**FY 2016: Q3 Report (April 1 – June 30, 2016) – Team Michigan**

**Reducing Emissions Using Methanotrophic Organisms For Transportation Energy (REMOTE)**

**Anaerobic Bioconversion Of Methane To Methanol**

**A.    Executive summary**:

Our REMOTE project aims to develop transformational technologies for bioconversion of methane to liquid fuels. We plan to engineer the genetically tractable methanogen, *Methanococcus maripaludis*, with genes that encode the conversion of methane to methanol. Because this process is thermodynamically unfavorable, we also will include the genes encoding sulfate or nitrate reduction.

Enlisting researchers from a government laboratory, a research institute, and two universities, the project involves a team of four investigators with complementary expertise and the skills needed to successfully execute the project plan: John Leigh (Univ. Washington), Nathan Price (Institute for Systems Biology), Stephen Ragsdale (Univ. Michigan) and Simone Raugei (PNNL). Nadine Wong in our technology transfer office is our T2M liason.Briefly, our scientific specific aims are:

1. To actively express the gene clusters encoding the MCRs from *M. marburgensis* and from the anaerobic methane oxidizers, ANME-1 and ANME-2, in a genetically tractable methanogen; to genetically engineer the pathways for converting methane to methanol and to couple this pathway to an appropriate reduction path, which makes this process thermodynamically favorable.
2. To use genome-scale metabolic flux modeling of the genetically engineered *M. maripaludis* strains, thus, ensuring that enzymes in the engineered pathway can operate at high efficiency in the new organism. We will also reengineer other aspects of *M. maripaludis* metabolism by performing genetic alterations to optimize the methanol pathway in its new metabolic context and perform metabolomic and metabolic flux experiments to iteratively test, refine, and validate the metabolic model.
3. To purify the newly introduced enzymes from *M. maripaludis* and conduct in vitro biochemical studies to test their functionality. We will also conduct pathway flux measurements and efficiency determinations aimed at optimizing methane oxidation to liquid fuels, and
4. To use computational studies to understand the mechanism of the anaerobic methane oxidizing enzyme, MCR, and to predict the effects of site-directed mutagenesis on the reaction mechanism.

We have made progress on each of these aims and all scientific aims are on track to timely completion. Below, I have outlined challenges and risks associated with the work and identified alternative strategies should any of the current plans fail. Although the major aims (above) remain intact, I proposed some changes in approach that should facilitate accomplishing the GTL goals. We also are on budget.

**Aim 1:** To date, our expression of heterologous proteins in M. maripaludis has been too low to achieve our goals with respect to metabolic flux. Therefore we have turned our attention to measures that should substantially increase expression levels. The results presented in this update suggest that we should be able to increase expression by using a promoter from the gene for the highly abundant S-layer protein rather than the histone promoter. Results also suggest that we should continue to use our replicative vector rather than integrate constructs into the chromosome. To improve protein stability, we have deleted the proteasome and added a copy of the chaperonin, and the effects of these measures will be tested. In addition, although not part of the milestones, we have been able to replace the native M. maripaludis McrA gene with a “codon-optimized” gene that contains a different DNA sequence but encodes the same protein sequence. This demonstrates one approach we might use to express a variant of Mcr that could have more favorable kinetic properties. This approach would depend on the enzyme remaining functional for normal methanogenesis.

**Aim 2:** Our completed genome scale flux balance model of *M. maripaludis* has already reached its accuracy targets for predicting gene knockout lethality and growth yields over a range of methane secretion rates. Moreover, we have previously reported that adding a ferredoxin:hydrogen oxidoreductase allows us to predict stoichiometrically feasible conversion of methane to methanol. The remaining challenge is to use our model to predict energetically feasible methane oxidation via the addition of reduction pathways. In the past quarter, we addressed this challenge by simulating methane oxidation in concert with three different reduction pathways commonly found in nature: sulfate reduction to sulfide, nitrate reduction to nitrite, and ferric ion reduction to ferrous ions. Based upon these simulations, we predicted only nitrate reduction to be both stoichiometrically and energetically feasible for coupling to methane oxidation. Our model suggests that at standard 1 mM metabolite effective concentrations, 0.66 mol NO3 reduced/mol CH4 oxidized would be sufficient to drive our desired conversion.

**Aim 4:** With respect to Aim 4, we have continued our investigation on residues at the active site that potentially modulate the catalytic activity of methanogenic MCR and started the study of the methanotropic ANME-1 MCR. Specifically, we performed an extensive calculation of the binding free energy of CoBSH and CoBS- using force field-based molecular dynamics simulations and we developed a force field to describe the F430 cofactor and non-standard aminoacidic residues of ANME-1 MCR. At the same time, we performed quantum mechanical calculations of the energetics of the initial steps of methane formation on a model of ANME-1 MCR active site similar to that used previously for *Maripaludis* MCR. This study indicates that the chemical differences of F430 are not responsible for the different reactivity of ANME-1 MCR and *Maripaludis* MCR as reaction free energies for breaking the S-CH3 bond of CoM and the subsequent formation of CoB-SS-CoM are, within our computational error, identical. We are currently exploring if and how the cysteine-rich patch between F430 and the surface of the protein can facilitate the reduction of ANME-1 MCR from the inactive Ni2+ form to the catalytic competent Ni+ oxidation state.

**Aim 5:** Prepare a process feasibility analysis for the anaerobic bioconversion of methane to methanol.

**B.    Status of milestones due in the current quarter (Q1FY16) and status of any overdue milestones**. I included all milestones that being currently worked on.

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| **WBS** | **Due Date** | **Status** | **Summary** |
| M1.3 | 4/31/2016 | 100% complete | **Determine best promoter for expression of heterologous proteins. Compare constructs where lacZ is driven by the hmv promoter, the mcr promoter, and the S-layer promoter**. We found that the S-layer promoter improves expression over the hmv promoter by about 75%. We did not test the mcr promoter due to technical complications. |
| M1.6 | 5/31/2016 | 100% complete | **Complete construction of a host strain for enhanced protein stability and solubility. Complete knockout of proteasome-associated factors and overexpression of the chaperonin.** |
| M1.4 | 8/1/16 | ?% complete | **Determine the best site in M. maripaludis for protein expression. Construct his-tagged M. maripaludis McrA, his-tagged M. acetivorans MtaBC, or lacZ in the replicative vector and integrated into three sites in the genome. Compare expression levels**. MtaA expressed on the replicative plasmid was more abundant than from a site in the chromosome. We are still constructing a strain where MtaA will be added to an existing operon of genes encoding abundant proteins. |
| M1.5 | 6/1/15 | ?% complete | **Achieve mRNA levels similar to native Mcr** |
| M1.8 | 8/31/16 | ?% complete | **Demonstrate inducible expression by IPTG, tetracycline, nitrogen starvation, or phosphate starvation.** |
| M2.1 | 12/1/14 | 100% complete | **Build draft genome scale flux balance and core metabolic flux models for *M. maripaludis* metabolism:** **Deliver first genome-scale metabolic model capable of simulating growth and byproduct section with >75% accuracy.** |
| M2.3 | 6/1/15 | 100% complete | **Deliver improved model that enables predictions with >85% predictive accuracy for knockout lethality, and <20% error for wildtype growth and byproduct yield predictions.** |
| M2.4 | 10/1/15 | 80% complete | **Deliver top 5-10 strain design predictions based on methanol yield to team for implementation.** After testing multiple possible electron sinks, simulations with our model have suggested that nitrate reduction to nitrite could feasibly couple to methane oxidation, provided that a ferredoxin:hydrogen oxidoreductase is also added to the organism. Other reduction pathways commonly found in methanotrophic consortia were predicted as infeasible, thus our next step will be to push beyond these previously observed pathways to predict additional feasible strain designs. |
| M3.3 | 12/1/15 | 50% complete | **Deliver an MeTr with an activity of >10 units/mg for methanol production from methyl-SCoM**: Determine kinetic and physical properties of the heterologously expressed methyl-SCoM:methanol MeTr in the forward & reverse directions. We have measured expression and activity and aim to increase expression by 10-fold. |
| M4.2 | 10/1/15 | 95% Complete | **Identify at least 10 amino acids modulating substrate binding.** We have identified the most enzyme residues that mostly contribute to the binding of CoBSH. Free energy calculations are underway to estimate the relative contribution of each residue to substrates binding both in metahnogenic and methanotropic MCR. |
| M4.3 |  | 90% Complete | **Validate activation energies in MCR pathways using experimental data from Ragsdale’s lab.** We have extended the QM calculations of the breaking of the S-CH3 bond and the formation of CH4 to ANME-1 MCR. The results show that the chemical differences of F430 between enzymes are likely not responsible for the different reactivity of the MCRs of ANME-1 and *M. maripaludis or M. marburgensis*. |
| M5.7 | 2/1/15 | 80% Complete | **Develop a pitch deck.** Ppt presentation tailored to use in meetings with potential partners, funders, etc. Slides will focus more on the market/commercial aspect rather than the technical. This would include market size, value proposition etc. |
| M5.8. | 2/1/15 | 40% Complete | **Meet with potential industrial partners**. Though we did have conversations with several potential partners, mostly venture funds people, we agree with the advice of Rich Zwosec, our consultant, that we should not initiate meetings with potential partners until we have demonstrated robust conversion of methanol to methane. This can be accomplished as soon as we can heterologously express adequate levels of the methanol:CoM MeTr. This is currently a major focus and progress toward this end is described under the Milestone 1.X sections. |
| M5.9 | 8/1/15 | 0% complete | **Write proposal for follow-up funding of the project**. We have identified one potential source of follow-up funding and will be discussing this as soon as we are able to show conversion of methane to methanol. |
| M5.10 | 9/1/14 | 50% complete | **Adjust the technoeconomic model to fit the methane-to-methanol proposal so that it is ready for data inputs.**  We have developed a preliminary model and will discuss this with ARPA-E project officers at the Remote meeting to ensure this meets their criteria. As described above, we are awaiting some experimental results on the conversion of methanol to methane and the targeted reverse reaction before proceeding with this. It will be helpful as the models from other groups become available to share these. |
| M5.11 | 9/1/15 | 0% complete | **Add data to the technoeconomic model and perform sensitivity analysis**  As described above, we are awaiting some experimental results on the conversion of methanol to methane and the targeted reverse reaction before proceeding with this. It will be helpful as the models from other groups become available to share these. |

**C.    Supporting data & additional information**

**Milestones 1.x**

M1.3. We fused lacZ to three promoters, the Methanococcus voltae histone (hmv) promoter, the M. voltae S layer protein promoter including 278 nucleotides upstream of the translation start site, and the M. voltae S layer promoter including 385 nucleotides upstream of the translation start site. We introduced these constructs into M. maripaludis on a replicative plasmid and measured expression. The results were as follows:

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| **Promoter** | **Beta-galactosidase values** |
| Methanococcus voltae histone (hmv) promoter | 416 |
| Methanococcus voltae S-layer promoter (long) | 714 |
| Methanococcus voltae S-layer promoter (short) | 23 |

The results show that the S-layer promoter gives somewhat better expression than the histone promoter that we have been using. The longer upstream region of the S-layer promoter is necessary. Use of the S-layer promoter may improve expression by about 75%. We did not test the mcr promoter since recombination with already-existing mcr promoters on the vectors could make the constructs unstable.

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| M1.4. We chose the MtaA protein from *Methanosarcina acetivorans* to compare protein levels expressed from a replicative plasmid and expressed from a site in the chromosome. A Western blot is shown in Fig. 1. The results indicated that the protein expressed from the replicative plasmid was more abundant than the protein expressed after integration of the construct into the chromosome. These results suggest that the best strategy going forward will be to express heterologous genes on the replicative plasmid. We are still working on another strategy, to add MtaA to the native Mcr operon.  **Milestones 2.x**  **Milestones 2.3-2.4: Build and refine a draft metabolic model for *M. maripaludis.***  We have previously described our metabolic model of M. | | Fig. 1. Western blot comparing Mta expression levels |
| *maripaludis S2* and the iterative process of manual curation. In the previous quarter, we also described our model’s ability to robustly predict measured growth yields within or close to the 95% confidence interval across a range of methane evolution rates (Fig. 2). This growth yield validation falls well within our goal of <20% prediction error and, together with our previously described knockout validation data, demonstrates the high quality of our model. | Figure 2*: Growth yield predictions compared to 95% confidence interval of measured values* | |

Having already demonstrated that adding a ferredoxin:H2 oxidoreductase could stoichiometrically but not energetically enable reverse methanogenesis, we tested hypothetical additions to our solution. Namely, we tested 3 reduction pathways previously found in methanotrophic consortia: sulfate reduction to sulfide; nitrate reduction to nitrite; and ferric ion reduction to ferrous ions. Of these possible pathways, shown below in Figure 3, we predicted that only nitrate reduction would be a feasible strain design. Although iron reduction would be the most thermodynamically favorable of these pathways (ΔG ≈ -207 kJ/mol), the absence of water in this reaction creates a mass imbalance in our model. Thus, even though

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| we achieved energetic feasibility, we could not simultaneously achieve stoichiometric feasibility. Sulfate reduction did not suffer from this same problem as each mole of reduced sulfate produced 4 moles of water. However, the initial reaction in this pathway consumes 1 mole of ATP, the metabolite most prominently required for biomass. Because sulfate reduction produces the least negative free energy (ΔG ≈ -92 kJ/mol), the amount of sulfate that must be reduced in order to offset the energy of methane oxidation actually consumes all the ATP | Macintosh HD:Users:sragsdal:DARWIN MacBookPro:Grants:ARPA-E Methane oxidation:Quarterly Reports:Quarter 2_2016:Fig3.tif  Fig. 3: Candidate reduction pathways for conversion of methane to methanol |

produced through reverse methanogenesis, rendering this pathway infeasible in the model.

Unlike the aforementioned pathways, nitrate reduction was predicted to be feasible because it produces water to achieve mass balance in the model and has a sufficiently negative free energy (ΔG ≈ -146 kJ/mol) to offset the free energy of reverse methanogenesis without using up too many electron carriers. At standard effective metabolite concentrations of 1 mM, we predict that 0.66 moles NO3 reduced per mole CH4 oxidized would be sufficient to cross the threshold of ΔG ≤ 0 and would produce about 0.88 moles of CH3OH. Furthermore, a higher flux bound on nitrate or more favorable concentration gradient could easily drive this value much lower. This provides the first strain design predicted by our model to be completely feasible for methane oxidation to methanol. Notably, our predictions thus far have generally been restricted to the realm of transformations known to occur in methanotrophic consortia in nature. These predictions have informed our possible future candidate pathways by demonstrating the importance of generating water and of managing electron carrier usage in our solutions. Moving forward, we plan to test many more possible pathways while adhering to these criteria.

**3.3 Deliver an MeTr with an activity of >10 units/mg for methanol production from methyl-SCoM*.***

We have been working closely with the Leigh lab to quantify levels of expression of the MCR and the MtaABC. We have used two ways to quantify the amount of the various expressed proteins, purification and comparative Western blots using antibody against the his tag. Purification gives us absolute protein abundance. Western blotting allows us to compare levels of the same his-tagged protein in different

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| expression strategies, but is not a reliable quantification method when comparing two different proteins.  We have purified the ANME 2c and *M. marburgensis* MCR, which had been heterologously expressed in *M. maripaludis* (Fig 4), using a Ni- affinity column and observed the expected three | Table 1. Expression of MCRs in *M. maripaludis*  Expression Table.tif |
| bands for the three subunits of the enzyme. The total amounts of purified protein per 1 L culture are given in Table 1. This corresponds to the following amounts (mg protein/g cell dw) of purified proteins expressed: ANME 2c, 0.20; *M. marburgensis*, 0.50. Protein quantitation showed that both enzymes are present in quantities exceeding the milestone goal of 0.1 mg protein/g cell dw. However, the levels are significantly lower than those of the native *M. maripaludis* (1.7 mg/L culture) and *M. marburgensis* (18.4 mg/L culture). Spectrophotometric measurements demonstrated that all proteins contained the F430 cofactor. | Macintosh HD:Users:sragsdal:DARWIN MacBookPro:Grants:ARPA-E Methane oxidation:milestones and updates:Purified ANME&marburgensis MCRs.jpg  Fig. 4: Expression of the different MCRs |

**Expression of *M. acetivorans* methanol:coenzyme M methyltransferase (mtaABC) in *M. maripaludis***

Expression of the His-tagged methanol MeTr was quantified by both protein and antibody (anti-His tag) staining of protein purified from the Ni-NTA column. 0.6 l of culture was grown and the cells were harvested, lysed, and purified by affinity chromatography (Fig. 5). The Coomassie-stained gel shows that the protein is nearly pure. Quantification by Western blot and protein assays revealed that the protein is present at a level of 0.1% of cell protein, or approximately 0.5 mg/ g cdw. Activity assays are consistent with his number and reveal that the expressed protein appears to be fully active. For example, Mta, with a cell extract activity of 220 mU/mg, is present at ~5% of cell protein in *M. acetivorans*. This would be roughly equivalent to 3.3 mU/mg of Mta activity in cell extracts of *M. maripaludis* where it is present at 0.1% cell protein (we would expect 4.4 mU/mg). Thus, it is very important to increase levels of expression of both Mta and MCR by ~10-fold to achieve our GTL goals.

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| *Macintosh HD:Users:sragsdal:DARWIN MacBookPro:Grants:ARPA-E Methane oxidation:milestones and updates:Mta_JM_33116.tif* | *Macintosh HD:Users:sragsdal:DARWIN MacBookPro:Grants:ARPA-E Methane oxidation:milestones and updates:MtaActivity_33116.tif*  Fig. 5: Protein stained gels, and Western analysis showing levels of expression of the different MeTrs |

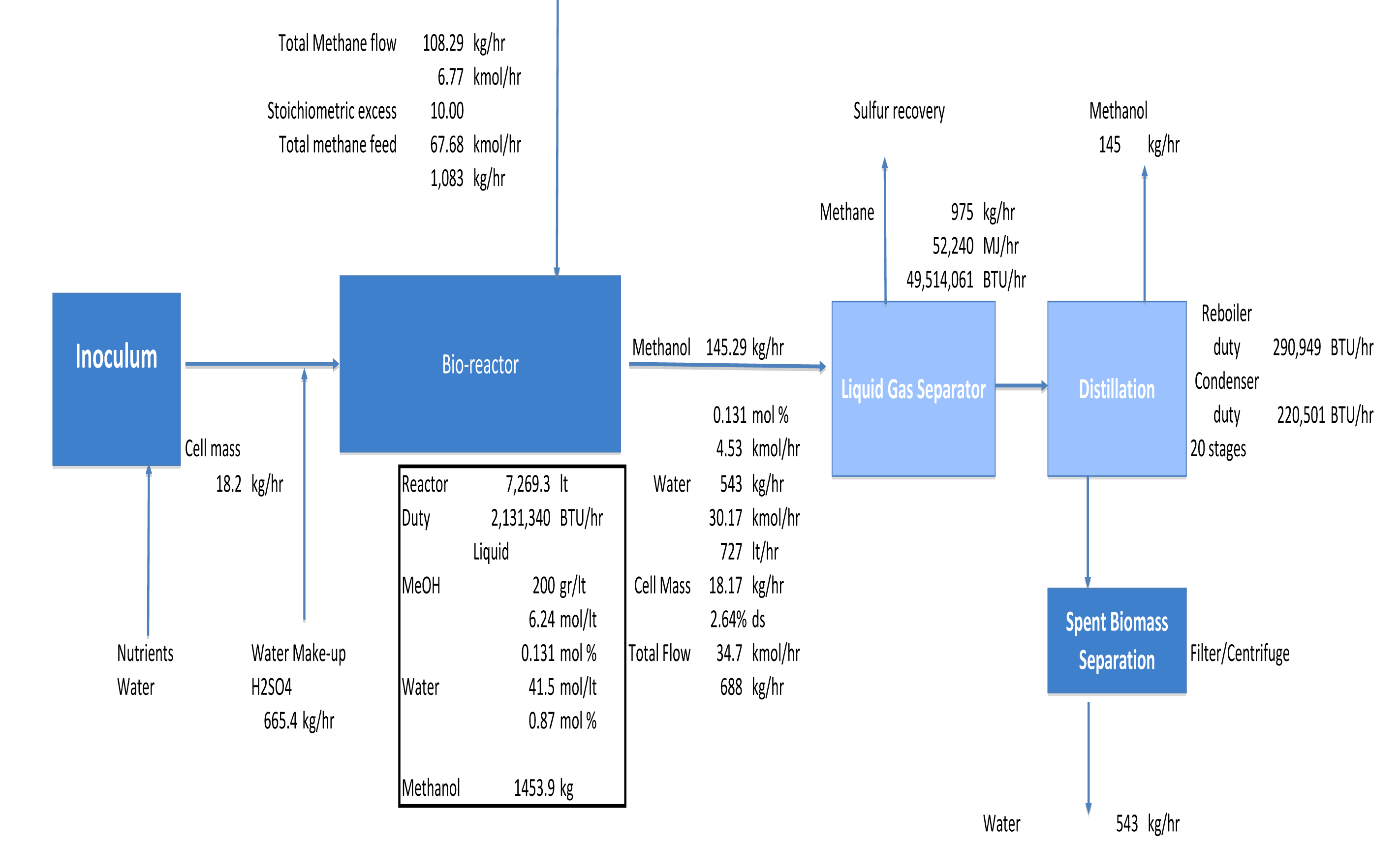
Working closely with the Raugei laboratory, we were successful in determining the mechanism of MCR and establishing the involvement of a methyl radical (1,2). We also have been successful in elucidating each of the steps in the reverse reaction (in preparation & again in close work with PNNL) and determining the experimental binding constants to benchmark the computational work described under section 4.

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| **Milestones 4.x**  **Milestone 4.3: Validate activation energies in MCR pathways using experimental data from Ragsdale’s lab.** The QM calculations on the *Maripaludis* MCR completed in the previous quarter were extended to ANME-1 MCR. This study indicates that the chemical differences of F430 in the two enzymes are likely not responsible for the different reactivity of ANME-1 MCR and *Maripaludis* MCR since the reaction free energies for breaking the S-CH3 bond of CoM and the subsequent formation of CoB-SS-CoM are, within our computational error, identical (differences within 1 kcal/mol). This result suggests the different enzymatic activity could be due either to a different substrate (CoBSH, | Macintosh HD:Users:raug510:Desktop:Untitled.png |
| CoM and/or CoB-SS-CoM) binding affinity or/and to a modulation of the F430 redox properties by the cysteine rich patch connecting F430 to the surface of the protein. We are currently exploring both possibilities as part of the (proposed) revised Milestone 4.4  **Milestone 4.4: Understand how structural and dynamical differences in methanogenic and methanotrophic (ANME) MCR regulate activity.**  We aim at understanding if and how the residues interacting with CoBSH, CoM and CoB-SS-CoM influence their binding affinity, as reflected in their *K*­M or *K*­D values, in turn, altering catalytic activity. More specifically we would like to understand how to change the binding affinity of substrates and intermediates by mutating the residues interacting with them, and see if it is possible to shift the binding affinity in order to make the reverse reaction faster. The calculation of the binding affinity of CoBSH and CoBS-in *M. marburgensis* MCR that we completed in the previous | **Fig. 6. Binding of substrates to MCR.** Top panel: key interactions stabilizing the substrates in the catalytic pocket. Bottom panel: Free energy profiles for the binding/dissociation of CoBSH and CoB- (free energy reported as a function of the distance of the phosphate tail from the surface of the proteins). |

quarter allowed us to identify the residues that contribute the most to stabilize CoBSH in the substrate channel (Fig. 6). We are extending these past simulations to the binding of CoM. In particular, we are focusing on residues Arg270 (see figure) and Trp318. Indeed, Arg270 is always methylated in methanogenic MCRs but not in ANME-1 MCR (Arg285 according ANME-1 MCR numbering) and Trp318 is found to have a hydroxyl function at the position C7 in ANME-1 MCR (Trp333 in ANME-1 MCR). Extending the calculation of the binding affinity of CoBSH to ANME-1 MCR will reveal if this modifications influence the binding of COB (and possibly of CoM). If they do, we will be able to rationalize one of the contributions to the different reactivity of the two MCR and suggest a clear strategy to alter substrate binding.

**Milestone 5.4: Technoeconomic model**

Rich Svocek of High Hurdles, LLC prepared a process feasibility analysis for the anaerobic bioconversion of methane to methanol (Fig. 7).  We engaged the services of VerdeNero for the engineering calculations of this process. The process is energetically feasible assuming we can meet the operational targets set forth in the milestones. In this analysis, which was sent to the project managers at ARPA-E, a conventional bioprocess was found to be feasible from the perspective of energy efficiency. Achieving the process targets with respect to final methanol titer, cell concentration and rate will be critical to develop a commercially feasible process.  Using 1 g/l/hr productivity and 67% carbon yield, a 64% overall energy efficiency is achievable to produce a final product of 90% methanol/10% water. The major use of methane as energy (vs. feedstock) would be in the reboiler at a rate of 14 BTUs of energy per 100 BTUs of energy content in the output methanol. It was found that, if the sulfate to sulfide pathway is chosen as the electron acceptor, a strategy for recovery and re-use of sulfur is likely to be necessary based on the equimolar consumption of sulfuric acid.

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**Fig. 7. Process flow diagram**

**D.    Major risks to future milestones**:

**Milestones 1.x.** With strategies for improved expression and stability of heterologous proteins in place, we hope to be able to demonstrate metabolic flux from methanol to methane, and eventually from methane to methanol. There is still a potential for toxicity of heterologous proteins in M. maripaludis, and our plan to design an inducible expression system should help in that case. With regard to heterologous (ANME) Mcr, our ability to activate the enzyme in M. maripaludis remains an unknown.

**Milestones 2.4.** With our model essentially completed, our chief remaining challenges lies in generating reasonable strain designs. We have had difficulties implementing the SimOptStrain strain design method and using Kbase gap-filling as a means for generating new designs. also with using the Kbase for gapfilling. We will continue to work with these platforms to try to quickly generate new strain designs and test their energetic feasibility. However, rather than relying solely on these tools, we will also be extending our manual efforts to identify and test promising candidate pathways to pair with methane oxidation.

**Milestone 3.x.** The major risk relates to whether or not we are able to achieve a sufficiently high level of expression of the MCR and MtaABC.

**Milestone 4.x:** The riskiest aspect of M4.3 is the calculation of barriers between reaction intermediates, which is highly sensitive to the initial geometry of the proposed reaction intermediate structure. As stated in the previous reports, there is also a risk of being “scooped” by competing scientists, however the computational resources and the approach we are using is more sophisticated than others previously applied to this system.

**Milestones 5.x.** The riskiest aspect of a commercially feasible process is whether or not we can meet our operational targets: 1 g methanol/liter/hr productivity.

REFERENCES:

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