CAAP Quarterly Report, FY '25 Q2
Mar. 30, 2025

Project Name: Rhamnolipid: a Bio-based, Ecologically Friendly, Corrosion Inhibitor and SRB Biocide for Crude Pipelines

Contract Number: 693JK32350001CAAP

Prime University: University of Akron

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Reporting Period: Jan. 1, 2025 thru Mar. 30, 2025.

Project Activities for Reporting Period:

Students / Hiring.

Current graduate students: Jan. 2025, Elaheh Mozayan, M.S. Biochem., University of Kashan, Iran; Jan. 2025 or May 2025: Kingsford Duah Agyemang, B.S. Petroleum Eng., Kwame Univ. Uddipta Mondal, B.S. Chem. Eng., BUET and Tijani Abdul-Gafaru B.S. Petroleum Eng., Kwame Univ.

Current undergraduate students: Ellie Zimmerer (Chem. Eng.), Rosemary Sterling (Chem. Eng.), Callie Lewis, (Chem. Eng.), Lily Clemente (Chem. Eng.).

New undergraduate students: Jack Schulze (Corr. Eng.).

Previous undergraduate students: Joseph Botzman (Corr. Eng) and Mikey Markov (Corr. Eng.).

Milestones

Table 1 lists the milestones for this project and their approximate status. As seen in this table, we are nearing completion or our produced water experiments, approximately 90% complete. These were our initial "proof of concept experiments" which were very successful showing that RhL is an effective inhibitor in simulated produced water. There has been a delay on starting two milestones, Flow System Modifications and PW SRB Attachment in Flow. These two milestones are related in that we must first modify our existing system/instrumentation before beginning the attachment investigation. This delay owes to a delay in graduate student hiring which was remedied in the fall and we do not expect any further delays on these milestones.

Table 1. List of milestones (from proposal) and approximate status. Note PW stands for produced water. *Italics* indicate a schedule change and new dates.

	Status	Sched. Begin	Sched. End
1. *RhL Fermentation*	ongoing	10/9/23	5/30/26
2. *Corrosion, %IE and Mechanism*	50% complete	10/9/23	5/25/26
Purchase consumables, cell mods.	complete	10/9/23	12/11/23
Produced Water Exp,	90% complete	12/18/23	8/31/25
Crude Surrogate Exp.	*delayed*	*6/1/25*	*12/1/25*
Actual Crude Exp.		9/22/25	5/25/26
3. *SRB-MIC*		9/1/24	6/1/26
Flow System Mods	*delayed*	1/06/25	*9/1/25*

PW-SRB CorrRate (static)	50% complete	9/1/24	*9/1/25*
PW-SRB Attachment, CorrRate (flow)	*delayed*	*9/1/25*	5/12/26
4. *Di-RhL vs. Mono-RhL*		9/2/25	6/1/26
Separation of Di and Mono		9/2/25	12/31/25
CorrRate IE		1/1/26	5/30/26

Milestone 1, RhL Fermentation

To successfully produce RhL on a repeted basis, our goal is to identify the limiting non-carbon (C), non-nitrogen (N) nutrient in RhL fermentation. Understanding this limitation will aid in optimizing RhL productivity by adjusting mineral nutrient concentrations. To reach this goal, we carried out four batches of experiments in this quarter. Their rationales, designs, and key findings are described below.

Because the focus is on identifying the limiting non-C, non-N nutrient, potential limiting substrates were shortlisted. Yeast extract and peptone are in the original production medium, mainly as organic N sources. These ill-defined substances were removed from the media used in the study, and ammonium nitrate was used to make up the combined N amount they provided. KH_2SO_4, Na_2HPO_4, and NaCl are apparently provided in excess in the original production medium. They were included in the media used in this study but were excluded from the list of potential limiting substrates. The five major substrates considered as potential limiting factors were $MgSO_4$, $FeSO_4$, $CaCl_2$, $MnCl_2$, and trace elements. To identify which of these is limiting, experiments were conducted in shake flasks. All media were prepared to support only approximately 0.5-1 g/L of cell growth, to minimize the possibility of reaching limiting oxygen transfer and/or pH. Continuous oxygen transfer was maintained by covering the shake flasks/beakers with cotton sandwiched in two layers of cheesecloth. C and N sources were provided in excess.

In the first batch of experiments, we used 12 shake flasks to evaluate six systems in duplicate. One was the control system with concentrations of all nutrients being proportionally reduced from those in the original production medium, to limit the maximum cell concentration to 0.5-1 g/L. In the other five systems, the concentration of one of the five potential limiting substrates was reduced to 50% of that in the control system to determine its impact on cell growth. Samples were collected at varying intervals and analyzed for pH and intracellular protein (IP) and cell dry weight (CDW) concentrations. It was anticipated that systems with limiting nutrients, due to the 50% concentration reduction, would show distinct variations in profiles of pH and IP and CDW concentrations. However, results from this batch of experiments did not allow us to make conclusions. During the initial hours, pH remained in the acceptable range (>5.7), and all systems showed similar cell growth patterns and reached the stationary phase very quickly. However, pH later dropped below the acceptable range and showed different metabolic shifts. These findings suggested that further investigation was necessary.

In the second batch of experiments, the following modifications were made. (1) We limited the investigation to three systems: control, 25% $MnSO_4$, and 25% trace elements. This time we used 9 shake flasks to make each system triplicate. The lower (25%, instead of 50% in the previous experiments) potential limiting substrate concentration was to make the limitation more pronounced. (2) The inoculated seed culture volume was reduced from 10% to 2.5% of the medium volume, to extend the growth phase for clearer observation. (3) The phosphate buffer strength was

increased (>20 g/L) to maintain pH within the suitable range. Samples were collected throughout the expected growth phase based on previous experimental observations. This time we were able to solve the pH declining issue. However, the growth curves for different systems were not distinguishable and had large standard deviations to allow drawing any conclusions. One mistake was identified in the experimental procedure, i.e., the samples taken were stored in a refrigerator and processed for analysis later. This allows further cell metabolism and/or decaying during storage, leading to uncertainties and large standard deviations.

The mistake was corrected in the third batch of experiments conducted with similar setup. The experiments again involved three systems. But, besides the control, the two potential limiting substrate systems had 33% (instead of 50% or 25% in the previous batches) $FeSO_4$ and 33% trace elements, respectively. In addition, the inoculum level was raised to 4% (from the 2.5% used in the second batch). Processing samples immediately after collection significantly reduced the standard deviations. However, while the potential limiting systems showed slightly less cell growth than the control system, the differences were too small to indicate clear growth limitation (**Fig 1**).

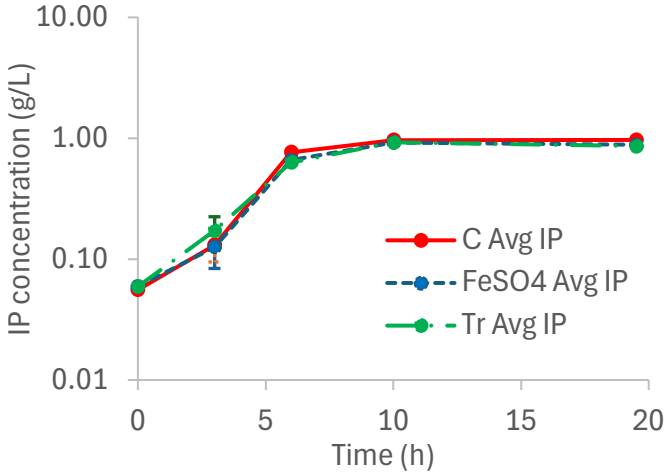

Figure 1 Profiles of average intercellular protein concentration for control (C) system, 33% FeSO4 system, and 33% trace elements (Tr) system observed in the third batch of experiments.

Results of the third batch of experiments led us to consider the possible interference due to the nutrients introduced with the seed culture, which was grown in tryptic soy broth (TSB). Therefore, the fourth batch of experiments were made to compare two systems. One system, denoted as non-TSB C in **Fig 2**, was made with the control medium (without any reduced substrate concentrations) but was inoculated with washed seed culture (using centrifugation). The other system, denoted as TSB C, was inoculated with unwashed seed culture into a medium with only C and N sources and the phosphate buffer (i.e., without any non-C, non-N, non-P nutrients). The results showed that the TSB-grown seed culture, if unwashed, indeed carried nutrients to enable cell growth, but only to about 0.2 g/L of IP (**Fig 2**). In future experiments, washed seed culture will be used to eliminate this interference.

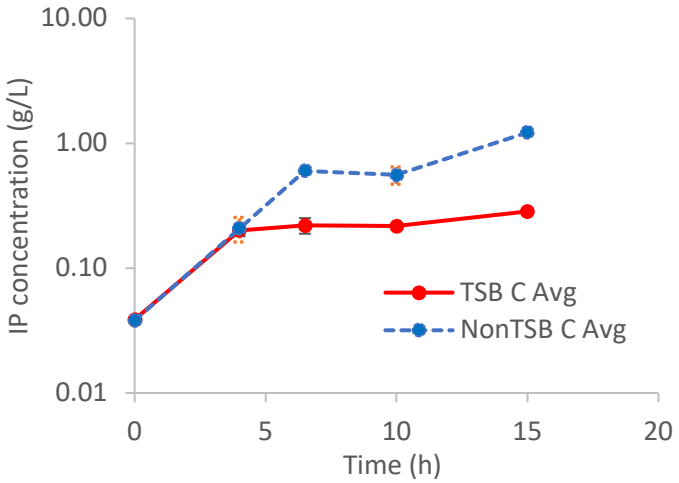

Figure 2 Profiles of average intercellular protein concentration for two systems: TSB C – unwashed TSB-grown seed culture in medium with only soybean oil, N source, and phosphate buffer, and NonTSB C – washed seed culture in the control medium with all nutrients.

Milestone 2, Corrosion in PW simulant.

For the past two quarters we have been collecting long-term exposure data for C1018 specimens in a produced water simulant (1% NaCl, RCE 1000 RPM, CO_2 purged) with and without RhL. In these experiements we are recording OCP data, polarization resistance via LPR measurements as well as polarization resistance via EIS data at E_{corr}. In the previous quarterly report (FY '25, Q1), we discussed the LPR results for the RhL and non-RhL cases. Here we discuss our initial analysis of the EIS data in the absence of RhL while the EIS data for solutions with RhL will be discussed in the next report.

A typical Nyquist plot from the EIS vs. time data in presented in **Figure 3** which plots the imaginary impedance (Z") vs. real impedance (Z'). The data are characterized by a capacitive loop at high frequencies ($Z = -1/j\omega C$) and an inductive loop at low frequencies ($Z = j\omega L$). Similar data has been obtained by other investigators for steel in NaCl / CO_2 (Zeng, Lillard, Cong: *Corrosion* 2016). The data in **Figure 3** can be modeled by the equivalent circuit shown in **Figure 4** where: R_s is equal the geometric solution resistance between the specimen and reference electrode, C_{dl} is the double layer capacitance associated with the electrochemical interface, R_t and R_L are charge transfer resistances associated with iron oxidation and L is an inductance associated with surface adsorption of the intermediate species who's charge transfer resistance is associated with R_L. Specifically, for the CO_2 system R_t is proportional to the oxidation of Fe(M) to Fe(I) via the reaction:

$$Fe(M) + HCO_3^-{}_{ads} \rightarrow FeHCO_3^-{}_{ads} + e^-$$

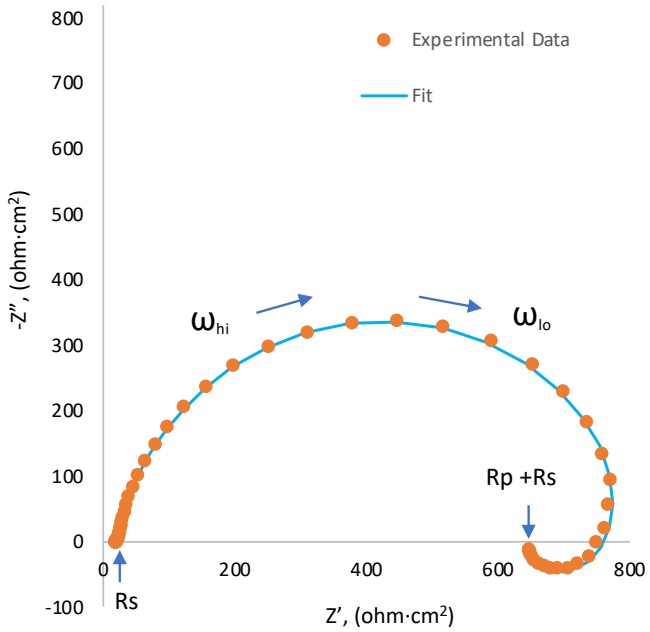

Figure 3 Typical Nyquist plot obtained from the EIS vs time data (here, at=12 hrs.) for C1018 steel in a RCE experiment at 1000 RPM. Solution was 1% NaCl saturated with CO_2. CNLS fit is also shown. The data were collected over the frequency range of 100 kHz to 0.01 Hz; in the figure, high frequencies (ω) are to the left and low frequencies are to the right. Graphical values for R_p and R_s are also shown.

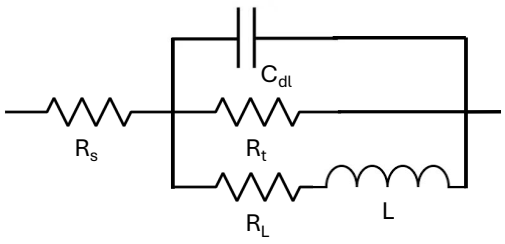

Figure 4 Equivalent circuit model used to fit the EIS data for the non-RhL case. Elements are defined in the text.

while L and RL are associated with the accumulation of adsorbed Fe(I) carbonate and its oxidation to Fe(II) via the reaction:

$$FeHCO_3^-{}_{ads} \rightarrow FeHCO_3^+ + e^-$$

A fit of the experimental data to the equivalent circuit in **Figure 4** is also presented in **Figure 3** and a summary of the values from all of the experiments are presented in **Table 2**. The polarization resistance, R_p (inversely proportional to the corrosion rate), in **Table 2** was calculated from the EIS fit using the relationship:

$$\frac{1}{R_p} = \frac{1}{R_t} + \frac{1}{R_L} \ .$$

Also in **Table 2**, the values obtained for R_p from EIS are compared with those obtained from LPR (reported on in Q1 FY '25). As seen in this table, good agreement exists between the two separate methods.

Table 2: Values obtained from CNLS fitting experimental EIS data vs. time to the equivalent circuit in **Figure 4**.

Fitted parameter	1% NaCl			
	t = 0 hr	**t = 6hrs**	**t = 12 hrs**	**t = 18 hrs**
$R_s(\Omega \cdot cm^2)$	18.6	18.7	19.45	19.30
$R_t(\Omega \cdot cm^2)$	296.8	684.3	819.7	829.2
$R_L(\Omega \cdot cm^2)$	859.5	1503	2669	4363
C_{dl} ($\mu F/cm^2$)	185.2	105.1	129	154.2
$L(\Omega \cdot cm^2)$	1263	1488	3353	6267
$\mathbf{Rp^{EIS}}(\Omega \cdot cm^2)$	219. 8	469.6	627.1	696.76
$\mathbf{Rp^{LPR}}(\Omega \cdot cm^2)$	201.4	460.3	617.6	691.7

Milestone 3, SRB-MIC.

The sulfate-reducing bacterial strain *Desulfovibrio vulgaris* (ATCC 7757) was revived and cultured in anaerobic Postgate C medium, purged with nitrogen. To maintain an anoxic environment, 0.1 mL of 7.66 wt % sodium sulfite was added to the culture, and this was replenished daily along with fresh medium in equal volume (5 mL). The culture was incubated in sterile serum bottles, and pH of the culture solution was monitored and adjusted to a value of 7 using 0.1 M sulfuric acid or 0.1 M sodium hydroxide. Optical density (OD) readings of the bacterial suspension were recorded to track growth. Titration analysis using barium chloride was performed to confirm successful bacterial revival by tracking sulfate reduction. Fresh Postgate C medium required approximately 1 mL of $BaCl_2$ to fully precipitate the available sulfate, while the revived SRB culture required only 0.6 mL, indicating a significant reduction in sulfate concentration. This confirmed metabolic activity and sulfate reduction by the revived SRBs.

An initial attachment study in quasi-static conditions is currently underway. The primary goals of these experiments are 1) to evaluate the concentration of nutrients that that need to be added to the produced water simulant to sustain SRB growth and 2) to investigate the effect of RhL on cell attachment / bio-film formation and planktonic growth. In these experiments, the Postgate C solution was prepared at 1/6 concentration and added to the produced water simulant, 1% NaCl solution with and without 100 ppm RhL. The solutions, cells and associated apparatus were autoclaved followed by purging with CO_2 for 24 hrs. After purging, the cells were inoculated with ATCC 7757 SRB in a glove-bag and C1018 mass loss specimens were then introduced followed by sealing the cells. At the end of the 30 day exposure period, corrosion rate, SRB growth and attachment will be evaluated for both cells (with and without RhL).

Project Financial Activities Incurred during the Reporting Period:
Since the beginning of the project, we have spent $151,313.46 (with encumbrances): $87,735.47 salary, $14,704.84 fringe, $8,740.86 supplies $50,909.87 indirect cost. The spending break down is shown below.

Ledger Account	Budget	LTD Actuals	Total Spend (with Encumbrances)	Remaining Balance	% Remaining
Salary	$234,584.00	$74,914.91	$87,735.47	$146,848.53	62.60%
Fringe Benefits	$20,599.00	$14,281.80	$14,704.84	$5,894.16	28.61%
Supplies & Services	$18,600.00	$8,706.88	$8,740.86	$9,859.14	53.01%
Student Aid	$30,000.00	$2,500.00	$2,500.00	$27,500.00	91.67%
Travel	$10,000.00	$0.00	$0.00	$10,000.00	100.00%
Total Direct Costs	$313,783.00	$100,403.59	$113,681.17	$200,101.83	63.77%
Indirect Cost	$147,567.00	$50,909.87	$50,909.87	$96,657.13	65.50%
Total Direct & Indirect Costs	$461,350.00	$151,313.46	$164,591.04	$296,758.96	64.32%

Project Activities with Cost Share Partners:
None.

Project Activities with External Partners:
None.

Potential Project Risks:
None.

Future Project Work:
During Q3 of FY ’25, the PIs anticipate working on the following topics:

1) Identifying the limiting substrate in the production medium. Implementing the findings in future fermentor runs to improve RhL production.
2) Analysis of EIS data from RhL
3) The corrosion behavior of carbon steel coupons under various medium conditions, both with and without reducing agent and RhL, will continue to be investigated. A growth curve for *D. vulgaris* will be generated, along with monitoring of the oxygen content and pH of the medium. To track the corrosion behavior of carbon steel in the presence of sulfate-reducing bacteria (SRB), a semi-flow system utilizing serum bottles will be employed, with periodic replenishment of fresh Postgate C medium while maintaining under the anoxic condition.

Potential Impacts to Pipeline Safety:
FY ’25, Q2 – The results, thus far, are extremely encouraging. We have verified our initial experiments that show RhL may be an effective inhibitor crude pipelines.