Application of the ATP Bioluminescence Method in Rapid Detection of Microorganisms in Jet Fuel

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Abstract: *Rapid detection of microorganisms in jet fuel can effectively prevent a series of problems caused by microbial contamination. In this paper, the application of the ATP bioluminescence method developed by the research group is systematically studied in the rapid detection of microorganisms in jet fuel. The results show that the ATP standard curve of the ATP bioluminescence method has good linear relationships with the standard curves of the colonies of five major contamination fungi and mixed bacteria (R^2 is all above 0.96), and that the ATP content in each single colony is on the order of magnitude of 10^{-17} mol, indicating that this method can achieve rapid and effective measurement of the total number of microorganisms in jet fuel. The method also proves t have good measuring performance in various aspects – the method detection limit is 8.8×10^{-17} mol and the repeatability and reproducibility is 29.35%, which are within the acceptable range; the fluorescence reaction is highly stable, and a fluorescence decay of 5% can be guaranteed within 60 s. This method has little difference in field detection with the HY-LITE® JET A1 fuel test method recommended by IATA, and what is more, it is accurate, stable and lost-cost, making it effective as an alternative method. It is an independent intellectual property of China and can be recommended as a method for rapid detection of microorganisms in jet fuel in national standards.*

Keywords: *ATP bioluminescence method, Jet fuel, Contamination fungi, Detection.*

Introduction

The rapid detection of microorganisms is of great significance for the safe storage and use of jet fuel [10, 21]. Currently, the internationally accepted detection methods for microorganisms in jet fuel are IP 385 [7] (United Kingdom Petroleum Industry Association) and ASTM D6974 (American Society for Testing and Materials) [1]. Despite their accuracy and effectiveness, these methods still cannot meet the needs of our military in the field detection due to the complicated operations, long culture period and high requirements for the operators. It is in urgent need to develop a rapid detection method for microorganisms in jet fuel that is fast, accurate and easy to operate. The ATP bioluminescence method has been gradually adopted by researchers because of its simple operations and accurate test results ever since its discovery in the 1940s [11, 15]. After more than half a century of development, the ATP bioluminescence method has become relatively mature in both theory and equipment, and thus it is widely used in food hygiene, medicine and other fields [5, 6, 22].

The luciferase-catalyzed luminescence reaction of the ATP bioluminescence method is a multi-step catalytic reaction [17]. Its basic reaction principle [2, 4, 8] is that, under the joint catalytic action of luciferase and Mg^{2+}, ATP, fluorescein and oxygen molecules react and produce fluorescence with a certain wavelength range (562-570 nm). Adenosine triphosphate (ATP), as a source of energy for life activities, exist extensively in all living organisms. Studies have found that [12, 16] different types of microorganisms all contain stable contents of ATP, which means the content of ATP can reflect the number of microorganisms. In addition, there is a good linear relationship between the viable cell count and the ATP luminescence value when ATP is within a certain range [13, 14], so the ATP bioluminescence method can be applied for rapid detection of microorganisms in jet fuel. This study, by taking the five major contamination microorganisms in jet fuel [9] as the objects, attempts to explore the application effect of the ATP bioluminescence system developed and optimized by the research group in the rapid detection of microorganisms in jet fuel.

Test materials and methods

Materials

No.3 jet fuel used in the test, sampled from an oil depot in Chongqing; amorphotheca resinae, alternaria alternate, penicillium restrictum, aspergillus penicillioides and chaetomium globasum, from China Center of Industrial Culture Collection; 0.1 mol/L ATP reference material, from Sigma-Aldrich (U.S.); and Sabouraud's medium (4 g glucose, 1 g peptone, 1.5 g agar, 3rd grade water filled to 100 ml, and high temperature sterilization at 115 ℃ for 30 min), were prepared for later use in the lab.

HY-LITE 2 fluorescence detector, from Shanghai Office of Merck Group (Germany); ATP fluorescent detection pen, from Hefei Dianfeng Biotechnology Co., Ltd.; Tomy SX autoclave sterilizer, from Tomy Digital Biology (Japan); Genex pipette (20 μL, 100 μL and 100 Ml), from MicroShine Scientific Instruments; SPX-150 thermostatic incubator, from Beijing Houhui Laboratory Instrument Technology Co. Ltd.; ZCZY-CS stackable incubator shaker, from Shanghai Zhichu Instruments Co., Ltd.; Milli-Q pure water filter, from Millipore (U.S.); and SJ-CJ-3FD clean bench, from Su Jie Medical Equipment (Suzhou, China) Co., Ltd.

The compositions of the microbial extraction agent, the cracking agent [20] and the luciferase reaction system are as follows (Tables 1-3).

Table 1. Composition of the microbial extraction agent

Component	Mass fraction	Manufacturer
Tween 80	0.12%	Shanghai Aladdin Bio-Chem Technology Co., Ltd.
Dimethicone	0.08%	
Brilliant green	0.02%	

Table 2. Composition of the microbial cracking agent

Component	Mass fraction	pH	Manufacturer
BAB	0.05%	8.5	Shanghai Aladdin Bio-Chem Technology Co., Ltd.
Chlorhexidine	0.025%		Shanghai Macklin Biochemical Co., Ltd.
$CuSO_4$	0.015%		

Table 3. Composition of the luciferase reaction system

Component	Mass fraction/ concentration	pH	Manufacturer
Luciferase	0.05 mg/mL	7	Hefei Dianfeng Biotechnology Co., Ltd.
Fluorescein	0.08 mg/mL		Sangon Biotech (Shanghai) Co.,Ltd.
Glycylglycine	25 mmol/L		
BSA	0.5 mg/mL		
DEAE-Dx	0.1%		
$MgSO_4$	5 mmol/L		Shanghai Aladdin Bio-Chem Technology Co., Ltd.
DTT	7.5 mmol/L		

Test methods

Plotting of the ATP standard curve

Prepare a series of ATP standard solutions (3E-8, 1E-8, 3E-9, 1E-9, 3E-10, 1E-10, 3E-11 and 1E-11 mol/L) and divide them into two groups, one containing only ATP reference material (RM), and the other containing both ATP RM and the cracking agent. In each test, mix 25 μL of ATP standard solution with 400 μL of the optimum luciferase reaction system and oscillate it for 10 s, and then measure the relative luminescence intensity. For each group, the test is repeated 3 times, with the results averaged as the basis for plotting of the ATP standard curve.

Plotting of the standard curves of colonies of the five major contamination fungi and mixed fungi

Take amorphotheca resinae for example. Take out the culture solution of amorphotheca resinae cultivated in the 35 ℃ oscillating table for 3 days, and dilute it to fungal solutions at different ratios (1:2, 1:10, 1:20, 1:100 and 1:200 respectively) with high-temperature sterilized normal saline. Take out 1 mL of fungal solutions with different dilutabilities and let them react with 1 mL of cracking agent for 2 min, and then take 50 μL of the mixture using the pipette for fluorescence detection, and at the same time perform plate count of the amorphotheca resinae solutions with different dilutabilities by reference to the method provided in GB 4789.2-2016 *National Food Safety Standard – Microbiological Examination of Food: Aerobic Plate Count* [3]. Based on the above, plot the standard curves of the amorphotheca resinae colony count and the relative luminous intensity.

Steps of detection by the ATP bioluminescence method

The steps of the ATP bioluminescence method to detect microorganisms in jet fuel are as follows:

1. Sampling. Each time take 1 L of jet fuel sample from the bottom of the oil depot or fuel tanker into the sample bottle. To ensure the reliability and stability of the test results, samples shall be taken from the same position each time.
2. Microbial extraction. Absorb 1 mL of the prepared extraction agent with the pipette and add it all to the 1 L sample bottle, close the lid and then shake it hard for 30 s. Afterwards let the sample bottle stand for 5 min, and then absorb all of the blue extraction agent precipitated at the bottom of the bottle with a long pipette.
3. Microbial cracking. Mix the 1 mL microbial extraction agent fully absorbed out with the 1 mL microbial cracking agent for 2 min to fully achieve the microbial cracking effect and make all the intracellular soluble matters (including ATP) outflow to minimize test errors.

4. ATP fluorescence reaction. Extract 50 μL of the mixture of the extraction agent and the cracking agent into a test tube, and then add 400 μL of the prepared luciferase reaction liquid, shake it for 10 s and then place it in the fluorescence detector for determination of the relative luminous intensity.

Notices:

1. Where the test conditions permit, the microbial extraction and cracking process should be carried out on a clean bench, and UV sterilization shall be performed for more than half an hour beforehand. The testers should wear gloves to prevent the environment and the bacteria on the skin of the hands from impacting the microorganisms in jet fuel and making the measurement results high.
2. The microbial extraction, cracking and ATP fluorescence reactions all involve shaking or oscillating operations. The testers should achieve sufficient oscillation effect within the specified time, and try to keep the oscillation amplitude and frequency consistent.
3. The microbial extraction agent, cracking agent, and luciferase reaction solution should be used within their respective specified time periods to avoid any reagent failure, which may lead to the inability to detect fluorescence values.

Measuring performance characterization of this method

(1) Method detection limit (MDL)

MDL is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the response signal of the analyte can be distinguished from that of the blank sample [18]. In this test, the MDL was determined through blank tests. The cracking agent and the luciferase reaction system were added successively according to the operation specifications. A total of 10 blank parallel determinations were performed, and the MDL was calculated according to the Eq. (1) below.

$$MDL = t_{(n-1,0.99)} \times S, \tag{1}$$

where t is the t-distribution with a degree of freedom (DOF) of $n - 1$ and a confidence level of 99%; n – number of sample parallel determinations; S – standard deviation of the parallel determinations.

(2) Repeatability and reproducibility (*R&R*)

Combining repeatability and reproducibility [19] in detection is to reduce the measurement errors and make the detection value as close as possible to the true value. Repeatability refers to the measurement variation under the "same four" conditions – the same target materials measured by the same operating personnel, with the same operating instrument, according to the same steps. Reproducibility refers to the variation of the mean value when the same analyte is measured with the same operating instrument, according to the same steps, but by different operating personnel.

In this study, the laboratory-simulated jet fuel contaminated by microorganisms were taken as the objects. Students from 3 different disciplines were asked to measure each of the 10 contaminated fuel samples 3 times respectively, and calculate the %*R&R* using Eqs. (2)-(5):

$$EV = \bar{R} \times K_1, \tag{2}$$

$$AV = \sqrt{(\bar{X}_{DIFF} \times K_2)^2 - (EV^2/nr)}, \tag{3}$$

$$R\&R = \sqrt{EV^2 + AV^2}\,, \tag{4}$$

$$\%R\&R = \frac{R\&R}{TV} \times 100\,, \tag{5}$$

where EV is the repeatability; AV – reproducibility; $\bar{R}$ – overall mean of ranges; X_{DIFF} – range of mean values; K_1, K_2 – constants, which can be obtained from the constant table of the (X-R) chart; TV – total variation.

(3) Fluorescence stability (FS)
FS is a measure of the fluorescence decay of the relative luminous intensity over time. With the lab-simulated jet fuel contaminated by microorganisms as the objects, measurements were carried out for 15 consecutive times without the detection tube being taken out according to the steps of detection by ATP bioluminescence method, and the result was expressed as the fluorescence decay rate. The calculation formula is shown in Eq. (6):

$$\text{Decay Rate (\%)} = (RLU_1 - RLU_2)/RLU_1 \times 100\%, \tag{6}$$

where RLU_1 is the detection value of the maximum relative luminous intensity and RLU_2 is the detection value of the relative luminous intensity at another time.

Application of the ATP bioluminescence method in actual detection
With both the ATP RM and the jet fuel samples taken from the military oil depot, this paper explored the adaptability and accuracy of this ATP bioluminescence method in actual detection applications, and compared it with the HY-LITE® JET A1 fuel test method recommended by IATA.

(1) ATP RM
ATP standard solutions with different concentrations (3E-8, 1E-8, 3E-9, 1E-9, 3E-10, 1E-10, 3E-11 and 1E-11 mol/L) were prepared, and then underwent fluorescence detection by this ATP bioluminescence method and the HY-LITE® JET A1 fuel test steps (the cracking agent was involved in both cases). Each group was tested 3 times and the results were averaged.

(2) Jet fuel samples from a military oil depot
The jet fuel samples taken from the bottom of Tank #5, #6 and #7 in an oil depot in Chongqing were respectively tested by the ATP bioluminescence method and the HY-LITE® JET A1 fuel test steps to explore the adaptability and accuracy of this method in the actual detection of microorganisms in jet fuel and its differences with the HY-LITE® JET A1 fuel test method.

Results and discussion

Plotting of ATP standard curve

As can be seen from Fig. 1, the luciferase reaction system maintained good enzymatic activity regardless of the presence or absence of the cracking agent. In the range of 10^{-11} to 10^{-8} mol/L, the ATP concentration showed a good linear relationship with the relative luminous intensity, and it can be seen from the test data that 1 RLU was roughly equal to the fluorescence produced by 10^{-17} mol of ATP. By referring to this curve, we can obtain the ATP concentration of the reaction substrate using the fluorescence values obtained in the subsequent tests. The linear correlation coefficient of the ATP standard curve without the

cracking agent was R^2 = 0.9946, while that of the ATP standard curve with the cracking agent was R^2 = 0.9803, which shows that the luciferase reaction system has good adaptability to the cracking agent; in addition, the two-sample T test was performed using the software Origin on the two groups of relative luminous intensity measured without and with the cracking agent, respectively, and it was found that there was no significant difference between the two groups of data (P = 0.6877 > 0.05). Therefore, it can be concluded that this luciferase reaction system can well address inhibitory effect of the cracking agent on the luciferase activity, improving the accuracy of the ATP bioluminescence method in detection of microorganisms in the jet fuel.

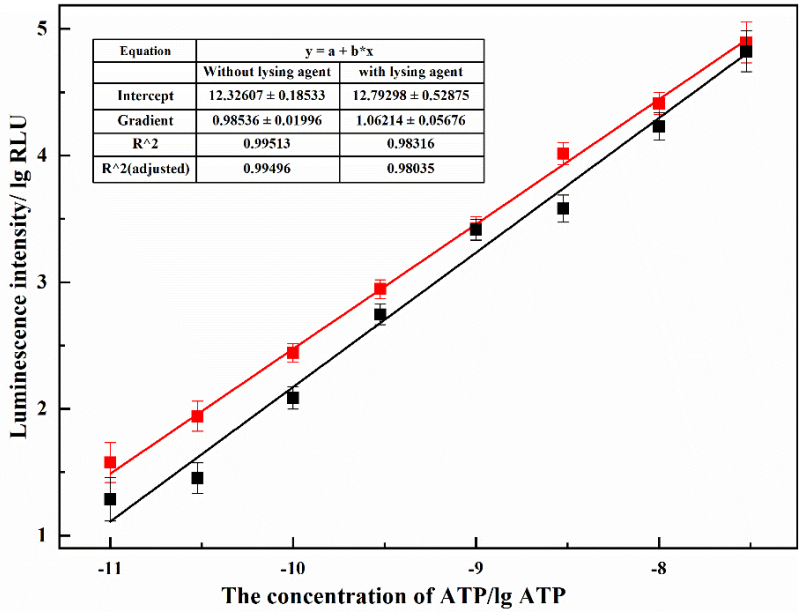

Fig. 1 ATP standard curve.
The red line is the ATP standard curve of the solution with no cracking agent, while the black line is that of the solution with the cracking agent.

Plotting of the standard curves of colonies of the five major contamination fungi and the mixed fungi

The standard curves of colonies of the five major contamination fungi and the mixed fungi were plotted respectively. First take *Amorphotheca resinae* for example. From Fig. 2, it can be seen that the colony count of *Amorphotheca resinae* was in a good linear relationship with the relative luminous intensity (R^2 = 0.9816), presenting a high linear fitting degree. The data obtained and the analysis in Fig. 2 show that the ATP content of a single *Amorphotheca resinae* colony was approximately 4×10^{-17} mol.

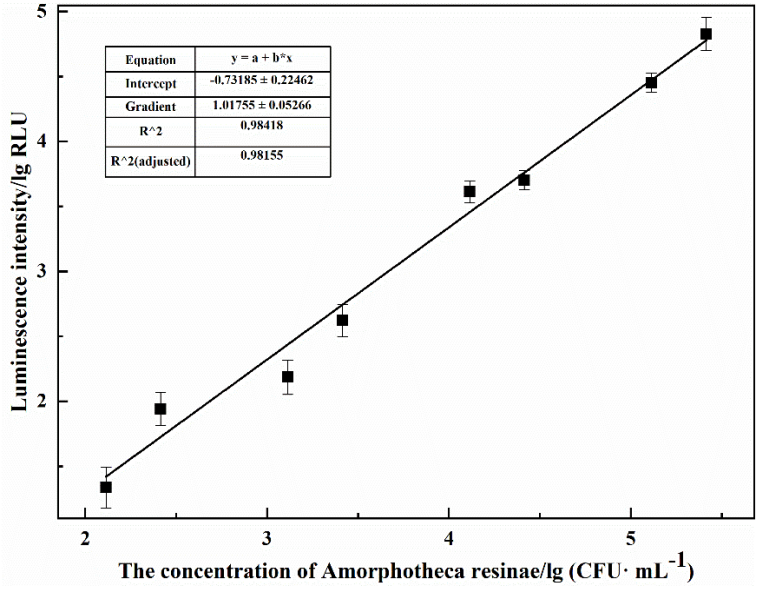

Fig. 2 Standard curve of the *Amorphotheca resinae* colony

It can be seen from Figs. 3-6 that there were also good linear relationships between the colony counts of *Penicillium restrictum*, *Aspergillus penicillioides*, *Alternaria alternate* and *Chaetomium globasum* and the relative luminous intensity (R^2 is all between 0.96 and 0.98), and the contents of ATP in different types of fungi were on the same order of magnitude, except with some slight differences. The ATP content of a single *Penicillium restrictum* colony was about 4.5×10^{-17} mol, that for *Aspergillus penicillioides* roughly 5×10^{-17}mol, that for *Alternaria alternate* about 4.5×10^{-17} mol, and that for *Chaetomium globasum* is 3.5×10^{-17} mol. At the same time, it can be seen from Fig. 7 that there was also a good linear relationship between the colony count of mixed fungi and the relative luminous intensity (R^2 = 0.97559), and that the average ATP content of mixed fungi was 4×10^{-17} mol/CFU. From the above, it can be concluded that the major contamination fungi in jet fuel all have good linear relationships with the ATP fluorescence value, and that the ATP content is all on the order of magnitude of 10^{-17} mol/CFU. Therefore, the ATP bioluminescence method is highly reliable and accurate in the rapid detection of microorganisms in jet fuel. It avoids the under-estimation of microbial contamination caused by different ATP contents of fungi, and is able to quickly, effectively and accurately estimate the total number of contamination microorganisms in jet fuel.

Measuring performance characterization of the method

(1) MDL

As can be seen from Table 4, the minimum substance concentration that could be measured with 99% confidence that the fluorescence signal generated by ATP detection could be distinguished from that of the blank sample was 8.8×10^{-17} mol, and the difference between any detection values in the blank test was within "the result of the mean determined in the blank test ± 1/2 of the estimated detection limit" [18], which was statistically significant. Therefore, it is concluded that the MDL of the ATP bioluminescence method in the rapid detection of microorganisms in jet fuel is 8.8×10^{-17} mol at the 99% confidence level.

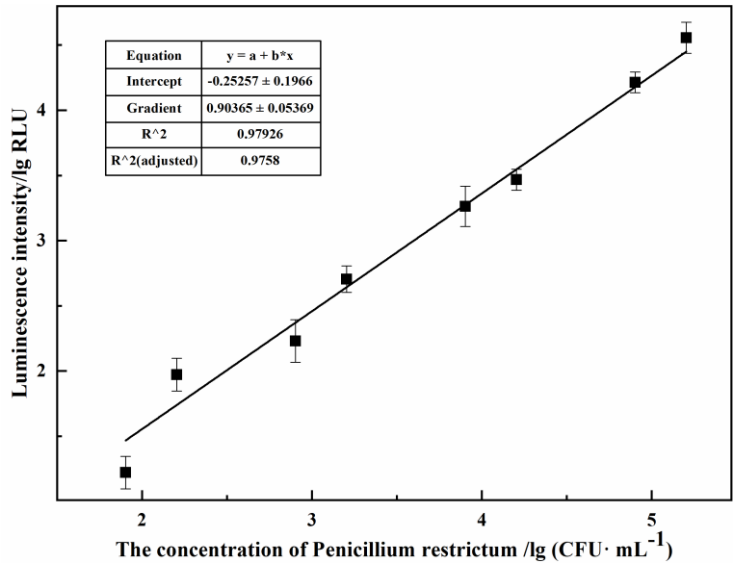

Fig. 3 Standard curve of the *Penicillium restrictum* colony

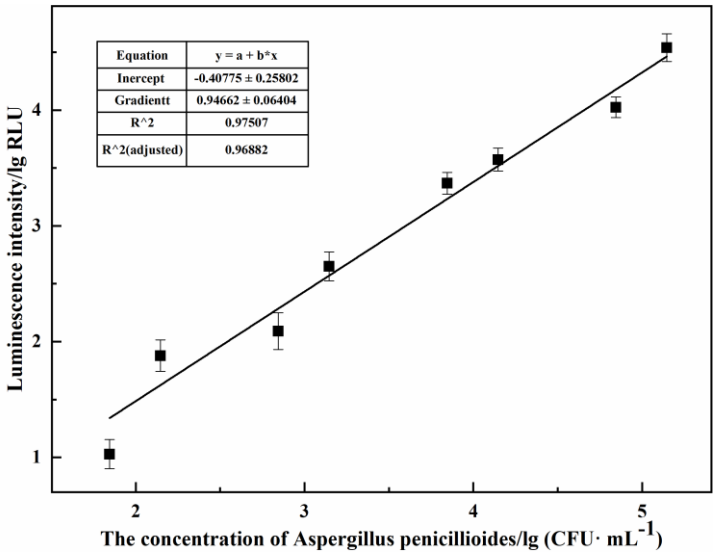

Fig. 4 Standard curve of the *Aspergillus penicillioides* colony

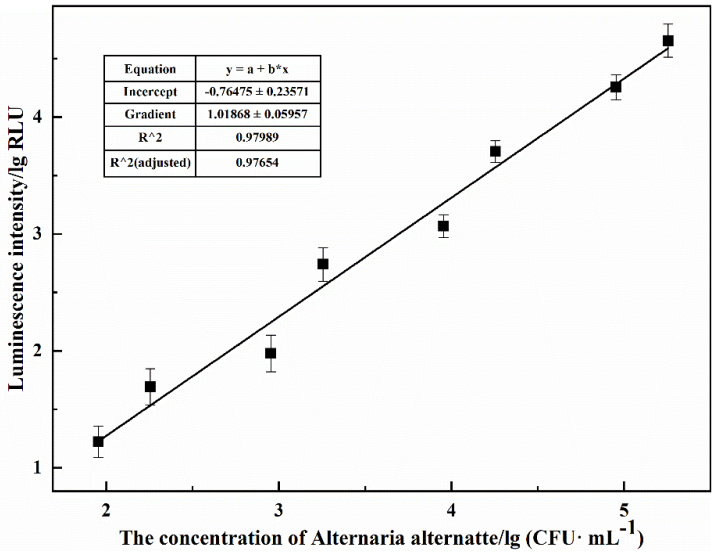

Fig. 5 Standard curve of the *Alternaria alternate* colony

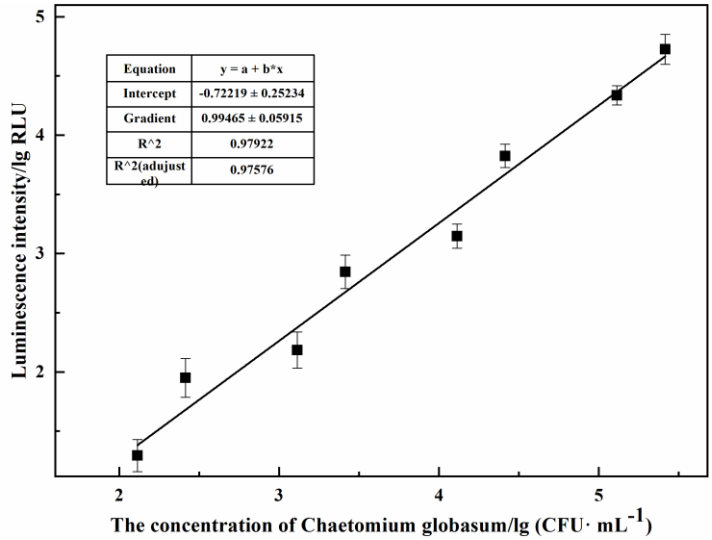

Fig. 6 Standard curve of the *Chaetomium globasum* colony

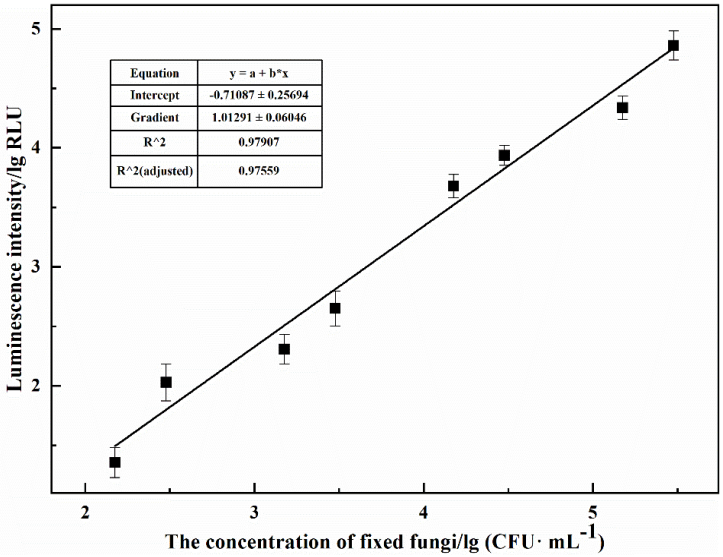

Fig. 7 Standard curve of the mixed fungi colony

Table 4. MDL calculation table

No.	RLU	T, (9, 0.99)	S	MDL, (RLU)	MDL, (ATP/mol)
1	18	2.821	3.134	8.841	8.8×10^{-17}
2	26				
3	24				
4	21				
5	18				
6	22				
7	23				
8	25				
9	26				
10	19				

(2) *R&R*

According to Table 5 and Table 6, the repeatability (%EV) was 24.82%, and the reproducibility (%AV) 15.65%, and the percentage of repeatability variation in total variation was significantly higher than that of reproducibility variation, which indicates that the variation caused by equipment was significantly higher than that caused by personnel, probably due to the fact that the HY-LITE 2 fluorescence detector displayed only two significant digits in the thousands (the figure was shrunk by 100 times), leading to slight fluctuations in the detection data of the same sample in multiple tests. The %*R&R* was 29.35%, which was between 10% and 30%. According to the acceptance criteria, and the importance of application, the costs of the measuring devices and the maintenance costs and the prospect that the instrument accuracy and the standardization of the detection method will be further improved, this detection system can be considered acceptable.

Table 5. *R&R* datasheet

Testing personnel	**Number of tests**	**Number of contaminated fuel samples n/each**										**Mean**
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	
A	1	38	38	40	38	39	39	36	34	39	39	38
	2	39	38	38	37	37	39	38	34	38	40	37.9
	3	36	38	38	39	38	39	38	36	37	40	37.9
	X_n	37.7	38	38.7	38	38	39	37.3	34.7	38	39.7	$\bar{X}_A$ = 37.9
	R_n	3	0	2	2	2	1	2	2	2	1	$\bar{R}_A$ = 1.6
B	1	36	38	38	37	39	39	38	35	37	39	37.6
	2	36	38	38	39	38	39	36	34	37	40	37.5
	3	37	37	38	39	38	38	38	34	37	38	37.4
	$\bar{X}_n$	36.3	37.7	38	38.3	38.3	38.7	37.3	34.3	37	39	$\bar{X}_B$ = 37.5
	R_n	1	1	0	2	1	1	2	1	0	2	$\bar{R}_B$ = 1.1
C	1	38	39	38	36	36	36	37	34	38	38	37
	2	38	38	38	36	38	38	38	34	38	40	37.6
	3	37	39	39	36	38	36	38	35	38	40	37.6
	$\bar{X}_n$	37.7	38.7	38.3	36	37.3	36.7	37.7	34.3	38	39.3	$\bar{X}_C$ = 37.4
	R_n	1	1	1	0	2	2	1	1	0	2	$\bar{R}_C$ = 1.1
$\bar{\bar{X}}_n$		37.2	38.1	38.3	37.4	37.9	38.1	37.4	34.4	37.7	39.3	
Overall average $\bar{\bar{X}} = \left(\bar{X}_A + \bar{X}_B + \bar{X}_C\right)/m = (37.9 + 37.5 + 37.4)/3 = 37.6$												
Mean of overall range $R = \left(\bar{R}_A + \bar{R}_B + \bar{R}_C\right)/m = (1.6 + 1.1 + 1.1)/3 = 1.27$												
Sample range $R_P = \max\{\bar{\bar{X}}_n\} - \min\{\bar{\bar{X}}_n\} = 39.3 - 34.4 = 4.9$												
Range of mean values $X_{DIFF} = \max\{\bar{X}_{A,B,C}\} - \min\{\bar{X}_{A,B,C}\} = 37.9 - 37.4 = 0.5$												

Table 6. *R&R* report

<table>
<tr><th colspan="3">Test unit analysis</th><th>Total variation, % (TV)</th></tr>
<tr><td colspan="2">Repeatability (EV)</td><td>Variation caused by equipment</td><td rowspan="4">%EV = 100(EV/TV) =
= 100×(2.056/8.285) = 24.82%</td></tr>
<tr><td rowspan="3">$EV = \bar{R} \times K_1 =$
$= 1.27 \times 3.05 = 2.056$</td><td>Number of tests r</td><td>K_1</td></tr>
<tr><td>2</td><td>4.56</td></tr>
<tr><td>3</td><td>3.05</td></tr>
<tr><td colspan="2">Reproducibility (AV)</td><td>Variation caused by personnel</td><td rowspan="4">%AV = 100(EV/TV) =
= 100×(1.297/8.285) = 15.65%</td></tr>
<tr><td colspan="3">$AV = \sqrt{(\bar{X}_{DIFF} \times K_2)^2 - (EV^2 / nr)} =$
$= \sqrt{(0.5 \times 2.7)^2 - 2.056^2 / 30} = 1.297$</td></tr>
<tr><td rowspan="3"></td><td>Testing personnel m</td><td>K_2</td></tr>
<tr><td>2</td><td>3.65</td></tr>
<tr><td>3</td><td>2.70</td></tr>
<tr><td colspan="3">R&R</td><td rowspan="2">%R&R = 100(R&R/TV) =
= 100×(2.431/8.285) = 29.35%</td></tr>
<tr><td colspan="3">$R\&R = \sqrt{EV^2 + AV^2} =$
$= \sqrt{1.297^2 + 2.056^2} = 2.431$</td></tr>
<tr><td rowspan="4">Between-sample variation (PV)
$PV = R_P \times K_3 = 4.9 \times 1.62 = 7.92$</td><td>Sample size</td><td>K_3</td><td rowspan="11">%PV=100(PV/TV) =
= 100×(7.92/8.285) = 95.60%</td></tr>
<tr><td>2</td><td>3.65</td></tr>
<tr><td>3</td><td>2.70</td></tr>
<tr><td>4</td><td>2.30</td></tr>
<tr><td rowspan="7">Total variation (TV)
$TV = \sqrt{(P\&R)^2 + (PV)^2}$
$= \sqrt{2.431^2 + 7.92^2} = 8.285$</td><td>5</td><td>2.08</td></tr>
<tr><td>6</td><td>1.93</td></tr>
<tr><td>7</td><td>1.82</td></tr>
<tr><td>8</td><td>1.74</td></tr>
<tr><td>9</td><td>1.67</td></tr>
<tr><td>10</td><td>1.62</td></tr>
</table>

Note: 1. All calculations in the table are predictions based on 5.15σ (99.0% of the area of the normal distribution curve).

2. Acceptance criteria:

- (%*R&R*) if the error is less than 10%, the measuring system is usually considered acceptable;
- (%*R&R*) if the error is between 10% and 30%, the system may be considered acceptable considering the importance of application, the costs of the measuring devices and the maintenance costs;
- (%*R&R*) if the reading is over 30%, the system is considered unacceptable, and various efforts should be made to improve the measuring system.

(3) FS

It can be seen from Fig. 8 that, for the mixed fungi, the first detection result was not the maximum value of the relative luminous intensity, but the third or fourth detection result reached the peak instead, regardless of the concentration of the mixed fungi. The peak lasted in 3 consecutive detections and for about 30 s (about 10 s in each detection). Therefore, 3 to 5

detections are required to pick the maximum value as the final test result. The fluorescence decay requirement of 5% can be met in the 2nd to the 8th detections of the relative luminous intensity, with an FS duration of 60 s, which basically met the "glow" type illumination requirement and reduced the test error brought by time. From the detection data, it can be seen that in the decay process after the relative luminous intensity reaches the maximum value, the trend showed a certain pattern: when the detection value was in the place of ten thousands, the difference in the detection values between adjacent detections was about 1000, and when the detection value was in the place of thousands, the difference was around 100; and when the detection value was in the place of hundreds, the difference was around 10. This may have something to do with the data processing method of the HY-LITE 2 fluorescence detector, in which, only two significant digits are retained.

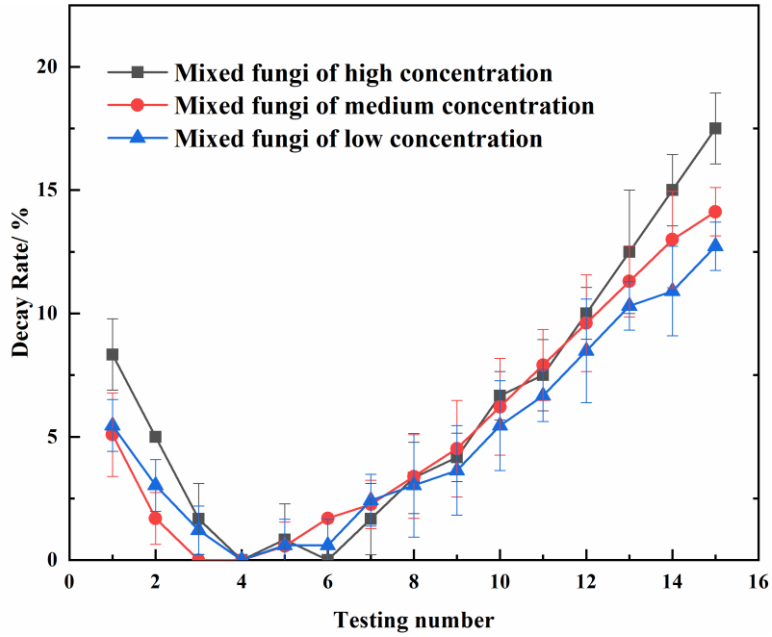

Fig. 8 Fluorescence decay trend chart

Application of the ATP bioluminescence method in actual detection

(1) ATP RM

As can be seen from Table 7, the measurement results of the HY-LITE® JET A1 fuel test method were generally higher than those of the ATP bioluminescence method, but the linear correlation coefficient of the latter (R^2 = 0.98949) was slightly higher than that of the former (R^2 = 0.98035). The reason may be that the ATP bioluminescence method used 1 mL of cracking agent and 1 mL of microbial-containing extraction agent for microbial cracking and extracted the ATP in vivo, and thus the cracking agent inevitably appeared in the luciferase reaction system, thereby bringing less impact on the luciferase activity (it has already proved that whether there is a cracking agent brings no significant difference in fluorescence detection). In the HY-LITE® JET A1 fuel test method, on the other hand, cracking occurred in the groove of the detection pen, and the cracking agent would not flow into the luciferase reaction system, which separated the cracking agent that already worked and the luciferase reaction system and thus reduced the impact of the cracking agent on the activity of luciferase. Therefore, the detection pen for the ATP bioluminescence method should be later designed to incorporate this advantage to minimize the inhibitory effect of the cracking agent on the activity of luciferase.

Table 7. ATP RM analysis and comparison table

ATP concentration, mol/L	ATP bioluminescence method, RLU	HY-LITE® JET A1 fuel test, RLU	Linear equations and correlation coefficients
3E-8	66333.33	68333.33	ATP bioluminescence method: Lg(RLU) = = 0.94167×Lg(ATP) + 11.83613 R^2 = 0.98949
1E-8	17000	21333.33	
3E-9	6033.33	6733.33	
1E-9	1633.33	1866.67	
3E-10	556.67	816.67	HY-LITE® JET A1 fuel test: Lg(RLU) = = 1.06214×Lg(ATP) + 12.79298 R^2 = 0.98035
1E-10	153.33	206.67	
3E-11	56.67	96.67	
1E-11	19.33	51	

(2) Jet fuel samples from the military oil depot

As can be seen from Table 8, the ATP bioluminescence method has good adaptability, accuracy and repeatability in the detection of jet fuel samples from the military oil depot. On the whole, this method delivered lower detection results than those of the HY-LITE® JET A1 fuel test method, for which the reason is preliminarily believed to lie in the microbial extraction effect of the extraction agent. The previous experiments of the research group showed that the extraction efficiency of the extraction agent was 73.8%, which needed to be further improved. In addition, the coefficient of variation CV of the ATP bioluminescence method was lower than that of the HY-LITE® JET A1 fuel test, with a good measurement repeatability. This study verified the application effect of the ATP bioluminescence method in the field detection of microorganisms in jet fuel, which laid a good foundation for the development of subsequent supporting instruments and the popularization of the method.

Table 8. Actual detection datasheet of jet fuel samples

Fuel sample	Method	1	2	3	CV, %
Tank 5#	ATP bioluminescence method	2700	2700	2600	2.2
	HY-LITE® JET A1fuel test	2900	2800	2900	2.0
Tank 6#	ATP bioluminescence method	2900	2900	2700	4.1
	HY-LITE® JET A1fuel test	3000	2900	2900	5.3
Tank 7#	ATP bioluminescence method	810	800	780	1.9
	HY-LITE® JET A1fuel test	900	850	850	3.3

Conclusions

This paper performed lab studies and field detection for the application of the ATP bioluminescence method in the rapid detection of microorganisms in jet fuel. The test results are as follows:

(1) Where the cracking agent existed, the linear correlation coefficient of the ATP standard curve was R^2 = 0.9946, and it was not significantly different from that when there is no cracking agent. This optimized luciferase reaction system can well reduce the inhibitory effect of the cracking agent on the luciferase activity.

(2) The standard curves of colonies of the five major contamination fungi and the mixed fungi in jet fuel were plotted. The ATP content of a single colony was on

the order of magnitude of 10^{-17} mol, and the linear relationship was good, with a linear correlation coefficient of over 0.96. Therefore, this method can quickly and accurately measure the total number of microorganisms in jet fuel.

(3) A set of steps was preliminarily developed to detect the microorganisms in jet fuel by the ATP bioluminescence method, and several notices were proposed.

(4) The detection limit of this method was 8.8×10^{-17} mol, and the *%R&R* 29.35%, which were within the acceptable range; the fluorescence reaction stability was high, and the FS duration was up to 60 s with a guaranteed 5% fluorescence decay rate, which basically met the "glow" type illumination requirement.

(5) Whether for ATP RM or the jet fuel samples from the military oil depot, the detection results delivered by this ATP bioluminescence method were not significantly different from those by the HY-LITE® JET A1 fuel test method, indicating that it basically meets the detection accuracy and stability requirements. What is more, it is also low-cost. Therefore, this method has the potential to be the alternative method to the HY-LITE® JET A1 fuel test, and it also provides theoretic basis for the development of China's own rapid detection method for microorganisms in jet fuel in the future.

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