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ADENOSINE TRIPHOSPHATE TESTING – RECENT ADVANCES IN THE DIFFERENTION BETWEEN BACTERIAL AND FUNGAL CONTAMINATION AND DETECTION OF DORMANT MICROBES IN FUEL AND FUEL-ASSOCIATED WATER SAMPLES

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ABSTRACT

Quantification of adenosine triphosphate (ATP) in fuels and fuel-associated waters was first presented at the Technische Akademie Esslingen 6th International Fuels Colloquium in 2007. At the time, two issues limited the overall usefulness of ATP as a test parameter: inability to differentiate between bacteria and fungi and inability to detect dormant microbes. Recent research has addressed both of these issues. This paper presents protocols for detecting dormant microbes – identified as microbes that are not metabolically active in the sampled fluid, but which can become active under appropriate conditions – and for differentiating fungi from bacteria. The newly developed protocols achieve >90% detection of bacterial endospores in fluid samples. They also provide >90% differentiation between bacterial and fungal contaminants in these fluids.

INTRODUCTION

The measurement of adenosine triphosphate (ATP) in fuels and fuel-associated water, using ASTM Method D7687 [1], has proven to be a fast, accurate and precise tool for quantifying microbial contamination in fuel systems [2, 3]. However, the ASTM test method has two limitations: it does not detect microbes that are dormant in the sample; nor does it differentiate between bacteria and fungi. Dormant microbes include fungal spores, bacterial endospores and persistor cells. This paper presents protocols for detecting dormant cells and for differentiating between bacteria and fungi.

MATERIALS AND METHODS

Adenosine Triphosphate Testing

ASTM Method D7687 was used for all ATP tests. Briefly, a 5 mL sample was pressure filtered through a 0.7 µm in-line, glass-fiber, depth filter. The retentate was then washed with a proprietary rinsing solution (all ATP test reagents are from LuminUltra Technologies, Ltd., Fredericton, NB, Canada) and air dried. A proprietary lysing regent was then used to extract ATP into a 17 x150 mm reaction tube. The 1.0 mL extract was then diluted with 9.0 mL of a proprietary buffer. Finally, 100 µL of diluted ATP extract was mixed with 100 µL of Luciferin-Luciferase reagent in a 12 x 55 mm cuvette, and the cuvette was placed into a luminometer to obtain a relative light unit (RLU) reading. All RLU data were converted to pg ATP mL^{-1} by comparison with the RUL of a 1.0 ng mL^{-1} ATP reference solution. For the dormant cell detection effort, pg ATP mL^{-1} data were transformed to Log_{10} pg ATP mL^{-1}.

In order to investigate the effect of the surfactant selected for the bacterial-fungal differentiation protocol, a 1:1 mixture of fungi and bacteria (see *Bacterial and Fungal Contaminant Differentiation; Test Microbes*, below) was tested by two protocols: ASTM D7687 and a second protocol in which 1.0 mL aliquants of the mixture are dispensed into each of two reaction tubes. One reaction tube contained 1.0 mL of the aforementioned proprietary lysing agent. The second reaction tube contained 9.0 mL of a proprietary stabilizing reagent. After vigorous shaking and one-minute standing, the contents of the first reaction tube were transferred to a reaction tube containing 8 mL of the proprietary buffer mentioned in the previous paragraph plus resin beads. This tube is inverted several times to ensure complete but gentle mixing. Once the resin beads have settled, a 100 µL portion was transferred to a 12 x 55 mm cuvette and reacted with 100 µL Luciferin-Luciferase reagent to obtain RLU and pg ATP mL^{-1}, as described above. Similarly, a 100 µL portion of the stabilizer-diluted subsample was transferred to a 12 x 55 mm cuvette and reacted with 100 µL Luciferin-Luciferase reagent to obtain RLU and pg ATP mL^{-1}, as described above. The results yielded *Total* ATP and *Dissolved* ATP, respectively. In context of this work,

Total ATP – tATP – is defined as the ATP detected in a sample from which no effort is made to separate whole cells from cell-fragments or extracellular ATP before the lysis step. *Dissolved* ATP – dATP – is ATP detected in a sample in which cell lysis has been inhibited. *Cellular* ATP – cATP – is computed as the difference between tATP and dATP, and *biomass stress index* (BSI%) is computed as the percentage ratio of dATP to tATP.

Detecting Dormant Cells

A commercially available, *Bacillus thermogenesis*, spore paste preparation ((BTK; Safer's BTK Biological Insecticide; Woodstream Corp, Brampton ON) was used to model a dormant bacterial population. Based on the protocol described by Min *et al.* [4], BTK was suspended in liquid media to give ~1.3 x 10^7 IU mL^{-1}. During preliminary experiments, ATP was tested immediately after BTK suspensions were prepared and after suspensions had incubated in a 37° C water bath for 15 min. Ultimately, the 37°C immersion period was optimized at 30 min. Four media were compared for their effect on endospore germination: brain-heart infusion broth (BHI; DIFCO, Becton Dickinson, Franklin Lakes, NJ), LiquiCult ^{TM} broth (LCB; MCE, Inc., Lake Placid, NY), nutrient broth (DIFCO) and trypticase soy broth (TSB; DIFCO). After determining the broth that produced the greatest post-induction yield, ATP was tested as a function of germination induction time at 37°C in TSB. All testing was performed in duplicate or triplicate.

Bacterial and Fungal Contaminant Differentiation
Test Microbes

Preliminary method development experiments were conducted using *Saccharomyces cerevisiae;* selected for its size (5 to 10 µm dia), single-cell morphology and commercially available as baker's yeast (Fleischmann's – Active Dry Yeast; ACH Food Companies, Inc., Memphis, TN, USA), suspended in Sabouraud-dextrose broth (Oxoid, Ltd, Basingstoke, Hampshire, UK) as the representative fungus. An uncharacterized, Gram negative bacterium, previously isolated from a contaminated metalworking fluid (MWF) sample, was used as the representative bacterium. For subsequent experiments, two filamentous fungal species (tentatively identified as *Aspergillus niger* and *Penicillium chrysogenum*) which had previously been isolated from contaminated MWF were used.

Filtration Media

The following filter media were evaluated: glass fiber filters mixed cellulose esters (MCE; 5 µm; EMD Millipore Corporation, Billerica, MA, USA), polycarbonate (PC; 12 µm, 10 µm and 8 µm; EMD Millipore Corporation, Billerica, MA, USA), polyvinylidene difluoride (PVDF; 5.0 µm;

EMD Millipore Corporation, Billerica, MA, USA) and polytetrafluoroethylene (PTFE; 5.0 µm; EMD Millipore Corporation, Billerica, MA, USA). The PVDF and PTFE filters were supplied as in-line, syringe filters in housings. The other filtration media were 25 mm dia filter disks. Filter disks were placed into Millipore in-line filter housings for use. During testing, each of the described filters were placed in series with the ASTM D7687 standard in-line filter so that the larger pore-size filters were positioned upstream of the standard D7687 filter. In order to test filtration efficiency, sample ATP was tested on both filtered and unfiltered portions. Efficiencies were computed as ratios of duplicate tests before and after filtration. The target filtration efficiencies were <10 % of total ATP being recovered in the yeast filtrate (>90% in the yeast retentate) and >90% of total ATP being recovered in the bacterial filtrate (>90% passing through the larger pore-size filter and being captured by the D7687 filter).

Surfactants

Two surfactants were evaluated for their ability to disaggregate bacterial flocs without lysing either bacteria or fungi. Surfactant A was tested at 1.8%, 0.9%, 0.45% and 0.045% (v/v), and surfactant B) was tested at 0.8%, 0.4%, 0.2% and 0.1% (v/v). In all cases, 10x working stocks were prepared in deionized water. Performance was evaluated by comparing biomass retention of 5.0 mL untreated MWF with biomass retention of 5.0 mL of MWF to which 0.5 mL of surfactant solution had been added and shaken vigorously for 30 sec, before filtration.

RESULTS

Detecting Dormant Cells

Four growth media were tested for BTK ATP concentration before and after heat-treatment (30 min at 37 °C). Fig 1 shows the Log_{10} pg ATP mL^{-1} data. One-way analysis of variance (ANOVA) of the initial and post heat-treatment data sets indicated that ATP recoveries varied significantly among the growth media. In this series, TSB provided the greatest increased ATP-yield between pre and post treated testing. The BHI medium provided the highest post-treatment ATP yields. Consequently, BHI and TSB were selected for further testing.

Duplicate series of BHI and TSB were spiked with BTK at 2, 3, 4, 5, 6 and 7Log_{10} dilutions of the spore paste (the 1Log dilution was unfilterable). Log pg ATP mL^{-1} versus Log dilution factor (DF) was compared by two-way ANOVA and by comparison of the dilution curves. The ANOVA indicated that the results were significantly different between the two media (F_{obs} = 148; $F_{crit[0.95]}$ = 4.1). The dilution curves (Fig 2) showed that TSB's trend line slope was closer to -1.0 than that for BHI. At lower DF, ATP recovery from TSB was greater than from BHI. Based on these results, TSB was selected as the recovery medium.

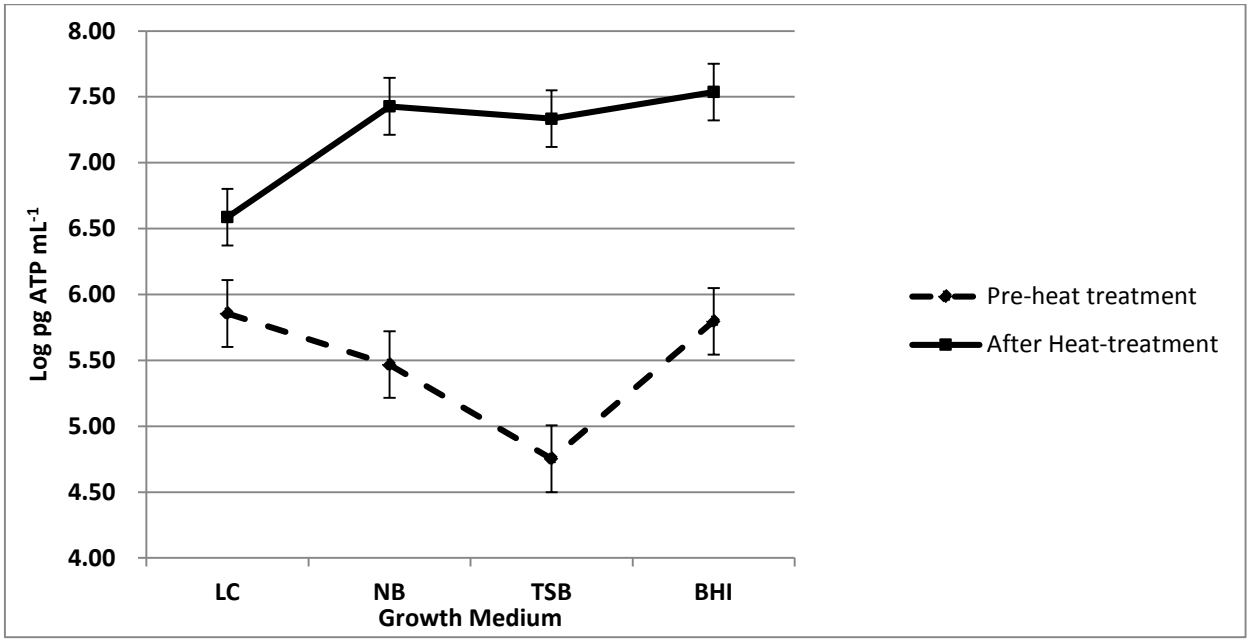

Fig 1. ATP concentration as a function of growth medium.

The final development step was to determine the optimal heating period. The objective was to provide maximum opportunity for germination before the first generation of germinated endospores reproduced. Duplicate runs were tested and each sample was tested in duplicate; generating ATP results for BTK in TSB at each time interval. The data are presented in Fig 3 without error bars. Coefficients of variation were all ≤0.9%. Based on the results, a 30 min incubation period was selected.

Differentiating Between Bacteria and Fungi

Nominal pore size (NPS), electrostatic properties and flow-path geometry affect size separation significantly. Consequently, our strategy was to identify a filtration medium that would consistently retain >90% of fungal cells and <10% of bacterial cells suspended in samples. We selected *S. cerevisiae* as the model fungus for our method development effort because it is a single-cell of its relatively uniform, ovoid shape and size (5 to 10 μm dia). Filter media that retained *S. cerevisiae* could reasonably be expected to retain filamentous fungi. We selected the uncharacterized Gram negative rod, primarily as a matter of convenience and secondarily, because of its tendency to form aggregate masses (flocs). Again, our assumption was that if we could effectively disaggregate flocs of our test bacterium, the protocol would have a good chance of being broadly applicable.

Table 1 summarizes the results of the filtration medium evaluation on *S. cerevisiae* suspensions. In these experiments, 5.0 μm MCE, PTFE and PVDF met or approximated our criterion for *S.*

cerevisiae ATP retention. Total pg ATP mL^{-1} were measured per ASTM D7687. Filtrate pg ATP mL^{-1} were obtained by performing D7687 on samples that had been filtered through test media. Percent retention was computed as:

$$\frac{[ATP]_{Total} - [ATP]_{Filtrate}}{[ATP]_{Total}} \times 100 = \% \text{ Retention} \quad (1)$$

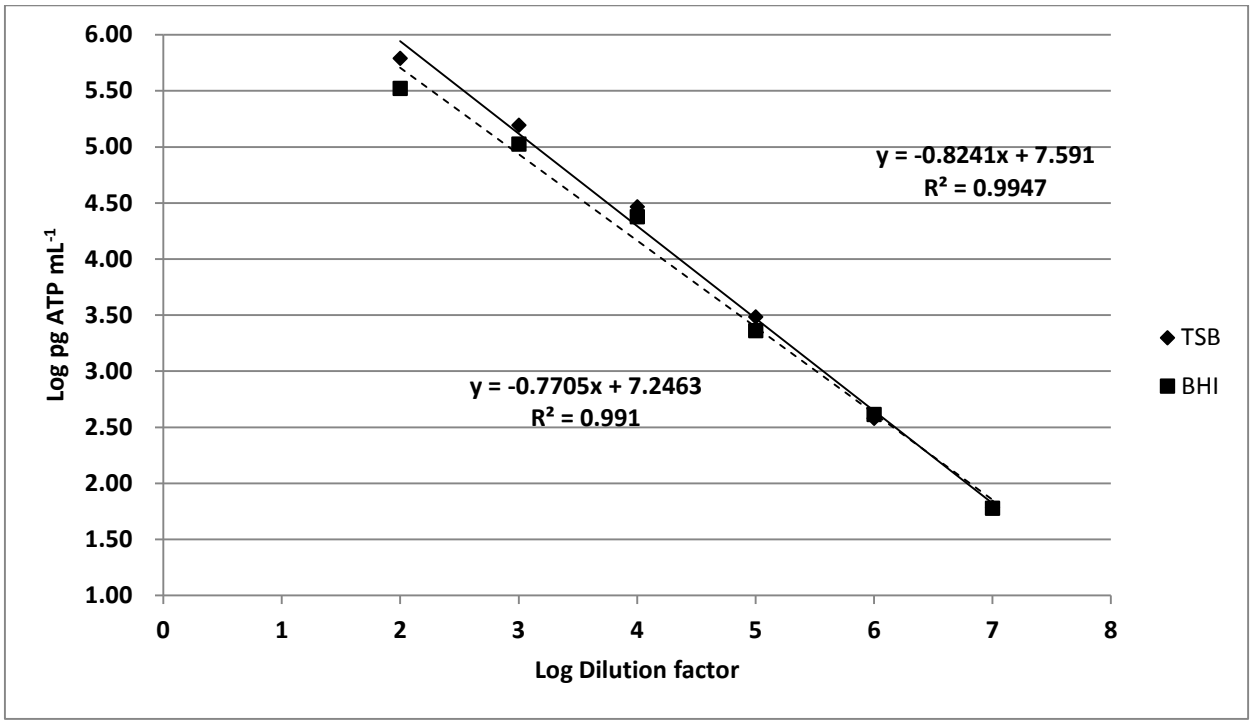

Fig 2. Comparison of ATP concentration versus Log_{10} dilution factor for BTK in BHI and TSB.

The PTFE evaluation was performed on a different day from the other tests. Consequently, the total *S. cerevisiae* ATP biomass differed from the totals reported for the other filtration media.

Subsequent studies focused on PTFE because it retained nearly 100% of the *S. cerevisiae* ATP, and PVDF because data variability was among replicates was smallest (coefficient of variation – CV% – =7%).

Having demonstrated good fungal biomass retention, the next experiment evaluated bacterial retention. Table 2 summarises the results of these tests. Again, *Total* ATP reflects ASTM D7687 results and *Filtrate* ATP reflects D7687 run on 0.5µm PTFE filter filtrates. On a percentage basis, the 5.0 µm PTFE medium retained more than double the bacterial biomass as did the PVDF medium. Consequently, PVDF was selected as the candidate medium for method

refinement. Although 5.0 µm PVDF worked well for bacteria and fungi individually, we decided to evaluate its efficacy for separating mixed suspensions. As demonstrated in Table 3, the medium quantitatively retained *S. cerevisiae* and permitted the bacteria to pass.

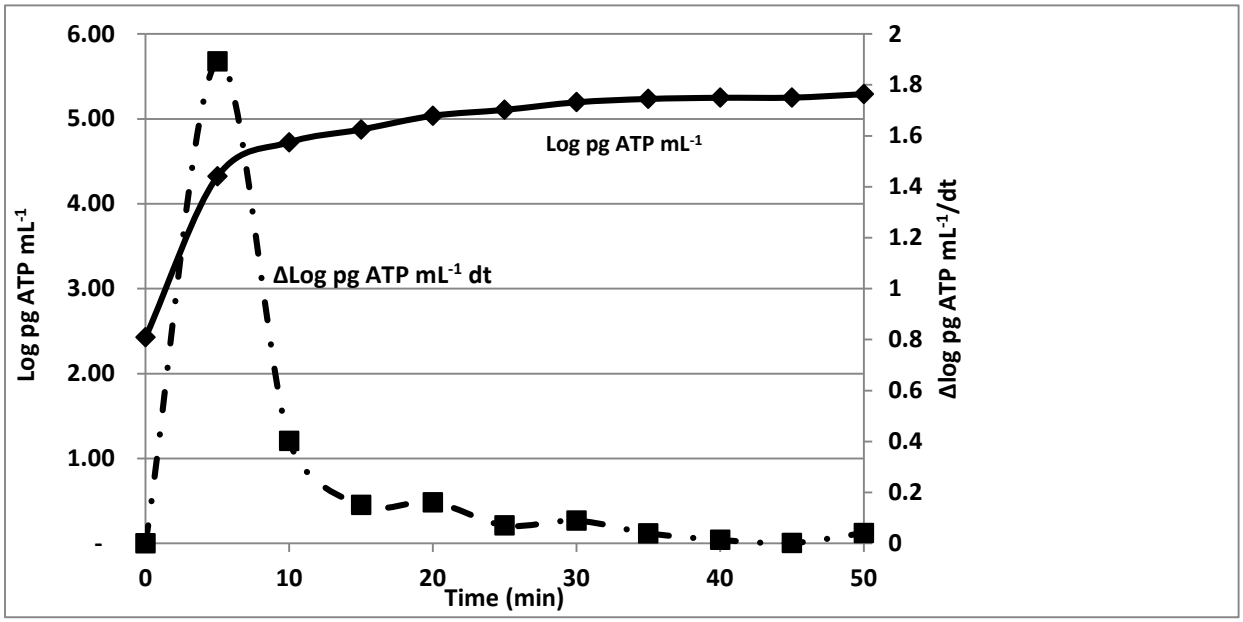

Fig 3. Log$_{10}$ pg ATP mL^{-1} as a function of BTK incubation time in TSB at 37° C.

Table 1. Effect of filter medium and pore-size on *S. cerevisiae* ATP retention.

Fitler medium	Pore size (µm)	pg ATP mL^{-1}				AVG % Retention
		Total		Filtrate		
		AVG	SD	AVG	SD	
MCE	**5.0**	**679**	**107**	**98**	**42**	**86%**
PC	12.0	679	107	527	323	22%
	10.0	679	107	444	47	35%
	8.0	679	107	392	60	42%
PTFE	**5.0**	**986**	**225**	**12**	**225**	**99%**
PVDF	**5.0**	**679**	**107**	**88**	**8**	**87%**

Table 2. Effect of filter medium on bacterial ATP retention

Fitler medium	Microbe	pg ATP mL^{-1}				% Retention
		Total		Filtrate		
		AVG	SD	AVG	SD	
PTFE	Bactera	3,928	N.D.	1,029	36	74%
	Yeast	1,435	123	12	4	99%
PVDF	Bactera	1,485	36	977	5	34%
	Yeast	986	225	18	4	98%

Table 3. Size differential filtration to separate bacteria and fungi.

Microbe	pg ATP mL^{-1}				Observed % Retention	Expected % Retention	Criterion % Retention
	Total		Filtrate				
	AVG	SD	AVG	SD			
100% B	788	78	806	56	-2%	0%	<10%
75% B + 25% Y	800	62	651	30	19%	25%	15 to 35%
50% B + 50% Y	806	58	474	50	41%	50%	40 to 60%
25% B + 75% Y	703	16	239	46	66%	75%	65 to 85%
100% Y	469	29	8	2	98%	100%	>90%

As noted above, floc formation confounds size-separation, filtration strategies to differentiate fungal from bacterial contamination in fluid samples. Consequently, the next step was to identify a surfactant that would effectively disperse flocs without lysing bacterial or fungal cells. Experiments with Surfactant B at end-dilutions of 0.1 to 0.8% (v/v) indicated that at all of the concentrations tested, the surfactant lysed bacteria (data not shown). The proprietary Surfactant A was tested at concentrations of 0.045 to 1.8% (v/v). At 0.45% the product effectively dispersed bacterial flocs without lysing a significant number of cells (Table 4).

Table 4. Effect of Surfactant A on cellular ATP recovery and bacterial floc disaggregation.

Surfactant A Treatment (% v/v)	pg ATPmL				
	Undifferentiated Total	Differential Method			Total -Sum
		≥5.0 μm	<5.0 μm	Sum	
0	12,000±269	2,500±690	8,000±1,000	10,500	1,500
1.8	9,400±320	2,100±320	7,600±35	9,700	(300)
0.9	11,000±270	740±33	9,000±440	9,740	1,260
0.45	**10,700±320**	**890±145**	**9800±470**	**10,690**	**10**
0.045	12,600±180	2,300±1,220	9,300±200	11,600	1,000

Although preliminary studies, using *S. cerevisiae* s our model fungus, yielded promising results, there wanted to confirm that we'd obtain comparable results with filamentous fungi. We also wanted to further evaluate the impact of the proprietary surfactant on fungal cell integrity.

In the first stage of a two-stage investigation, *A. niger* and *P. chrysogenum* and the uncharacterized gram negative bacterium that had been used in the initial experiments were each grown in an emulsifiable oil (EO) MWF, diluted 5% (v/v) in deionized water. The two fungal populations were combined and the diluted MWF containing the fungal mixture were blended 1:1 with the MWF containing the bacterial growth. The microbial mixture was then split into two portions. One was treated with surfactant the other was not. Each portion was tested in triplicate by each of the two (filtration and non-filtration) ATP test protocols described above. In this test series, there was no attempt to differentiate between bacterial and fungal ATP.

The test results are summarized in Table 5. The ATP concentrations obtained with and without surfactant were analysed using ANOVA and Student's T statistics. The results of both computations supported the hypothesis that surfactant treatment had no significant effect on ATP recoveries. However, ASTM D7687 consistently recovered more ATP than did the non-filtration method (F_{obs} = 16.7; $F_{crit\ [1,11;\ \alpha = 0.05]}$ = 5.32). Routinely, D7687 results are reported in Log_{10} pg ATP mL^{-1}. The D7687 and non-filtration protocol yields were 3.99±0.07 Log_{10} pg ATP mL^{-1} and 3.78±0.11 Log_{10} pg ATP mL^{-1}, respectively. Although these yields were significantly different, the 0.21 Log gap between the two values would not normally affect result interpretation.

Due to the BSI variability, although BSI% averaged 18±5% for the surfactant-treated samples and 9.0±5% for the untreated samples, the effect of surfactant on BSI% was not significant at the 95% confidence level (F_{obs} = 4.37; $F_{crit\ [1,5;\ \alpha = 0.05]}$ = 7.71).

Table 5. Effect of surfactant treatment on total ATP recoveries by two ATP test methods (all results in pg ATP mL^{-1})

Parameter	Treatment	
	No-surfactant	**Surfactant**
tATP	7,000 ± 1,300	7,000 ± 1,800
dATP	600 ± 260	1,200 ± 150
cATP	6,000 ± 1,400	6,000 ± 1,800
BSI%	9 ± 5.3	18 ± 4.6
D7687 cATP	11,000 ±1,000	8,600 ± 520
F-ratio		2.16
F-crit $_{[1,\ 11;\ a\ =\ 0.05]}$		5.32
t-Test		0.92
t-crit $_{[df\ =\ 10;\ \alpha\ =\ 0.05]}$		2.28

The ASTM D7687 protocol includes the use of a proprietary rinsing solution to remove chemicals that might otherwise interfere with test results in complex organic fluids such as fuels and lubricants. The final series of laboratory experiments was designed to determine whether there was a significant interaction effect between the surfactant and rinse solution. The untreated and surfactant treated samples used in the previous experiment were tested using the bacterial-fungal ATP differentiation protocol. Half of each samples set was tested by D7687 modified by eliminating the rinse and air-drying steps. For this experiment, a total of six subsamples were tested for cATP.

Tables 6 and 7 provide the results of two-way ANOVA analysis of individual and interaction effects of ATP test protocol and surfactant treatment on the ≥5 μm and <5 μm fractions respectively. As in the previous experiment, surfactant treatment did not affect ATP yields. However, the D7687 and non-filtration protocols showed a small but significantly (difference between average Log_{10} pg ATP mL^{-1} = 0.22; (F_{obs} = 7.10; $F_{crit\ [1, 11; \alpha = 0.05]}$ = 5.32) different ATP recoveries. Average Log_{10} pg ATP mL^{-1} by D7687 was 0.22 less than Log_{10} pg ATP mL^{-1} by the non-filtration protocol; suggesting that the rinse step caused some cell lysis and consequent cATP recovery.

Table 6. Effect of ATP test method and surfactant use on fungal ATP (≥ 5.0 μm size fraction) recovery.

SUMMARY	No Surfactant	Surfactant	Total
D7687 w/o Rinse			
Count	3	3	6
Sum	10.82	10.82	21.64
Average	3.61	3.61	3.61
Variance	0.14	0.16	0.12
D7687			
Count	3	3	6
Sum	10.84	10.60	21.45
Average	3.61	3.53	3.57
Variance	0.01	0.07	0.03
Total			
Count	6	6	
Sum	21.66	21.43	
Average	3.61	3.57	
Variance	0.06	0.09	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
ATP Protocol	0.003	1	0.003	0.033	0.860	5.318
Surfactant use	0.005	1	0.005	0.050	0.828	5.318
Interaction	0.005	1	0.005	0.053	0.824	5.318
Within	0.747	1	0.093			
Total	0.760	11				

Table 7. Effect of ATP test method and surfactant use on bacterial ATP (< 5.0 µm size fraction) recovery.

SUMMARY	No Surf	Surf	Total
D7687 w/o Rinse			
Count	3	3	6
Sum	9.63	10.08	19.71
Average	3.21	3.36	3.29
Variance	0.00	0.01	0.01
D7687			
Count	3	3	6
Sum	9.48	9.02	18.51
Average	3.16	3.01	3.08
Variance	0.00	0.05	0.03
Total			
Count	6	6	
Sum	19.11	19.11	
Average	3.18	3.18	
Variance	0.00	0.06	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
ATP protocol	0.121	1	0.121	7.098	0.029	5.318
Surfactant use	0.000	1	0.000	0.000	0.996	5.318
Interaction	0.070	1	0.070	4.107	0.077	5.318
Within	0.136	8	0.017			
Total	0.327	11.000				

Based on the results obtained using a 1:1 blend of mold and bacterial cultures, the final experiment in this series was analogous to the experiment described above for different ratios of the mold and bacterial suspensions.

DISCUSSION

Detecting Dormant Cells

The results that we obtained, corroborated those previously reported by Min *et al.* [4], in that we found TSB and BHI to be satisfactory recovery media and maximal germination rates were observed within 15 min after placing endospore suspensions at 37ºC. However, we opted to use a 30min germination period. We made this decision based on the hypothesis that dormant cells other than endospores might require more time to become revitalized in the recovery medium. During the current investigation, >90% of the [ATP] concentration increase was observed within 10min. We therefore anticipate that the threefold increase in incubation time (double the time used by Min and his team [4]) should provide a sufficint recovery period for most – if not all – dormant microbes in industial process fluid samples. During the next phase of our research, we will test this theory using fuel, fuel-associated water, MWF and oilfield water samples.

Differentiating between Bacteria and Fungi

Earlier reports [5,6] have confirmed that [ATP] covary strongly with culture data. However, although differentiaition between bacterial and fungal ATP is important for the selection of appropriate microbicides, it has not been previously reported. In a 1981 American Society of Microbiology (ASM) annual meeting presentation, Passman demonstrated that size-differential filtration could be used to separate fungi from bacteria for testing catalase activity in MWF. However, Passman used filamentous fungi in that investigation. During the preliminary phases of the current study, bacterial floc formation was determined to be a significant factor, contributing to the retention of bacteria on 5.0 µm filters. Moreover, we determined that both surfactant chemistry and concentration affected bacterial ATP recoveries. At concentrations sufficient to disaggregate flocs, Surfactant B caused significant cell lysis as evidenced by significantly reduced ATP recoveries in 5.0µm filtrates. Intact, individual bacteria would be retained by the 0.7µm filter, and the total of [ATP] in the 5.0µm and 0.7µm filter extracts would be equal to that in the original sample (undifferentiated total).

At 1.8% and 0.9% (v/v) Surfactant A caused significant cell lysis. However at 0.45% (v/v) Surfactant A effectively disaggregated flocs without lysing significant percentages of the bacterial population (Table 4). Having optimized the protocol using individual bacterial and fungal cultures, and culture blends, the next step will be to further validate the method in the same range of fluids as listed apropos of the dormant cell protocol.

CONCLUSIONS

In this paper we have presented two methods that extend the usefulness and applicability of ASTM methods D7687 [1] and E2694 [7]. We have demonstrated 30 min pre-incubation period in TSB is sufficient to ensure that dormant cells have become reactivated, but have not yet begun to proliferate. We have also demonstrated that we can successfully differentiate between bacterial and fungal contamination in fluids that are likely to be contaminated with bacteria, fungi or both.

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