage is the autotrophic regeneration of methanogens accompanied by CH₄ production [9,11–14]. In this process, methanogens exhal CH₄ as a metabolic end product of their energy conserving metabolism while fixing a variable part of CO₂ in the form of biomass [14–16]. Therefore, the production of CH₄ is essential for the survival of the organisms. The CO₂-BMP process can be carried out by an enrichment culture [17–23] or pure culture of methanogens [9,24] and benefits from its ability to convert CO₂ and H₂ to CH₄ at very high volumetric methane evolution rates (MERs) while in continuous culture [25,26]. An additional advantage is the mild bioprocessing conditions (e.g. temperatures from approx. 0 °C to 122 °C) that can be applied during CO₂-BMP [27,28].

High purity H₂ and CO₂ can be employed as substrates for the CO₂-BMP process [9,12,26,35]. It has also been shown that the CO₂ by-product of the anaerobic digestion process can be microbiologically transformed to CH₄ at different conversion efficiencies and MERs [13,21,24,29]. However, it has been noticed that the technology readiness level (TRL) of the different microbiological biogas converting technologies can vary tremendously [24]. Although direct microbiological biogas conversion in anaerobic digesters was shown to be possible, the MER and CH₄ concentration in the offgas remained negligible [20,24]. On the contrary, microbiological biogas conversion by using pure [13] or enrichment cultures [21,23] of methanogens was shown to be efficient. Drawbacks of using enrichment cultures for microbiological biogas conversion are the ambiguous adaptation procedures, the time it takes for the culture to adapt to certain conditions, and unintended side reactions that occur within the enrichment [24,30]. Eventually, pure cultures of methanogens were not only applied in microbiological biogas upgrading [13,31], but were also utilized for conversion of CO₂ from industrial flue gases [13]. While pure cultures have been used for the conversion of chemical species, it should be noted that the CO₂-BMP process results in a different product formation kinetic [32,33] when compared to liquid-based continuous culture bioprocessing [6,34]. Therefore, many challenges in the analysis of production kinetics, physiology, scale-up, and modelling of the CO₂-BMP process have emerged [8].

The first aim of this study was to comprehensively assess the effects of key process parameters (KPP) on several CO₂-BMP process variables, which were obtained from literature, on continuous culture bioprocessing. Second, this study discusses the multivariate dependencies inherently attributable to CO₂-BMP gas-to-gas conversion bioprocesses. Third, it is shown that the presented models possess limits that prevent a simple analysis of the CO₂-BMP process. Fourth, the application of multivariate data analysis and modelling CO₂-BMP process is thoroughly discussed. It was of great interest to review and refine the understanding of the kinetic aspects involved in gas converting bioprocess technologies and to better control and avoid undesired or uncontrolled limitations of the CO₂-BMP kinetics.

The novelty of this contribution goes beyond bioprocess modelling. Here, a critical analysis of literature on CO₂-BMP in pure culture was performed. It is shown that both liquid and gas limitations need to be carefully considered when attempting CO₂-BMP bioprocessing. Examples on how to model the CO₂-BMP processes are given and it is shown that wrong conclusions have often been drawn due to an application of erroneous results. It is discussed that during CO₂-BMP modelling an in depth understanding of the biology and the process is required and that the physiology of the target organism must be carefully considered to cope with the multivariate nature of this process. Finally, it is shown that biological gas-to-gas conversion and energy storage processes must be scaled by linking kinetics, modelling, and physiology.

2. Material and methods

First, the existing literature of pure culture CO₂-BMP, independent of bioreactor conditions and scale, was reviewed with an in depth examination of methanogenic strains, bioprocess setup, and growth conditions. Second, pure culture CO₂-BMP data was extracted from literature [11,12,25,26,32,35–44]. Third, the data was applied for qualitative and quantitative assessment and subsequent modelling. A list of comprehensively extracted results from literature is provided in Supplementary Material 1. From all literature reports on pure culture CO₂-BMP, only the data on continuous culture experiments were analyzed as the stability of process variables in steady state allowed for a precise quantification. Closed batch and fed-batch CO₂-BMP experiments were not considered.

2.1. Definition of parameters and units

The following variables and KPPs were extracted or calculated based on the information provided in literature: the gassing rate per working volume per minute (vvm [L L⁻¹ h⁻¹]), temperature [°C], the pH, oxidation reduction potential (ORP [mV]), agitation [rpm], sulphide dilution rate (DS [d⁻¹]), trace element concentration (TE), medium dilution rate (D [h⁻¹]), the gassing rate, and the reactor pressure [barg]. Additionally, the following variables relating to production and/or yield were extracted from literature: methane evolution rate (MER [mmol L⁻¹ h⁻¹]), the specific CH₄ evolution rate (qCH₄ [mmol g⁻¹ (gram cell dry weight) h⁻¹]), the CH₄ offgas concentration [Vol-%], biomass concentration (x [g (gram cell dry weight) L⁻¹]), the specific growth rate (μ [h⁻¹]), and the growth yield (YCH₄ [g (gram cell dry weight) mol⁻¹]), or, where attainable, the growth to product yield (Y(CH₄)CH₄ [C-mol mol⁻¹]), Y(CH₄)CH₄ was used to assess the flux of the carbon into biomass and into CH₄ on a C-molar level for all the cultivations performed with Methanotrophs versus Methanobacter marburgensis [11,12,26]. Although the analysis of Y(CH₄)CH₄ was possible for experiments reported before [11,12,26,35], Y(CH₄)CH₄ could not be retrieved or calculated from all of the experiments presented in Supplementary Material 1 because C-molar biomass productivity (τCH₄) [C-mmol L⁻¹ h⁻¹] had not been reported. However, Y(CH₄)CH₄ that was defined as the quotient of μ to qCH₄ [15] could be retrieved from literature. Most KPPs and variables could be directly extracted from literature without the necessity to convert results [11,12,26,35]. In some cases the conversion of extracted literature data into aforementioned molar units was performed.

2.2. Data validation procedure

Data was curated according to the degree of reduction balance (DoR-balance) and carbon balance (C-balance) by applying manual data quality control steps. These mass balance curation steps could only be performed were the relevant information was provided in literature. The relevant bioprocess and physiological parameters were then presented after a data quality assessment based on published methodologies [9,45]. Data curation also involved a thorough qualitative selection procedure where an assessment step analyzing the data by using the MER/MER_max concept was implemented. The MER/MER_max ratio presented is the dimensionless quotient of MER to the maximum possible
volumetric CH₄ production rate (MER_MERmax [mmol L⁻¹ h⁻¹]) according to the reaction stoichiometry and experimental settings neglecting biomass formation [11,12,26]. The MER/MERmax concept for apparent gas conversion to maximum theoretical gas conversion has been previously introduced [9,26]. The MER/MERmax concept was used to identify outliers according to the percentage of MER in relation to MERmax. The resulting quotient is referred to as MER/MERmax and is plotted against the CH₄ offgas content in Vol.-% (for data visualization, see Fig. 1). This characteristic graph changes with different H₂ to CO₂ gas inflow ratios. This is due to the fact that, based on the presented assumption(s), full gas conversion can only be achieved when a H₂ to CO₂ gassing ratio of 4:1 is applied [9]. Even though the data was extracted from literature for CO₂-BMP modelling purposes, re-calculation of the data was necessary to be able to equalize the entries for subsequent qualitative and quantitative analyses. This method overestimates MER for all other data that were not calculated based on the rinert correction factor [9,26]. The rinert correction factor accounts for the fact that stoichiometric gas contraction occurs during conversion. It is needed to calculate the MER based on the educt gas inflow and the CH₄ offgas composition [12,26]. However, if YCH4 to CH₄ is assumed to be 1–5% of the total carbon flux into the biomass, the error on MER quantification is relatively small [11,12,15,26,36,45]. This approach was applied to reject data with highly deviating DoR- or C-balances.

### 3. Results

Since the publication of the simple unstructured mathematical model for a continuous pure culture CO₂-BMP process [32], new approaches have been reported for the cultivation of methanogenic archaea converting CO₂. The model in Schill et al. [32] describes growth and productivity of *M. thermoautotrophicus* in a gas-limited state as function of KPPs such as D or gassing rate. In general, many studies on CO₂-BMP focused on fed-batch or continuous culture modes [26,32,33,36]. The primary goal of these studies was to induce gas-limited or liquid-limited conditions and derive quantitative physiological variables. In some cases, these studies also examined the underlying thermodynamic and metabolic constraints of biological methanogenesis [13,26,33,35,36,48,45]. However, the dual nature of limitations (gas transfer-based or liquid-based) or inhibitions that can be faced upon biomass growth pose challenges for the development of a robust and scalable technology [8].

Continuous culture CO₂-BMP data are shown in Fig. 1. These data were plotted according to the quotient of MER/MERmax to CH₄ offgas. *M. marburgensis* continuous culture data fits the MER/MERmax to CH₄ offgas relationship. This is a consequence of the method applied for the calculation of MER via rinert gas flow as described previously [9,12,26]. Although the rinert correction factor was shown to be fairly correct for low biomass concentrations between 1 and 5% [15,36], and the DoR-balances were shown to vary greatly [11,12,26], the calculation could become more erroneous if the YCH₄ to CH₄ is higher, as could be the case for other methanogenic archaea such as *Methanosarcina barkeri* [15]. In addition, it poses limitations in terms of quantification accuracy and the ability to identify physiologic effects during a process operation. Furthermore, if MER and MERmax Values are calculated by neglecting rinert, the proportion should be true. If MER is measured, but
**3.1. Gas transfer-limited versus liquid-limited biomass growth**

The appearance of dual (gas transfer-based or liquid-based) limitation mechanisms, which are inherent to CO$_2$-BMP processes pose challenges for process analytical technologies (PAT) and quantification towards the development of a robust, controlled, and automated bioprocess [8,9,35]. Therefore, in order to accurately quantify the kinetics of gas converting bioprocesses, it is important to know the actual limitation at either a given time point or as a function of the process parameters applied to allow for control of the biocatalytic activity [35]. This strategy allows scaled feeding of the organism according to physiological demand and avoids undesired limitations in the process reaction kinetics. Data from different CO$_2$-BMP processes [11,12,26,42,43] are shown as individual plots of qCH$_4$ as a function of D in Fig. 2.

As an example, data extracted from Peillex et al. shows that the data points calculated for qCH$_4$ have an unusually high variation at the same D [43]. Although data obtained from Peillex et al. fit the MER/MER$_{\text{max}}$ concept (Fig. 1), qCH$_4$ data values were found to be more than 400% above the qCH$_4_{\text{max}}$ reported for *M. marburgensis* in continuous culture [12,35]. Although qCH$_4_{\text{max}}$ estimation of *M. marburgensis* was performed by using dynamic process conditions [12,48,49] or via a controlled liquid-limited condition [35], it can be considered that a maximum standard deviation of 10% is expected on the reported values. Therefore, qCH$_4$ values obtained by Peillex et al. are not likely to reflect physiological characteristics of *M. marburgensis* and will therefore not be used for modelling. A possible cause for the qCH$_4$ deviation in Peillex et al. could be an erroneous determination of biomass. In fact, if biomass concentration was under-evaluated by a factor of ten, then the qCH$_4$ values would be underestimated because the calculated MER$_{\text{max}}$ is higher than the real MER$_{\text{max}}$. Therefore, it must be noted, whether or not the MER was calculated from literature data or taken directly from the publication.

Physiological effects cannot be quantified if the C-balance variability is too high. In fact, the accuracy required for C-balance neglecting r(x) is too high. Therefore, enhanced C- and DoR-balancing would benefit the modelling of MER and Y(x) as a function of KPP. Calculations of MER/MER$_{\text{max}}$ from literature data were expected to possess a slight offset from the concept graph line as some systematic differences are inherent to the calculation process. However, even with this in mind, some data could not be closely fitted as can be seen in Fig. 1. An interval around full conversion of reactive gases was set in order to compensate for neglecting r(x). Subsequently, only data fitting within this interval were presented and retained in the final data set. The final data set (Supplementary Material 1) will be used for the modelling of CO$_2$-BMP and the multivariate data analysis of process variables.

**Fig. 3.** qCH$_4$ plotted against D for CO$_2$-BMP continuous cultures under different types of limitations. The $Y_{\text{co2/CH4}}$-range is indicated. (a) liquid-limited cultures at $Y_{\text{co2/CH4}} < 0.3 \, \text{C-mol mol}^{-1}$, (b) gas-limited cultures. For gas-limited cultures at different $Y_{\text{co2/CH4}}$ only selected steady states from [26,35] were used.

When analyzing the model proposed by Schill et al. during gas-limited CO$_2$-BMP, a linear relationship between qCH$_4$ and D is expected. This characteristic is associated to the autobiot catalytic reactions at a fixed $Y_{\text{co2/CH4}}$ and gas transfer rate (GTR). Essentially, a culture will reach different values for x at equilibrium with different applied D (i.e. x decreasing with an increasing D as a consequence of wash out) [12,32]. This phenomenon can be observed in Fig. 3, where data from *M. marburgensis* [11,12,26,35] can be dissected into two broad trends for qCH$_4$ as a function of D depending on the type of limitation affecting biomass growth.

Although simple linear relationships generally describe liquid substrate based bioprocess development [51], the specific product formation with respect to D in a gas- or liquid-limited bioprocess is different [12,32,36,52]. In Fig. 3, it can be seen that linear relationships cannot be trivially applied to CO$_2$-BMP processes and that this relation depends mostly on the limitation faced by r(x). It is well known that qCH$_4$ can...
vary greatly at a given GTR independent of D when a liquid-limitation or inhibition occurs [11,35]. However, under gas-limited conditions qCH4 is linearly dependent on D at a slope proportional to $Y_{(CH4)}$ [12,32,36]. This is also shown in Fig. 3.

The maintenance energy of a liquid-limited pure culture can be determined by plotting the specific product productivity as a function of D. However, maintenance energy is defined as the energy for metabolic functions not related to growth. Hence, for CO2-BMP it would correspond to the Gibbs free energy inherent to the material flow used neither for biomass or product formation. A $Y_{(CH4)}$ of zero would be experimentally required to allow quantification of the maintenance-related metabolism. Additionally, the kind of limitation (gas or liquid) or inhibition in Fig. 3 could be elucidated when analyzing qCH4 as a function of D. However, it is extremely challenging to assure that the examined cultures are solely gas- or liquid-limited, since not only proper biomass, vvm, CH4 of gas quantification, and subsequently analytics must be taken into account, but a priori knowledge about the setup of interest is also required.

3.2. Bioreactor setup – another limitation towards high MER

The correlation of the MER as a function of the volume dependent gassing rate, vvm, is another relationship that is often presented for analyzing CO2-BMP [12,25,26,42,53]. An analysis of MER as a function of vvm is shown for the data obtained from literature in Fig. 4.

In literature, linear relations were often shown for MER as a function of vvm [26,36]. However, this relation is not only strictly setup dependent, but also only holds true within a limited range of vvm increase and is furthermore known to impact the CH4 offgas. In a specific CO2-BMP setup with a specified GTR, a maximum gassing rate can be applied. At higher gassing rates, increases of MER will not occur. In continuously stirred tank reactors (CSTRs), this is caused by flooding of the stirrer [54]. Flooding of the stirrer describes the phenomenon that for a given agitation speed with increasing vvm a set-up specific gas to liquid mass transfer maximum (flooding point) will be reached. Beyond the flooding point additional gas supplied to the bioreactor will not anymore be able to be transferred to the liquid phase. The additionally supplied gas will cause the aggregation of bigger-sized gas bubbles that ascend around the stirrer axis and escape the bioreactor. Fig. 4 shows the comparison of the relation between vvm and MER in two different CSTR setups with deviating mixing systems and therefore different mixing efficiencies. While in one system (Fig. 4a), due to flooding of the stirrer, additional gassing did not contribute to an increase of the MER, the other setup showed a steady increase of MER over vvm as additionally provided gas could also be effectively transferred into the liquid phase (Fig. 4b). However, a trend towards a maximum reachable MER can also be seen here as the curve is flattening with an increasing gassing rate [26].

The gas residence time should also be taken into consideration since at higher vvm the contact time between gas bubbles and liquid is consequently reduced. The mix of these physicochemical limitations implies that for a given reactor setup (with a defined maximum GTR) a maximum MER exists at which a targeted CH4 ofgas quality can be consequently reached. This is because offgas quality is always affected by the interplay of GTR and the average residence time of the reacting gases if sufficient biocatalyst is available [8]. These mechanistic constraints need to be considered and reflected in the models of gas converting bioprocesses particularly in the case of CO2-BMP. Unfortunately, such trends were not described or translated within the existing models available [11,26,32,36] which are often restricted to a limited operational space and are unsuited for extrapolating knowledge for the purpose of process operation or scale up activities in different reactor setups.

3.3. PCA of CO2-BMP continuous culture data

PCA is a statistical tool used for multivariate data analysis and that can be utilized to identify correlations and loadings among process parameters and dependent variables [46,47]. In this section PCA was applied to three different data sets. The data sets used for the PCs are available in Supplementary Material 1. An overview of all process parameters and dependent variables that could be applied in the PCA is shown in Table 1.

The PCA for Fig. 5a comprised 172 independent continuous culture steady state conditions performed with different methanogenic strains. A total of six principal components (PCs) are necessary to explain 77.70% of the total variability (Supplementary Material 3), which renders the cluster challenging to interpret due to the numerous dimensions involved. When analyzing PC 1 and PC 2, only 44.77% of the total variability of the dataset can be explained. This denotes a strong multivariate nature of variables and parameters in the CO2-BMP processes, which can generally be extended to all gas converting bioprocesses. This is because gas converting bioprocesses are not only dependent on the kinetics of gas to liquid mass transfer but also on the physiology of the biocatalyst. In Fig. 5a, it is difficult to recognize a clear clustering of KPPs with respect to MER or qCH4. For a CO2-BMP process performed at the same temperature for a given reactor setup, MER should tendentiously cluster with vvm, pressure, and agitation. Alternatively, qCH4 should share a correlation with D or $Y_{(CH4)}$, as it was shown in Fig. 3.

Such constraints could not be observed in Fig. 5a. The only conclusion that can be drawn from the CO2-BMP process data reported in literature is that great data variability might occur from the different experimental approaches and setups used by the different authors. This
led to the intention of retrieving a more compact PCA analysis. To obtain a clustering between the KPP of CO2-BMP and MER and qCH4 (please also refer to Fig. 1, a dataset using only data from M. marburgensis was applied (Supplementary Material 1). The results of this analysis showing PC1 and PC2 in a bi-plot are presented in Fig. 5b. Therein, three correlating clusters can be identified. Cluster 1 is composed of factors 1, 10, and 11, and therefore represents a combination of MER, H2/CO2 gassing rate, and pressure. Cluster 2 is composed of factors 8 and 12, wherein r(x) and TE are correlating. Cluster 3 is composed of factors 4, 7, 9, 16, and 18, where all liquid relevant process factors such as ORP, DS, and D are clustering with the dependent physiological variables qCH4 and Y(x/CH4). Nevertheless, this case also needs six PCs to explain 78.44% of the total variability. Hence, a detailed analysis would imply examining all of the combinations and permutations of PC1 to PC6. However, this exercise poses a certain challenge for the interpretation of results in such a multi-dimensional space. When considering the contribution of communalities to the individual PCs, it turned out that gas related process parameters (vvm, agitation, pressure, MER, CH4 offgas) as along with D and TE contribute to the first two PCs.

Finally, a third PCA was performed. This dataset consisted of data from gas-limited M. marburgensis cultures only. The relationship of GTR related process factors, aforementioned KPPs, and variables are shown in Fig. 6. In this PCA only two PCs were necessary in order to explain 68.49% of the total variability (Supplementary Material 3). In Fig. 6, a clustering of factors 1, 10, and 11 can be identified. The results presented in Fig. 6 are supported by findings reported in literature for gas-limited conditions during CO2-BMP in continuous culture operations [26,36,52]. The results of the PCA presented in Fig. 6 suggest that a proper multivariate model of continuous culture CO2-BMP could be obtained by applying MLR fitting methods. However, the presented data clearly showed that best results are obtained when using data restricted to a defined physiological state. The more variable the underlying dataset was in terms of physiological states, different reactor setups, or various strains used, the more difficult it was to obtain PCA results that could be set in a logical context to what is found in literature.

### 3.4. Modelling of MER

To highlight the complex interdependencies between process variables and responses in CO2-BMP, multivariate models are presented for MER and qCH4. Based on the results of the PCA analyses and the high number of data available for M. marburgensis continuous culture experiments, the latter dataset was subsequently used for MLR. The results are shown in Supplementary Material 2.

MLR analysis lead to the following results: vvm, pH, temperature, agitation, pressure, MER, CH4 offgas as along with D and TE contribute to the first two PCs.

Table 1: Overview of process parameters and dependent variables partially used for PCA.

| # | Process parameter/variable |
|---|---------------------------|
| 1 | H2 CO2 in (vvm)            |
| 2 | Temperature               |
| 3 | pH                        |
| 4 | ORP                       |
| 5 | Agitation                 |
| 6 | Gassing ratio             |
| 7 | DS                        |
| 8 | TE                        |
| 9 | D                         |
| 10| Pressure                  |
| 11| MER                       |
| 12| r(x)                      |
| 13| x                         |
| 14| CH4 offgas                |
| 15| Y                        |
| 16| qCH4                      |
| 17| Y(x/CH4)                  |
| 18| Y(x/CH4)                  |

Fig. 5. Bi-plots obtained from correlation PCA of CO2-BMP showing PC1 and PC2. PC3 – PC6 are not shown. In 5a the bi-plot shows clustering of KPP and variables, which cannot be related to findings published on CO2-BMP to literature. In 5b the bi-plot illustrates clustering of KPP and variables according to literature published on CO2-BMP continuous culture.

Fig. 6. Bi-plot from PCA of CO2-BMP showing PC1 and PC2. Only two PCs could explain 68.49% of the total variance of the data.
was established from continuous culture CO₂-BMP results that were based on different bioreactor setups and geometries [11,12,26,35]. Furthermore, the data density towards higher MER values decreases. In Fig. 7a, the MER of an *M. marburgensis* continuous culture utilized for CO₂-BMP is shown as a function of vvm and pressure for gas-limited conditions. The significant ANOVA model of MER of *M. marburgensis* is shown in Supplementary Material 2.

While the overall positive influence of vvm and reactor pressure on the MER is correctly reflected by the model plot presented in Fig. 7a, the exact correlation between vvm and MER is obviously wrong. The presented model predicts an exponential increase of the MER with vvm, while the data collected during experiments with a certain setup (Fig. 4) showed the opposite trend, a flattening of the curve towards higher vvm. As previously explained, this is an inevitable consequence of the flooding phenomena occurring in CSTR reactors at a certain vvm. However, since data from several different CSTR setups with different individual flooding points were used as input for the MLR analysis, the correlation between vvm and MER is erroneously predicted. To overcome this problem, a sub-dataset, consisting of data collected with a single bioreactor setup, was used to perform a new MLR analysis. The outcome is shown in Fig. 7b. In this case, the experimentally determined correlation between vvm and MER is now properly reflected but therefore only valid in the design space. This shows that modelling of gas transfer, and therefore setup dependent variables like MER, should be performed for specific bioreactor setups while taking into account the underlying mechanisms of gas-liquid mass transfer as well as the residence time of reacting species. GTR mechanisms can, among others, be affected by reactor geometry, operation mode, working volume, broth rheology, agitation system, and sparging.

### 3.5. Modelling of qCH₄

Another MLR model was established for qCH₄ (Supplementary Material 2) that depicts a coefficient of determination of 65.1% ($r² = 0.6148$). Significant factors of the qCH₄ MLR model are vvm, pH, agitation, TE, D, and pressure. A graph for qCH₄ as a function of vvm
and D is shown in Fig. 8 and the ANOVA models are presented in Supplementary Material 2. Temperature, ORP, gassing ratio, and DS were not found to be significant.

The model equation for qCH₄ (Supplementary Material 2) shows that agitation also affected qCH₄ in several ways. Agitation increases the kLa, which influences r(x) and MER. It has been observed that increasing agitation had negative effects on r(x) [11]. These significant qCH₄ model terms in the equation can be explained by the multivariate nature of external influences affecting the physiology of methanogens, e.g., pH, ORP, temperature or pressure [14–16]. Due to the multivariate analysis of existing CO₂-BMP data, it becomes obvious that such influences would require the employment of sensitive analytical methods (e.g., TE analytics) for the liquid phase [55] and fine quantification of gas flow and composition [8] to be able to enhance the overall accuracy of process elemental balancing. This would enable the resolution of small variations of Y(CH₄/CO₂) as a function of input parameters and/or to compensate for the eventual lysis of biomass which would significantly affect r(x) determination and subsequent Y(CH₄/CO₂) calculation [8].

4. Discussion

The above-mentioned constraints clearly show, that for a gas converting bioprocess, such as CO₂-BMP, the two main kinetic determining limitations, gas- and liquid-limitation, need to be considered for modelling the overall process kinetics. A summary of possible issues, their interpretation and tasks that could occur during analysis and modelling of the CO₂-BMP process is given in Table 2. However, it has to be noted that it is the gas to liquid mass transfer that is limiting MER and not the physiological capacity of the methanogens [11,12,26,32,33,35,56].

In a CO₂-BMP bioprocess, the biomass acts as an autobiocatalyst and needs to be properly handled to exploit the full biocatalytic activity of the organism. Therefore, inhibitory or limiting liquid-based compounds would need to be quantified with sophisticated PAT and methods [12,49,55]. After biomass is grown in CO₂-BMP fed-batch cultures [48,50], the continuous culture CO₂-BMP process will enter a H₂-based gas limitation phase [26]. Therefore, the growth medium for methanogens is generally aimed to be non-liquid limiting, and eventually non-inhibitory, as one of the main goals of CO₂-BMP is to achieve maximum MER for subsequent bioprocess scale-up. Therefore, overfeeding of minerals is often applied to avoid such liquid-based limitations. An example of such a constraint is presented in Fig. 9.

MER of M. marburgensis from continuous culture experiments was plotted as a function of feeding ratio with a sulphide flow rate (Sₓᵦ) to r(x). It clearly shows, that the highest MER values were obtained at a Sₓᵦ/r(x) feeding ratio of either > 0.001 and < 0.017 which is close to the elementary composition found in the biomass of methanogens [57]. Higher feeding rates are thus not necessary. This could also be an indication that sulphide overfeeding was affecting the quantification of physiological responses shown for CO₂-BMP in literature. Such findings could also be because of a variation observed in physiologic responses that could explain why none of the models shown above are valid. The equilibrium of sulphide species in aqueous phase and their interaction with TE needs to be dissected as a function of the pH, temperature, vvm, and ORP [58]. However, only one attempt has been made to account for H₂S/HS⁻/S²⁻ equilibrium when performing elemental balancing in CO₂-BMP [56]. The negative effect on either MER or r(x) could not be precisely determined for DS even though modelling indicates the possibility that the latter parameter was negatively influencing MER (Supplementary Material 2). Recently, sulphide and TE interactions were determined during CO₂-BMP fed-batch bioprocessing. This was done to avoid physiologically unfavorable KPP settings [48]. Even so, this attempt did not fully dissect the complex sulphide and TE interactions in CO₂-BMP processes. During CO₂-BMP modelling, the gas transfer limitations are also of concern. As it was shown before, gas transfer related variables, such as MER or CH₄ offgas, were found to be strongly setup dependent [59]. Modelling across different reactor setups can consequently lead to erroneous results if the influences on the system are not properly characterized.

Without a combination of PAT and experimental approaches it is difficult to unscramble liquid and gas transfer related influences, which could easily lead to misinterpretation of process factor correlations [11,12,48,49]. Proper modelling for CO₂-BMP therefore requires prior detailed knowledge about both the bioreactor setup and the physiology of the applied strain. This, however, creates the need for analytical tools that allow for the balancing of individual compounds, particularly for carbon and hydrogen molar fluxes, to a very accurate level. The presented results show that models based on literature data often lead to erroneous predictions and conclusions.

Previous approaches for modelling CO₂-BMP neglected parameters such as the influence of liquid limitations on the performance of the
system, assuming the culture to be solely gas-limited [32]. While this approach can deliver valuable results, it is very limited in applicability, since the constraints that need to be made to keep the assumptions valid are narrow and are difficult to achieve. This is especially true as several studies have shown a strict separation of gas transfer limitations and liquid limitation. This interdependency adds a great deal of complexity to the modelling of any gas converting bioprocess and is particularly true for CO2-BMP.

5. Conclusions

This work shows the inherent challenges faced in modelling CO2-BMP. The most important aspect is the dependency of the performance on both, gas transfer limitations and liquid-based limitations. Utilizing PAT is inevitable in order to discriminate between these two factors. Implementing a real-time biomass sensor to correct the \( f_{\text{iner}} \) calculation method for the MER, where biomass formation is currently neglected, could result in an improved C- and DoR-balancing and would allow performing accurate and timely \( k_{\text{L}}A \) determinations. Literature data often misses important information and/or the required accuracy for resolution of the underlying mechanistic effects, especially when modelling reactor dependent variables. Modelling can only be based on a mechanistic understanding of a particular process. Otherwise, modelling misinterpretation might occur. Understanding the mechanistic effects of CO2-BMP could therefore assist the analysis and modelling of other gas-to-gas conversion bioprocesses.

Competing interests

AHS and SB declare to have competing interests.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apenergy.2018.01.075.

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