PROJECT REPORT

Screening Test: Determine the Removal Efficiency of Enzymes against a *Pseudomonas aeruginosa* biofilm grown in the CDC Biofilm Reactor

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Summary: The goal of this screening test was to determine the removal efficiency of CV-700, a proprietary enzyme/phosphate remover, against a laboratory *Pseudomonas aeruginosa* biofilm grown according to ASTM Method E2562-07 in the CDC biofilm reactor. The CDC reactor generates biofilm under high fluid shear. The resulting biofilm is tightly adhered to the surface with mushroom shape architecture. The screening occurred in two phases. For phase 1, the removal efficiency of CV-700 was tested against fully established biofilm grown in the CDC reactor. Treatment occurred for 16 hours. Samples were collected at 2 and 16 hours. The four combinations of treatment tested were: 1) sterile buffered water and CV-700 at a ratio of 1 oz product per 1000 gal water (pH=7.2), 2) sterile buffered water (pH = 7.2), 3) sterile buffered water and 2 mg/L free chlorine (pH = 7.2), 4) sterile buffered water, CV-700 (at a ratio of 1 oz product per 1000 gal water) and 2 mg/L free chlorine (pH=7.2). Results showed that the 2 mg/L free chlorine treatment resulted in a slightly greater log reduction of bacteria during the first two hours of treatment, but by T=16, there was no difference between the treated reactors and the control reactor. For phase 2, biofilm was grown in the presence of the CV-700 during the continuous flow growth phase. CV-700 was added at a ratio of 5 oz per 1000 gal water to reactor 1 and 10 oz per 1000 gal water to reactor 4. Both reactors also contained 100 mg/L Trypic soy broth (TSB – standard bacterial growth media). After the growth phase, the biofilm was added to treatment reactors that contained: 1) equivalent of 5 oz CV-700/1000 gal water and 2 mg/L free chlorine, 2) sterile buffered water, 3) 2 mg/L free chlorine, and 4) equivalent of 10 oz CV-700/1000 gal water plus 2 mg/L free chlorine. The pH was adjusted to equal 7.2 in all of the reactors. Samples were collected at time equal to two and four hours. The results showed that the addition of CV-700 at a concentration equal to 10 oz/1000 gal reduced the biofilm that formed during the continuous growth phase of biofilm development by 2 logs, a difference of practical importance. Adding this biofilm into a treatment reactor containing both the CV-700 and 2 mg/L free chlorine did not result in an additional reduction of biofilm.

Project Dates: May 19, 2008 – June 27, 2008

Background
The goal of the screening project was to determine the removal efficiency of CV-700, a proprietary enzyme/phosphate remover, against a *Pseudomonas aeruginosa* biofilm grown in the CDC reactor under high fluid shear. Testing occurred against a laboratory biofilm that contained bacteria and the organic extracellular polymeric slime they generate as part of the biofilm matrix. Previous research has shown that bacteria within this biofilm exhibit a dense, tightly adhered mushroom shape architecture and are resistant to high levels of sodium hypochlorite when tested for a 10 minute contact time at neutral pH (Buckingham-Meyer *et al*., JMM 70:236-244, 2007). The screening occurred in two phases.

PHASE 1
Methods

1. A *Pseudomonas aeruginosa* biofilm was grown according to ASTM Method E2562-07, titled "Standard Test Method for the Quantification of a *Pseudomonas aeruginosa* Biofilm Grown with High Shear and Continuous Flow using a CDC Biofilm Reactor." The biofilm was grown on polycarbonate coupons in four separate reactors.

2. At the end of the 24 hour continuous flow growth phase, the top of each CDC reactor was removed, dipped into sterile buffered water to wash-off any loosely attached cells, and place into a treatment reactor that contained one of the following:
Reactor 1: CV-700 equivalent to 1 oz/1000 gal water
Reactor 2: sterile buffered water
Reactor 3: 2 mg/L free chlorine (reactor was covered with foil during treatment)
Reactor 4: CV-700 equivalent to 1 oz/1000 gal water and 2 mg/L free chlorine (reactor was covered with foil during treatment)

The pH in each reactor was adjusted to equal 7.2. Each treatment reactor sat on a stir plate set to 125 rpm.

3. At time equal to 2 and 16 hours, six coupons were collected.
- Three coupons were evaluated for viable bacteria according to the following procedure. Remove each coupon from rod and dip into sterile dilution water containing sodium thiosulfate (neutralizer). Scrape the biofilm from the coupon surface into a test tube containing sterile buffered water and neutralizer, disaggregate the sample, dilute and plate for viable cells.
- Three coupons were evaluated for total biomass present using the following procedure based upon the use of crystal violet. Remove each coupon from rod and dip into sterile dilution water containing neutralizer. Place coupon in sterile beaker and stain with 5 ml of 1% crystal violet (CV) for 10 minutes. Rinse coupons three times by dipping into sterile dilution water. Elute the CV from the coupons with 5 ml 95% ethanol for 10 minutes. Measure the absorbance of the elution solution on a spectrophotometer set to 540 nm.

4. At time equal to 2 and 16 hours, one bulk water sample was collected and evaluated for suspended bacteria according to the following procedure. Add 1 mL of sample to 9 mL sterile buffered water and neutralizer, disaggregate, dilute and plate for viable cells. Bulk water samples were also evaluated for pH and free chlorine concentration.

5. At time equal to 16 hours, the surface of one coupon from each reactor was qualitatively evaluated by viewing under a stereoscope.

Results

Water Chemistry

Table 1 shows the water chemistry results from the Phase 1 testing for each of the reactors. The pH was adjusted to equal 7.2 at time equal to zero in each reactor. The pH decreased by approximately 0.3 pH units in every reactor. The target free chlorine concentration was 2 mg/L for reactors 3 and 4. By time equal to 2 hours, there was no measurable chlorine in either reactor. Reactors 1 and 2 contained no chlorine.

Table 1. Water chemistry results for Phase 1.

	pH			Chlorine		
Reactor	T=0	T=2	T=16	T=0	T=2	T=16
1	7.21	7.00	6.95	NA	NA	NA
2	7.17	6.95	6.93	NA	NA	NA
3	7.23	6.86	6.86	1.65	0	0
4	7.18	6.88	6.87	1.92	0	0

Biomass Removal

Table 2 shows the percent biomass removal efficiency for reactors 1, 3 and 4. Removal efficiency was calculated by subtracting the mean absorbance reading for a treated reactor from the mean absorbance reading for the control reactor and dividing this amount by the mean absorbance reading for the control reactor. This number was multiplied by 100. At time equal to two hours, coupons from all of the treated reactors showed less biomass than the control reactor, but by time equal to 16 hours, there was no difference between the treated and control reactors.

Table 2. Removal efficiency found for reactors 1, 3 and 4.

	Percent Removal	
Reactor	T=2	T=16
1	31%	-21%
3	39%	-29%
4	22%	14%

Bacteria in the Bulk Water

Table 3 shows the log density of viable bacteria measured in the bulk water. The treatment vessels were sterile at the start of the experiment, and so any bacteria measured is detached biofilm. Slightly more bacteria were found in the bulk water of reactor 3. Reactors 1 and 4 were not different from the control reactor (#2).

Table 3. Log density of viable bacteria measured in the bulk fluid of each reactor.

	Log_{10}(cfu/mL)	
Reactor	T=2	T=16
1	7.12	7.65
2	7.29	7.84
3	7.48	8.03
4	7.13	7.52

Biofilm Bacteria

The biofilm results are presented in Table 4. The mean biofilm log density found in the four reactors at T=0 was equal to 8.33 $\pm$ 0.16. This is a typical biofilm density for the CDC biofilm reactor when operated according to ASTM Method E2562-07. Two different log reductions were calculated using the measured mean biofilm log densities. The first log reduction looked at a with-in reactor reduction at T=2 and T=16 hours by subtracting the mean log density at T=2 (or T=16) from the log density measured at T=0. At T=2, reactor 3 had the greatest reduction in biofilm cells. Although the difference is not of practical importance, the result is consistent with the results presented in Table 2. By T=16, though, the reduction in biofilm cells for the treated reactors were not different than the reduction calculated for the control reactor.

The second approach used to calculate the log reduction was to subtract the mean biofilm log density for the treated reactors from the mean biofilm log density found for the control reactor at the same point in time. Once again, at T=2, reactor 3 had the greatest log reduction, although the difference in not of practical importance. At T=16 the treated reactors were not different from the control reactor as demonstrated by the low reduction reported for reactor 1 (0.12) and the negative reductions reported for reactors 3 and 4.

Table 4. Phase 1 biofilm density and calculated log reduction values.

Reactor	Mean Biofilm Log Density for $\log_{10}$cfu/cm^2			Log Reduction			
						Reactor 2- Treated Reactor	
	T=0	T=2	T=16	(T=0) - (T=2)	(T=0) - (T=16)	T=2	T=16
1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
2	[illegible]	7.80	[illegible]	[illegible]	[illegible]		
3	[illegible]	7.41	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
4	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

At time equal to 16 hours, one coupon from each reactor was also inspected under the stereoscope; images are attached to this report as a power point file. A qualitative inspection of the coupon surfaces showed no difference among the coupons from the four reactors.

Conclusion

At time equal to 2 hours, the treatment vessel that contained only free chlorine showed a slightly greater removal efficiency, but by time equal to 16 hours, there was no difference between the treated and control reactors. Tested under the above described conditions, CV-700 did not remove biofilm.

PHASE 2
Methods

1. A *Pseudomonas aeruginosa* biofilm was grown according to ASTM Method E2562-07, titled "Standard Test Method for the Quantification of a *Pseudomonas aeruginosa* Biofilm Grown with High Shear and Continuous Flow using a CDC Biofilm Reactor" with the following modifications:
Reactor 1: add the equivalent of 5 oz CV-700/1000 gal to the continuous flow growth media
Reactor 4: add the equivalent of 10 oz CV-700/1000 gal to the continuous flow growth media

No changes were made to the continuous flow growth media for reactors 2 and 3. Polycarbonate coupons were used in all four reactors.

2. At the end of the 24 hour continuous flow growth phase, the top of each CDC reactor was removed, dipped into sterile buffered water to wash-off any loosely attached cells, and place into a treatment reactor that contained one of the following:

Reactor 1: CV-700 equivalent to 5 oz/1000 gal water and 2 mg/L free chlorine (reactor was covered with foil during treatment)
Reactor 2: sterile buffered water
Reactor 3: 2 mg/L free chlorine (reactor was covered with foil during treatment)
Reactor 4: CV-700 equivalent to 10 oz/1000 gal water and 2 mg/L free chlorine (reactor was covered with foil during treatment)

The pH in each reactor was adjusted to equal 7.2. Each treatment reactor sat on a stir plate set to 125 rpm.

3. At time equal to 2 and 4 hours, six coupons were collected.

- Three coupons were evaluated for viable bacteria according to the following procedure. Remove each coupon from rod and dip into sterile dilution water containing sodium thiosulfate (neutralizer). Scrape the biofilm from the coupon surface into a test tube containing sterile buffered water and neutralizer, disaggregate the sample, dilute and plate for viable cells.
- Three coupons were evaluated for total biomass present using the following procedure based upon the use of crystal violet. Remove each coupon from rod and dip into sterile dilution water containing neutralizer. Place coupon in sterile beaker and stain with 5 ml of 1% crystal violet (CV) for 10 minutes. Rinse coupons three times by dipping into sterile dilution water. Elute the CV from the coupons with 5 ml 95% ethanol for 10 minutes. Measure the absorbance of the elution solution on a spectrophotometer set to 540 nm.

4. At time equal to 2 and 4 hours, one bulk water sample was collected and evaluated for suspended bacteria according to the following procedure. Add 1 mL of sample to 9 mL sterile buffered water and neutralizer, disaggregate, dilute and plate for viable cells. Bulk water samples were also evaluated for pH and free chlorine concentration.

5. At time equal to 4 hours, the surface of one coupon from each reactor was qualitatively evaluated by viewing under a stereoscope.

Results

Water Chemistry
Table 5 shows the water chemistry results from the Phase 2 testing for each of the reactors. The pH was adjusted to equal 7.2 at time equal to zero. The pH decreased by approximately 0.2 – 0.3 pH units in every reactor. The target free chlorine concentration was 2 mg/L for reactors 1, 3 and 4. By time equal to 2 hours, there was no measurable free chlorine in any of the three reactors. Reactor 2 contained no chlorine.

Table 5. Water chemistry results for Phase 2.

	pH			Free Chlorine		
Reactor	T=0	T=2	T=4	T=0	T=2	T=4
1	7.17	6.87	6.80	2.07	0	0
2	7.10	6.91	6.87	NA	NA	NA
3	7.12	6.82	6.76	1.66	0	0
4	7.13	6.92	6.89	1.63	0	0

Biomass Removal
Table 6 shows the percent biomass removal efficiency for reactors 1, 3 and 4. Removal efficiency was calculated by subtracting the mean absorbance reading for a treated reactor from the mean absorbance reading for the control reactor and dividing this amount by the mean

absorbance reading for the control reactor. This number was multiplied by 100. At time equal to two hours, testing showed that coupons from reactors 1 and 4 contained less biomass than the control. Reactor 3 had a negative removal efficiency, meaning that the control coupon contained less biomass than a coupon collected from reactor 3. At T=4 hours, coupons collected from reactors 1 and 4 still contained less biomass than a coupon collected from the control reactor.

Table 6. Removal efficiency found for reactors 1, 3 and 4 during Phase 2 testing.

	Percent Removal	
Reactor	T=2	T=4
1	46%	32%
3	-40%	-12%
4	37%	24%

Bacteria in the Bulk Water

Table 7 shows the log density of viable bacteria measured in the bulk water. The treatment vessels were sterile at the start of the experiment, and so any bacteria measured is detached biofilm. The density of bacteria in the bulk fluid increased at a similar rate for all four reactors, although the rate of biofilm detachment (as measured by the increase of bacteria in the bulk fluid) was slightly greater for reactor 1. Reactor 4 contained 3.9 logs less bacteria than reactor 2, a difference of practical importance.

Table 7. Log density of viable bacteria measured in the bulk fluid of each reactor.

	Log_{10}(cfu/mL)	
Reactor	T=2	T=4
1	7.02	7.81
2	7.03	7.21
3	6.69	7.10
4	3.16	3.69

Biofilm Bacteria

The biofilm results are presented in Table 8. The mean biofilm log density measured in reactors 2 and 3 at T=0, was equal to 8.10 $\pm$ 0.16. Reactors 2 and 3 were operated according to the ASTM method with no modifications. This number is consistent with the mean biofilm log density measured at T=0 during Phase 1 testing (8.33 $\pm$ 0.16). The mean biofilm log density measured in reactor 1 at T=0 was equal to 8.13, indicating that the addition of CV-700 at a concentration equal to 5 oz/1000 gal to the reactor during the continuous growth phase of the biofilm development did not affect biofilm growth. The mean biofilm log density measured in reactor 4 at T=0 equaled 6.10 $\log_{10}(cfu/cm^2)$, a difference of 2 logs. This difference is of practical importance and implies that the addition of CV-700 at a concentration equal to 10 oz/1000 gal did reduce the biofilm that formed during the continuous growth phase of biofilm development.

Table 8. Phase 2 biofilm density and calculated log reduction values.

Reactor	Mean Biofilm Log Density $log_{10}(cfu/cm^2)$			Log Reduction				
						Reactor 2 – Treated Reactor		
	T=0	T=2	T=4	(T=0) – (T=2)	(T=0) – (T=4)	T=0	T=2	T=4
1	[illegible]	7.54	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
2	[illegible]	7.17	[illegible]	1.[illegible]	[illegible]	NA	NA	NA
3	[illegible]	[illegible]	[illegible]	-0.1[illegible]	-0.17	[illegible]	[illegible]	[illegible]
4	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Two different log reductions were calculated using the measured mean biofilm log densities. The first log reduction looked at a with-in reactor reduction at T=2 and T=4 hours by subtracting the mean log density at T=2 (or T=4) from the log density measured at T=0. At T=2, reactor 2 experience the greatest reduction in biofilm cells. A possible explanation is that the biofilm coupons that were sampled in the control reactor experienced a sloughing event. Sloughing events are really common and difficult to predict. Sloughing events are also why differences of one log or less are of little practical importance in biofilm efficacy testing.

The second approach used to calculate the log reduction was to subtract the mean biofilm log density for the treated reactors from the mean biofilm log density found for the control reactor at the same point in time. Reactor 4 had a log difference of between 1.5 and 2.1 logs at all points tested; a difference of practical importance indicating that was consistently less biofilm in this reactor. The results indicate that the decreased biofilm was not a result of the addition of chlorine to the reactor, but because less biofilm formed in the presence of the CV-700 (at a concentration equal to 10 oz/1000 gal). This also explains why fewer biofilm cells were found in the bulk fluid of reactor 4 (Table 7) and why the reactor was visibly clearer than the other treatment reactors.

At time equal to 4 hours, one coupon from each reactor was also inspected under the stereoscope; images are attached to this report as a power point file. A qualitative inspection of the coupon surfaces showed visible biofilm clumps on the coupon surfaces collected from reactors 1 and 3 and less clumping on the coupon surface collected from reactor 4. Interestingly, the surface of a coupon collected from reactor 2 had no visible clumps.

<u>Conclusion</u>

The results showed that the addition of CV-700 at a concentration equal to 10 oz/1000 gal reduced the biofilm that formed during the continuous growth phase of biofilm development by 2 logs, a difference of practical importance. Adding this biofilm into a treatment reactor containing both the CV-700 and 2 mg/L free chlorine did not result in an additional reduction of biofilm.