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Research article

Experimental workflow for developing a feed forward strategy to control biomass growth and exploit maximum specific methane productivity of *Methanothermobacter marburgensis* in a biological methane production process (BMPP)

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Abstract: Recently, interests for new biofuel generations allowing conversion of gaseous substrate(s) to gaseous product(s) arose for power to gas and waste to value applications. An example is biological methane production process (BMPP) with *Methanothermobacter marburgensis*. The latter, can convert carbon dioxide (CO_2) and hydrogen (H_2), having different origins and purities, to methane (CH_4), water and biomass. However, these gas converting bioprocesses are tendentiously gas limited processes and the specific methane productivity per biomass amount (qCH_4) tends to be low. Therefore, this contribution proposes a workflow for the development of a feed forward strategy to control biomass, growth (r_x) and q_{CH4} in a continuous gas limited BMPP. The proposed workflow starts with a design of experiment (DoE) to optimize media composition and search for a liquid based limitation to control selectively growth. From the DoE it came out that controlling biomass growth was possible independently of the dilution and gassing rate applied while not affecting methane evolution rates (MERs). This was done by shifting the process from a natural gas limited state to a controlled liquid limited growth. The latter allowed exploiting the maximum biocatalytic activity for methane formation of *Methanothermobacter marburgensis*. An increase of q_{CH4} from 42 to 129 $mmol_{CH4}\ g^{-1}\ h^{-1}$ was achieved by applying a liquid limitation compare with the reference state. Finally, a verification experiment was done to verify the feeding strategy transferability to a different

process configuration. This evidenced the ratio of the fed KH_2PO_4 to r_x ($R(F_{KH2PO4}/r_x)$) has an appropriate parameter for scaling feeds in a continuous gas limited BMPP. In the verification experiment CH_4 was produced in a single bioreactor step at a methane evolution rate (MER) of 132 $mmol_{CH4}*L^{-1}*h^{-1}$ at a CH_4 purity of 93 [Vol.%].

Keywords: design of experiments; chemostat; bioprocess quantification; carbon balance; biological methanogenesis; gas limited bioprocess; continuous bioprocess; power to gas; liquid limited growth; waste to value

Abbreviations

BMP	Biologic methane production
BMPP	Biologic methane production process
C-mol	Moles of carbon
P-mol	Moles of phosphate
CNH^{4+}	Ammonium concentration (mmol L^{-1})
Dbasal medium	Dilution rate of the basal medium (h^{-1})
DoE	Design of Experiments
DoR(-bal)	Degree of Reduction (balance)
C-bal	Carbon balance
MER	Methane Evolution Rate (mmol L^{-1} h^{-1})
MLR	Multiple Linear Regression
qCH4	Specific methane productivity (mmol g^{-1} h^{-1})
qCH4,max	Maximum specific methane productivity (mmol g^{-1} h^{-1})
rpm	Revolutions per minute
rx r(x)	Biomass production rate (C-mmol L^{-1} h^{-1})
TE	Concentration of trace elements in the basal medium
vvm	Volume of gas per reaction volume per minute (L L^{-1} min^{-1})
Y(x/CH4)	Growth to product yield (C-mol mol^{-1})
x	Biomass concentration (dry cell weight) (g L^{-1})

1. Introduction

In developed countries, interest towards modern technologies and efficient bio-energy conversion systems for the production of biofuels emerged with the aim of reducing the use of petroleum based fuels [1]. This growing interest is further sustained by the biofuels economy which is becoming competitive with fossil fuels [2,3]. Furthermore, a growing interest appeared over the past years for carbon capture and utilization processes by the mean of biologic systems [4–7]. However, biofuel is a broad term which includes several types of fuels such as bioethanol, biomethanol, biomethane, biodiesel, biogas, bio-syngas, or biohydrogen. In addition, for each of these biofuels different processing route exists [8–12]. Biofuel economy is expected to grow rapidly in the 21st century, bioprocesses aiming to use industrial, agricultural or municipal wastes following waste to a value concept will gain benefits in the long term [13,14]. Therefore, it emerges that an

important aspect for developing new bioprocessing routes resides in the substrates selection and their origins [15]. So, as a consequence of selecting sustainable and economically interesting substrates, an interest for gas based microbial processes aiming to upgrade, degrade or perform biologic remediation for industrial gas streams arose [16–20]. In this respect, biological methane production process (BMPP) proved to be a very promising 5th generation biofuel process [21] for upgrading gasses containing hydrogen (H_2) and carbon dioxide (CO_2) to methane (CH4), water (H_2O) and biomass. This bioprocess was already subject of an overall process efficiency simulation and proved to be efficient compare with existing methanation process [22].

BMPP was also well described in literature as a suitable process for different applications such as power to gas, biogas upgrade, and decentralized energy supply compatible with waste to value principles [23–27]. Recently, advances in genetics, biotechnology and bioprocess technology are enabling the emergence of new manufacturing concepts for converting renewable biomass to valuable fuels and products. This concept is generally referred as the biorefinery concept [28]. The integration of BMPP in order to improve the carbon impact and energy efficiency of a biorefinery was already reported in literature [21]. In addition, BMPP is an alternative to the well-known chemical methanation or more commonly called Sabatier process [26,27]. Hence, this bioprocessing route offers several advantages compare to the chemical process. The main advantages attributed to BMPP can be summarized by, a natural suitability for intermittent operations, milder process conditions in terms of operating pressures and temperatures, an intrinsic tolerance for pollutants contained in gaseous substrates and finally high volumetric conversion potential with a very stable reaction selectivity over time [21,22,31,32]. BMPP performed with *Methanothermobacter marburgensis* is a bioprocess performed under continuous operational modes facing tendentiously a mass transfer limitation of H_2 from the gas to the liquid phase. The quantification aspects of BMPPs are well described in existing literature [19,27,33–35].

In literature it is also explained, how gas limited continuous bioprocesses show a different behaviour compared with bioprocesses limited by a substrate supplemented with a liquid feed [36]. In fact, the dependency of product and biomass formation as function of the dilution rate is found to be significantly different [36]. The main differences with liquid limited bioprocesses can be summarized by a great stability for product formation rate as function of dilution rate [36]. On the other hand, an exponential increase of biomass concentration at decreased dilution rate is found until limitation or inhibition occurs. At low dilution rate in fact, biomass concentration tends to be very high because of a low wash-out [36]. Hence liquid substrates containing process nutrients need to be fed in a proportionally correct amount compare to biomass growth requirements, to maintain a stable catalytic activity. If a mineral or trace compound required in the multi-step enzymatic reactions is depleted, the enzymes activities are lost and methane formation stops [35]. Thus, rather than feeding more nutrients at reduced dilution rates to keep a gas limited state in the process, another approach might exist. This is the creation of a desired liquid limitation using a chemical compound not essentially required within the metabolic pathway for methane formation. This would allow decoupling methane formation and biomass growth as described in literature for similar microorganisms [37]. In addition, it allows controlling biomass growth within media development tasks where nutrients feeds need to be scaled based on growth attributes to sustain the biological system. Furthermore, gas limited bioprocesses are poorly exploiting the specific productivity allowed by biomass. Controlling biomass growth would allow to exploit also the maximum biocatalytic potential of methanogenic gas limited cultures by running the process close to $q_{CH4,max}$ values. In fact,

literature showed already that under liquid nickel depleted growth conditions localization of methane forming enzymes was affected [38]. However, this was never done before for gas limited continuous cultures in a state of the art bioprocess environment.

Different studies on substrates and nutrients depletion and their impact on the growth to product yield ($Y_{X/CH4}$) and methane evolution rate (MER) were already reported [33,34,35]. For example, the nitrogen source of the process was for example proven to vary $Y_{X/CH4}$. But it was also proved that at low ammonium concentrations (C_{NH4+}) a limitation hindering MER occurred [35]. This is explained by nitrogen being a main chemical element required in protein formation and therefore both for methane and biomass production. The possibility of controlling $Y_{X/CH4}$ was also described as a suitable strategy for improving carbon impact of the produced fuel in BMP systems [39]. However, it was never evaluated whether a defined substrate limitation could be used for controlling $Y_{X/CH4}$ while not affecting MER. In addition, theory anticipates risk for process stability at reduced dilution which induces high biomass concentrations due to long residence times. In fact, undesired limitations from compounds such as trace elements (TE) required for maintaining a stable biomass growth and MER are expected to occur if biomass growth is not controlled or feeds are not scaled for the specific growth attributes [36].

Therefore, the aim of this paper was to establish an experimental workflow consisting of a multivariate design of experiment (DoE) to optimize basal medium composition. The development strategy aimed at determining if the maximum specific methane productivity of *methanothermobacter marburgensis* in a continuous and stable bioprocess operation can be exploited by applying a defined liquid based limitation. This was done by investigating up to 20 times lower feeding rates of P, K, Na and Cl compare to referenced process operating conditions [24,32,33]. The experimental strategy revealed finally that a selective control of growth rate was possible while not impacting MER. Finally, it will also be evidenced by the verification strategy how to transfer, based on the ratio of fed KH_2PO_4 to r_x ($R(F_{KH2PO4}/r_x)$) given in [P-mol C-mol^{-1}]; a stable gas limited physiologic state to a BMPP performed with different gassing and dilution rates.

The presented experimental workflow allowed to reach a stable MER of 132 $mmol_{CH4}$ L^{-1} h^{-1} (3 $L_{n,CH4}$ L^{-1} h^{-1}) under a continuous and gas limited operational state with a CH_4 content in the wet offgas of 93 vol.% within a single passage of the reacting gases.

2. Materials and Method

2.1. BMPP setup and experimental description

The microorganism used in all the experiments presented in this work, is a hydrogenotrophic, thermophilic and methanogenic archaeon, strain *Methanothermobacter marburgensis* DSM 2133 [40–44]. The quality of reagent gasses, culture storage, cultivation conditions, analytics, medium composition, peripheral analytical instruments, monitoring, control and cultivation of *M. marburgensis* are described elsewhere [25,33]. All bioreactions were performed in a 10 L Biostat C+ laboratory reactor (Sartorius Stedim Biotech AG, Göttingen, Germany). The peripheral components of the bioreactors are described in existing literature [25,33,35]. The 10L bioreactor was controlled gravimetrically and the harvest was collected within the reaction vessel. All experiments were performed at 2 [bar] pressure. The dilution rate of the basal medium ($D_{basal\ medium}$) was controlled with a gravimetric flow controller to be constant for all experiments included in the DoE at

$D_{basal\ medium}$ = 0.05 [h^{-1}] and was defined as the reference dilution rate of the basal medium $D_{basal\ medium}$ = 0.05 [h^{-1}] = D_{ref}. The gassing rate applied to all experiment was G_{in} = 0.5 volume of gas per reaction volume per minute [L L^{-1} min^{-1}] (vvm) with an inlet ratio for H_2/CO_2 = 4 if not specified differently and was defined as the reference gassing rate G_{in} = 0.5 vvm = G_{ref}.

In the bioreactor the following parameters were maintained constant for each experiment:

- Agitation was set at 1500 rpm
- pH was set at pH=7
- Temperature was controlled at T=65 [°C]
- Working volume was maintained at V_w = 5 [L] if not specified differently

TE were maintained constant at 6X and are known to not be limiting for the growth rate (r_x) range reached at the given process conditions [25,35]. The only modifications compare to the reported feeding strategy in literature was the replacement of base by a NH_3 solution diluted to 1.5 [mol L^{-1}] used both for pH compensation and nitrogen source.

All process quantifications were carried out in bioreactor conditions, which fulfilled internally established steady-state prerequisites defined as follows:

- All culture parameters, inlet gassing rate and composition, offgas rates as well as concentrations had to be stable for a minimum of two complete bioreactor volume exchanges.
- Steady states were only assumed after a minimum of 5 bioreactor volume exchanges after a parameter change.

Mean elementary compositions, as well as ash content were used for the calculation of degree of reduction balance (DoR-bal) and carbon balance (C-bal) [35].

2.2. Establishment of the Design of Experiment (DoE)

The software MODDE 9.0 (UMETRICS, Umea, Sweden) was used to generate a bi-variate 3 level optimization CCF design. The choices for the design space are based on preliminary knowledge and described step wisely in the following section. The basal medium recipe was modified from the existing one to contain KH_2PO_4, NaCl and TE only. TE concentration was maintained constant in every experiment. $NaHCO_3$ was removed from the basal medium recipe as carbonate is supplemented by CO_2 gassing. Basal medium pH was adjusted with concentrated NaOH to the fermentation pH (pH = 7) [33]. The reference medium composition available in literature was set as the up-up edge of the design space. In the different experiment, the concentration of KH_2PO_4 and NaCl in basal medium was adjusted in order to provide at the fix dilution rate (D_{ref}) varying specific elemental feed rates in the bioreactor. For the low-low corner of the DoE a 1:20 reduction of KH_2PO_4 and NaCl concentrations in the basal medium was determined by a pre-screening dynamic experiment. This was however not reported in this paper as the only purpose was to set the DoE boundaries.

For the generation of the centre points a logarithmic transform was applied to the input parameters. This allowed placing centre point experiments at the logarithmic mean rather than at the arithmetic mean. This was done in order to enhance the resolution in the region of low feeding rates (region of interest) while not affecting the orthogonality of the design space. A bi-variate 3 level optimization composite face centred (CCF) design was selected. This allows also the resolution of interaction and quadratic terms in the model. The following two controlled factors were selected as input parameters: C_{KH2PO4} and C_{NaCl} in [g L^{-1}].

Because of the fixed D_{ref} applied to all DoE experiments the feed rate of each individual compounds could be calculated with: $F_i = C_i\ D_{ref}\ \ Mm_i^{-1}$ given in [mmol L^{-1} h^{-1}]. The worksheet generate originally by the software is accessible in supplementary material: original DoE worksheet.

2.3. In silico analysis

DoE was used to carry out a multivariate optimization of the basal medium composition. MODDE 9.0 (UMETRICS, Umea, Sweden) was also utilized for the subsequent data analysis and models generation. The following responses were chosen for the DoE because they reflect process performance and physiology:

- Biomass concentration (x) [g L^{-1}]
- Methane evolution rate (MER) [mmol L^{-1} h^{-1}]
- Specific methane evolution rate (qCH4) [mmol g^{-1} h^{-1}]
- Volumetric biomass production rate (r_x) [C-mmol L^{-1} h^{-1}]
- Growth to product yield Y(x/CH4) [C-mol mol^{-1}].

As a gage of quality for bioprocess quantification DoR-bal and C-bal are given in the result table. Multiple linear regression (MLR) was used for fitting each individual response with the logarithmic transform of the selected input parameters to generate an individual model for each of the selected responses.

3. Results

3.1. DoE result table, model generation and contour plot discussion

The main goal was controlling the catalytic activity of a gas limited biologic culture by applying a defined and controllable liquid limitation. For evaluating the possibility of limiting biomass growth while not impacting MER, a set of 13 *M. marburgensis* cultures reaching steady-state conditions was performed. The two parameters investigated were F_{NaCl} [mmol L^{-1} h^{-1}] and F_{KH2PO4} [mmol L^{-1} h^{-1}] according to the DoE worksheet which elaboration was discussed in section 2.3: generation of the DoE worksheet. The final DoE results sheet obtained after experimentation is shown in Table 1 underneath. Two additional experiments where included compare to the original worksheet elaborated by the software attached in the supplementary material: original DoE worksheet for enhancing model quality and resolution over the entire design space.

Table 1. CCF-DoE result table for each experiments showing selected input parameters (blue columns), responses (green columns), process quality parameters (orange columns).

Experiment	Run Order	Feed KH_2PO_4 [mmol/L*h]	Feed NaCl [mmol/L*h]	MER [mmol/L*h]	qCH4 [mmol/g*h]	r(x) [C-mmol/L*h]	$Y_{X/CH4}$ [C-mol/C-mol]	X [g/L]	C_{NH4+} [mmol/L]	DoR-balance	C-balance
1	1	0.12	0.10	242.9	124.6	3.9	0.016	1.95	27.6	98%	101%
2	10	2.50	0.10	253.2	48.1	10.6	0.042	5.26	69.6	98%	101%
3	7	0.12	1.97	248.2	129.3	3.9	0.016	1.92	25.7	101%	104%
4	5	1.84	1.97	244.7	41.3	12.8	0.053	5.92	85.4	101%	104%
5	12	0.12	0.44	234.1	127.9	3.8	0.016	1.83	38.4	101%	104%
6	11	2.50	0.44	253.6	46.4	11.8	0.046	5.46	69.0	101%	102%
7	9	0.56	0.10	254.6	55.2	9.7	0.038	4.61	54.8	99%	103%
8	8	0.56	1.97	254.1	53.7	9.9	0.039	4.73	53.7	101%	104%
9	3	0.56	0.44	246.9	46.7	12.1	0.049	5.29	56.7	101%	104%
10	13	0.56	0.44	242.0	46.0	11.0	0.045	5.26	75.7	101%	103%
11	4	0.56	0.44	247.7	44.7	11.5	0.046	5.54	68.6	101%	104%
12	2	0.12	0.10	242.1	115.3	4.2	0.017	2.10	26.3	98%	103%
13	6	2.50	1.97	241.4	42.0	12.5	0.052	5.75	91.3	98%	103%

From result Table 1 it can be seen that it was possible to keep MER constant while reducing by a factor 20 the fed NaCl and KH_2PO_4 to the bioreactor. It appears also that r_x varied while having no change in gas transfer and dilution rates. This gives a first valuable hint for a shift of the bioprocess to a liquid limited state [35,36]. The constant MER at different r_x in fact is finally reflected in the broad range of biocatalytic activities at which *Methanothermobacter marburgensis* cultures have been performing with a q_{CH4} ranging from 42 up to 129 [mmol g^{-1} h^{-1}]. The variations of $Y_{X/CH4}$ [C-mol C-mol^{-1}] are not attributed to the varying C_{NH4+} [mol L^{-1}] as the latter was never found in this range to be limiting nor inhibiting according to literature [34,35]. A summary of all factors and dependent variables is shown in Table 1. MLR was applied for data fitting and model generation for each of the selected response as function of the logarithmic transform of input parameters. Regression coefficients, model validity and standard errors are accessible in the supplementary material: ANOVA table for MLR model. The signal to noise ratios were exceeding threshold levels for each of the models and the freedom degrees of residuals were found comfortably high. Altogether this indicates a reliable model generation except for MER as shown in the supplementary material: model validity. The reason for the latter poor model validity is explained more in detail in the discussion of response contour plot A.

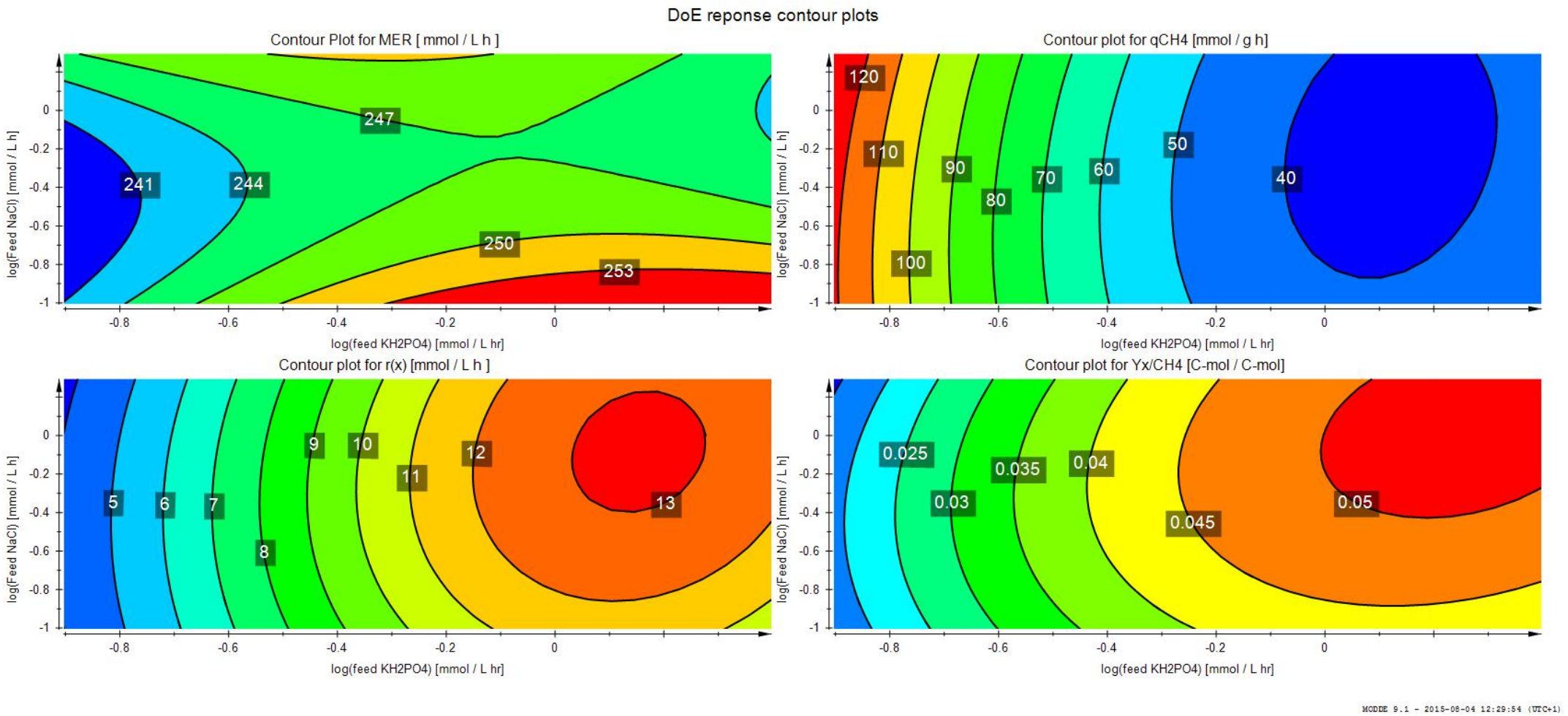

Figure 1. Reponses contour plots: (A—up left) methane evolution rate (MER values in [mmol L^{-1} h^{-1}]), (B—up right) specific methane productivity (q_{CH4} values in [mmol g^{-1} h^{-1}]), (C—down left) biomass growth rate ($r_x = r(x)$ values in [mmol L^{-1} h^{-1}] and (D—down right) biomass growth to product yield ($Y_{X/CH4}$ values in [C-mol C-mol^{-1}]) obtained by MLR for each individual selected response with two input variables (F_{NaCl} [mmol L^{-1} h^{-1}] and F_{KH2PO4} [mmol L^{-1} h^{-1}]). X and Y axis unit correspond respectively to the logarithmic transform of F_{KH2PO4} and F_{NaCl}.

3.1.1. Response contour plot A: MER = f(F_{KH2PO4}, F_{NaCl})

Response contour plot A shows that MER remained constant over the entire the design space. However, a poor model validity (supplementary material: ANOVA table for MLR model) was found and is explained by the fact that no statistically valid model could be generated with the small range of MER variations observed in this DoE space. It can be seen that the standard deviation reported on the mean MER value for all experiments is below 2.5%. So with a confidence interval $\alpha = 0.05$ applied to the MLR fitting a statistically valid model could not be obtained for MER though a good correlation coefficients was achieved. Thus, it can be stated that MER was constant in this DoE space with an $MER_{average}$ =246 ± 6 [mmol L^{-1} h^{-1}]. Therefore, it was here proven that process productivity (MER) could be maintained constant while applying up to 95% reduction for the fed NaCl and KH_2PO_4 in this BMPP configuration. A 95% reduction in fed NaCl and KH_2PO_4 gives an idea of the overfeeding applied if no rational media and feed design strategy is applied in the development phases of gas limited bioprocesses.

3.1.2. Response contour plot B: q_{CH4} = f(F_{KH2PO4}, F_{NaCl})

The second model describes how q_{CH4} varies as a function of DoE parameters. It can be seen in response contour plot B that q_{CH4} varied from 42 up to 129 [mmol g^{-1} h^{-1}]. This three-fold increase proofs the possibility of enhancing the process specific productivity also for gas limited bioprocesses by applying a defined feed strategy. It can clearly be seen looking to B that the parameter influencing mostly q_{CH4} was F_{KH2PO4}. On the other hand, F_{NaCl} shows an almost insignificant influence. The possibility of controlling the specific productivity of a given microorganism in a bioprocess is comparable to the control of the turnover rate of a solid catalytic particle. In this DoE it was observed that the catalytic "particle" here being a gram of microorganisms dry cell weight can turn over up to three times more substrates to product when the full catalytic activity is exploited. This means that it is possible to grow up to three times less cells while not affecting process performance. This also means, a potential three-fold decrease for nutrients feed compare to the uncontrolled reference gas limited state and evidence how tendentiously a gas limited bioprocess poorly exploit its full catalytic potential if no specific control strategy is applied.

3.1.3. Response contour plot C: r_x = f(F_{KH2PO4}, F_{NaCl})

On this plot, the specific variation of r_x can be seen for different feed rates of KH_2PO_4 and NaCl. It can be denoted that low values for F_{KH2PO4} implied a significant reduction of r_x inversely proportional to the increase observed previously in response contour plot B for q_{CH4}. The latter can be explained by the constant MER and the varying r_x. Biomass concentration is proportional to r_x in a continuous bioprocess with a fix dilution rate, therefore, it was expected that q_{CH4} increased with decreased values of r_x as being the ratio between MER and biomass concentration. However, when looking further to response contour plot C, some influences appeared to be a function of F_{NaCl}. It seems that a local optimum exists. This phenomenon could be explained by pushing further the understanding of the process with a more microbiologically oriented approach. In fact, NaCl is a salt not necessarily referred primarily to as a substrate. However, its concentration affects significantly

the osmolality observed in the bioprocess and so physiology of most living organisms. Therefore further investigation could be required to fully picture the influence of NaCl (and indirectly of osmolality) on process performance and growth attributes of *Methanothermobacter marburgensis*. Different osmolality might trigger metabolic changes and the microorganism would need to adapt to this varying environment according to literature [45,46,47]. The modification of intracellular pathways resulting from changing osmolality might then result in a modification of r_x and therefore an optimum for biomass growth might exist with respect to the process F_{NaCl} and indirectly of osmolality.

3.1.4. Response contour plot D: $Y_{x/CH4} = f(F_{KH2PO4}, F_{NaCl})$

Finally, response contour plot D shows the variation of $Y_{X/CH4}$ [C-mol C-mol^{-1}] which reflects the reaction selectivity for the carbon transformation [24,27]. For the first time, a tendentiously gas limited BMPP faced a growth limitation which did not impact MER. In fact, KH_2PO_4 was found to be suitable, as first approximation, to affect only growth and to not be deleterious in case of limitations for methane formation. The limitations on KH_2PO_4 allowed the uncoupling of growth and CH_4 production which was reflected in lower r_x for constant MERs. The trends observed in D is not surprisingly similar to the one observed in response contour plot C as $Y_{X/CH4}$ is the ratio between the varying growths rate observed in the design space with the constant MER values.

3.2. Parameter selection for feed strategy transfer

The following section focus on the possibility to control q_{CH4} through a selective reduction of r_x without impacting methane productivity. In addition, it was searched for a scalable feed related parameter to allow for transfer of the developed feeding strategy to process performed under different conditions. Therefore, q_{CH4} values from the DoE were plotted as function of the ratio of F_{KH2PO4} to r_x, $R(F_{KH2PO4}/r_x)$ given in [P-mol C-mol^{-1}]. As it can be seen on the following Figure 2 q_{CH4} increased significantly by reducing the F_{KH2PO4} for a given growth attribute while MER was never lowered at the given G_{ref}, D_{ref} and process conditions described previously.

Out of this graph, it emerged that variations in F_{KH2PO4} significantly influenced growth while not impacting MER. Because bioprocess technology seeks scalable parameters the ratio of F_{KH2PO4} to r_x ($R(F_{KH2PO4}/r_x)$) was selected for scaling a feeding strategy based on r_x. In gas limited state the latter is depending mostly on dilution and gas transfer rate. If $R(F_{KH2PO4}/r_x)$ is below 0.05 then a liquid limitation is expected to occur. Whereas a gas limited state is observed for values of $R(F_{KH2PO4}/r_x)$ higher than 0.15. The elementary composition anticipated the lowest feed to apply as the ratio of phosphate in biomass is found to 0.021 [P-mol C-mol^{-1}] [32,34]. Obviously this is only valid if other limitations and inhibitions of growth are avoided. On the other hand, transiently a lower ratio than 0.021 can also be applied if reducing the biomass concentration is desired. Nevertheless, this ratio was found as suitable for transferring a feeding strategy to other conditions in terms of dilution and gassing rate as not influencing methane formation but only limiting growth.

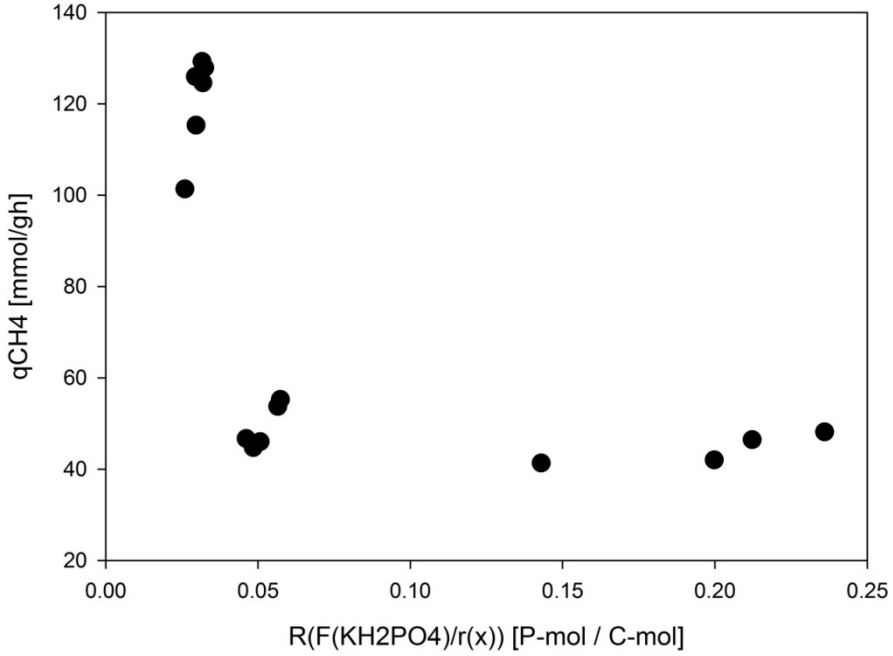

Figure 2. Specific methane production (q_{CH4}) given in [mmol g^{-1} h^{-1}] from the DoE experiments as a function of the ratio of F_{KH2PO4} to r_x, $R(F_{KH2PO4}/r_x)$ given in [P-mol C-mol^{-1}].

3.3. Verification experiment for feed transfer and high quality methane production

As introduced in the previous section, $R(F_{KH2PO4}/r_x)$ is a suitable parameter for scaling and transferring the feeding strategy of BMPPs. It allows based on growth to scale the feeding strategy independently of dilution and gassing applied to the process. In fact, to increase methane offgas content, gassing need to be reduced for a given reactor setup. Furthermore, this ratio could be used for controlling the desired physiologic state in a BMPP by applying a feed forward approach limiting selectively the microorganism growth. This shift of metabolism was identified in the DoE with the parameters $Y_{X/CH4}$ which under gas limited condition is expected to be close to 0.05 as literature explains for the given process conditions [33,35,48].

In this last section, a verification run is shown where the gas limited state of the process wanted to be maintained while reducing dilution and gassing rate applied to the process by 50% in order to produce high quality methane. Hence, in this experiment G_{in} and D_{in} were reduced by 50% compare to the reference values while keeping constant the feeding ratio $R(F_{KH2PO4}/r_x)$ to a value close to 0.2 [P-mol C-mol^{-1}]. Further, this experiment wanted also to show that high quality methane at reasonable rates can be produced in a single bioreactor step with CO_2 and H_2 as gaseous substrates and with only mineral elements as nutrients. The working volume was increased to 10 [L] and all other process parameters were scaled according to the volume modification. The results for the verification experiment and the reference experiment (DoE experiment 13) are given in Table 2. It can be seen that $R(F_{KH2PO4}/r_x)$ was kept constant compare to the reference experiment.

Table 2. Verification run with for CH_4 production at 93 [Vol.%] wet gas in a single reactor step at an MER of 131.9 [mmol L^{-1} h^{-1}].

	Reference DoE experiment n°13	**Verification experiment 50% reduction of G_{ref} and D_{ref}**
MER [mmol/Lh]	241.4	131.9
X [g/L]	5.7	5.2
qCH4 [mmol/gh]	42.0	25.6
r(x) [C-mmol/Lh]	12.5	5.4
YX/CH4 [C-mol/C-mol]	0.052	0.041
C NH4+ [mmol/L]	91.3	25.8
Dout [h-1]	0.067	0.033
CH4 [Vol.%]	64.0	93.0
R(FKH2PO4/r(X)) [P-mol/C-mol]	0.20	0.23
DoR-balance	101%	102%
C-balance	102%	103%

In this experiment it can be seen also that the gas limited state of the process was maintained. Though G_{in} and D_{in} were reduced by 50% the same concentration of biomass is found in the bioreactor at steady-state which aligns with prediction obtained with theoretical models [36]. This gas limited side is also reflected by the lowered q_{CH4} values. When looking to $Y_{X/CH4}$ it can be seen that this one is slightly lower compared with the reference state. However, this is not surprising according to results where the dependency of $Y_{X/CH4}$ on dilution rate was already reported [21,24]. The following graph (see Figure 3) represent the values obtained for H_2, CO_2, CH_4 [Vol.%] contained in the offgas as well as MER and gassing rate entering the reactor (G_{in}) at fixed H_2/CO_2 ratio of 4:1. The 100 hours signal record started once operation steady-state was assumed. Small fluctuation originates from sampling.

Out of the presented result graphs it was shown that by setting $R(F_{KH2PO4}/r_x)$ to a constant value of 0.2, it was possible to maintain the gas limited state while different process conditions where applied to the BMPP. In BMPP, r_x can be easily estimated knowing hydrogen transfer rate and dilution rate applied to the process through $Y_{x/CH4}$. Furthermore, it can be seen that if the natural gas limited state of a process is maintained, a poor exploitation of the catalytic activity of biomass is found. Therefore, this altogether suggests that natural gas limited states might not be the most optimized operating condition for running a BMPP. However, how far will be exploited the catalytic potential in an industrial BMPP application will need its own evaluation. Industrial process operations might prefer to guarantee process stability rather than minimizing slightly feeding costs. On the other hand, controlled growth is probably preferred for process development work such as media development or evaluation of process economic potential.

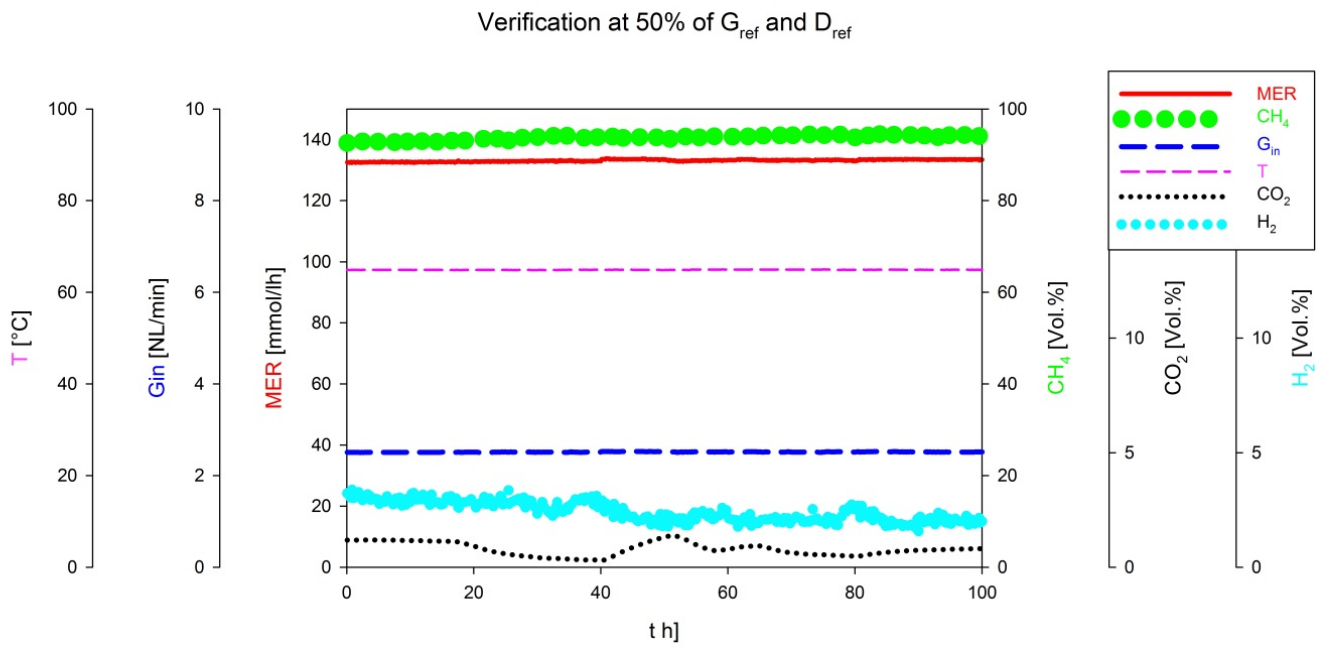

Figure 3. Signals obtained in the verification experiment. G_{in} [NL/min] (blue), CH_4 [Vol.%] (green), CO_2 [Vol.%] (black), H_2 [Vol.%] (cyan), MER [mmol*$L^{-1}h^{-1}$] (red).

4. Conclusion

This paper presented an experimental workflow consisting of an optimization DoE and a verification experiment for developing a scalable strategy for feeding gas limited bioprocess and exploiting their maximum biocatalytic activity.

The methodology consisted of adapting an optimization DoE by applying a logarithmic transform to the selected input parameters. This allowed having DoE centre points located at the logarithmic mean rather than at the arithmetic mean while keeping orthogonality of the design space for fitting the model by MLRs. This method could be applied both in up or low corners of a DoE space while allowing a greater resolution of the selected responses in the desired plane section of the design space.

In the DoE it was revealed also that the feed of KH_2PO_4 was mostly responsible for the growth rate reduction observed, while not impacting MER. This sustains under the view of DoR-balance and C-balance the possibility to decouple biomass growth and methane production in BMPPs.

Furthermore, it was possible to show that defined KH_2PO_4 feed could be applied for reducing growth rate selectively. This allows running the process exploiting the maximum specific methane productivity offered by the given microorganisms without impacting volumetric productivity or quality by shifting the process to a controlled liquid limited growth.

In addition, controlling growth in bioprocesses is a prior requirement for media development activities aiming to scale multiple nutrient feeds to sustain a defined growth. Hence, this method is also proposed for developing rational feeding strategies based on real biologic requirements in BMPPs.

Finally, the parameter $R(F_{KH2PO4}/r_x)$ was selected for scaling and transferring the feed strategy of this BMPP. A gas limited state was targeted, so the parameter $R(F_{KH2PO4}/r_x)$ was fixed to a value of

0.2 according to the reference experiment while applying a 50% reduction of dilution and gassing rates.

The successful extrapolation of the feeding strategy to a different process operation allowed stable production of CH_4 at a purity of 93 [Vol.%] wet gas in a single bioreactor step at a MER of 132 [mmol L^{-1} h^{-1}] (or 3 $L_{n,CH4}$ L^{-1} h^{-1}) while maintaining a desired gas limited state in the biologic system.

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Conflict of Interest

All authors disclose to have no conflict of interests.

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