Lysing Agents for Characteristic Polluting Microorganisms in Jet Fuels

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Abstract: *The adenosine triphosphate (ATP) bioluminescence assay is a feasible way to quantify the characteristic polluting microorganisms of the jet fuels. The effect of this method hinges on the release of ATP through the effective lysis of microorganisms. To achieve a good microbial lysis effect, this paper explores the fungi lysis effects of two quaternary ammonium salts, benzyldodecyldimethylammonium bromide (BAB) and benzalkonium chloride (BAC), and discusses the impacts of the two cationic surfactants on luciferase activity. The results show that BAB was more effective than BAC in the lysis of the characteristic polluting fungi, putting the optimal BAB concentration at 0.05%; traditional lysing agents like cetyl trimethyl ammonium bromide (CTAB) and trichloroacetic acid (TCA) had poor effects on fungi lysis; 0.025% Hibitine and 0.05% BAB exerted a synergistic effect on the lysis of characteristic polluting fungi, and were identified as the preliminary components of the lysing agent for the characteristic polluting microorganisms of jet fuels; the optimized luciferase-luciferin reaction system effectively mitigated the suppression effect of the lysis agent on enzymatic activity, and the ATP standard curves with or without lysing agent had no significant difference ($p = 0.9768 > 0.05$). The optimized luciferase-luciferin reaction system, coupled with the mixture of BAB and Hibitine, outshines the traditional plate culture method and HY-LiTE JET A1 Fuel Test in detection efficiency and cost.*

Keywords: *Jet fuels, Microbial contamination, Adenosine triphosphate, Adenosine triphosphate bioluminescence assay, Luciferase.*

Introduction

In recent years, microbial contamination has induced more and more problems in the use and storage of jet fuels. The presence of microorganisms in jet fuels may damage aircraft engines, clog fuel filters, cause fuel gauge failures, and corrode oil storage and transport equipment [4-6]. Our research team carried out a nationwide survey, and collected jet fuel samples with 98 pieces of filter membranes from eight provinces from north to south across China. The microbial species of the samples were analyzed by third-generation high-throughput sequencing, and the characteristic polluting microorganisms of the jet fuels were identified at the genus level. The results show that the top five fungal genera are: *Cladosporium* (25.03%), *Alternaria* (21.89%), *Penicillium* (18.54%), *Aspergillus* (10.57%) and *Chaetomium* (7.12%).

The high-throughput sequencing shows that the above five fungi account for over 80% of all polluting microorganisms of the jet fuels.

The adenosine triphosphate (ATP) bioluminescence assay is a feasible way to quantify the characteristic polluting microorganisms of the jet fuels, thanks to the good linearity between the number of living cells and ATP luminescence, when the ATP content remains in a certain range [12, 13]. In fact, the ATP bioluminescence assay is a combination between the light-emitting mechanism of fireflies with the positive correlation between ATP content and the number of microorganisms. For one thing, the fluorescence of fireflies, with a wavelength between 550 nm and 565 nm, is produced through the reaction between the ATP, fluorescein and oxygen molecules under the catalysis of luciferase and Mg^{2+} [2, 3, 11]; for another, the ATP content is positively correlated with the number of microorganisms because the content of the ATP, an energy currency in microbial cells, is stable in any type of microorganism, and the ATP released once a microorganism dies will be degraded by the ATPase in the microorganism or utilized by other microorganisms [8, 14].

The ATP assay directly relies on the release of ATP from cell lysis, which is currently driven by microbial lysing agents (surfactants). An ideal microbial lysing agent should enable fast and complete lysis, exert no significant impact on luciferase activity and inactivate ATPase irreversibly, without damaging the ATP [19]. The available microbial lysing agents include cetyl trimethyl ammonium bromide (CTAB) [7], trichloroacetic acid (TCA) [20], benzyldodecyldimethylammonium bromide (BAB) [15] and benzalkonium chloride (BAC) [16]. However, these agents have been mainly used to lyse bacteria, especially gram-positive bacteria, and rarely for fungi lysis. Neither are they frequently mentioned in the studies on detecting characteristic polluting microorganisms in jet fuels.

In actual applications, cationic surfactants, particularly quaternary ammonium salts like the BAB and the BAC, have gradually become the most popular microbial lysing agents. Studies have shown that the BAC is as effective as the TCA in ATP acquisition through bacteria lysis [9]. Therefore, this paper lyses the characteristic polluting fungi in jet fuels with quaternary ammonium salts, and examines the synergistic/antagonistic effect of the salts with other lysing agents. With the ATP standard curve, the author investigated the impacts of self-made lysing agents on the enzymatic activity of the luciferase-fluorescein reaction system, laying the basis for accurate determination of the number of the characteristic polluting microorganisms in jet fuels.

Materials and method

Main reagents and instruments

Cladosporium, lab preserved strain; *Penicillium restrictum*, *Aspergillus penicillioides*, *Alternaria* and *Chaetomium globasum*, China Center of Industrial Culture Collection (CICC); BAB, BAC & CTAB, Shanghai Aladdin Bio-Chem Technology Co., Ltd; TCA & chlorhexidine (Hibitane), Shanghai Macklin Biochemical Co., Ltd.; ATP standard, Sigma-Aldrich Corporation; ATP test pen, Hefei Peakedness Biological Technology Co., Ltd.; Sabouraud media (4 g glucose, 1 g peptone, 1.5 g agar, filled to 100 mL with Grade III water, 115 ℃, 30 min high-temperature sterilization), prepared in lab for further use.

HY-LiTE® 2 ATP Rapid Detection System, Beijing Office of Shanghai Xunjie Huazhi Co., Ltd.; TOMY SX-series autoclave sterilizer, Tomy Digital Biology; Genex pipette, MicroShine Scientific Instruments; SPX-150 constant temperature culture box, Beijing Houhui Experimental Instrument Co., Ltd.; ZCZY-CS superimposed vibration incubator, Shanghai

Zhichu Instrument Co., Ltd.; SJ-CJ-3FD ultra-clean workbench, Su Jie Medical Equipment (Suzhou) Co., Ltd.

Experimental method

Rejuvenation of five characteristic fungi

The five characteristic polluting fungi strains in jet fuels (*Cladosporium*, *Penicillium restrictum*, *Aspergillus penicillioides*, *Alternaria* and *Chaetomium globosum*), which were purchased and cryopreserved in the lab, were rejuvenated through streak cultivation on the autoclaved Sabouraud solid medium. The strains were cultured in the constant temperature culture box at 27-30 ℃ for 5-7 days. Then, the single colonies were relocated to Sabouraud liquid medium, and cultured in the superimposed vibration incubator at 28 ℃ and 180 r/min for 1 day. After that, the relative luminescence unit (RLU) was measured by HY-LiTE® 2 ATP Rapid Detection System and the compatible test pen. For each of the five characteristic fungi, 2 mL suspension was taken after 1 d culturing. The suspensions were mixed together, diluted to 100 mL with Sabouraud medium, and subjected to RLU measurement. The mixed fungi suspension and the suspensions of each of the five microbials (the single fungi suspensions) were placed at 4 °C to maintain their microbial activity.

Preparation of ATP standard solutions

The ATP standard solutions were diluted with sterile water from the ATP standard produced by Sigma-Aldrich Corporation. After dilution, the ATP concentrations were respectively 1E-11, 1E-10, 1E-9 and 1E-8 mol/L. The ATP standard solutions were stored at –20 °C. Before each use, the solutions were placed on the ultra-clean workbench to cool down naturally to room temperature.

BAB and BAC lysis tests on the five characteristic polluting fungi

The test instruments were autoclaved and placed on the ultra-clean bench, and sterilized by ultraviolet radiation. The five characteristic fungi were taken out from the 4 °C environment and allowed to stand. For each of the five characteristic fungi, 25 μL was collected and placed at the bottom of the ATP test pen, and added with 25 μL BAB or BAC solutions of different concentrations (0.02%, 0.04%, 0.06% and 0.08%). After shaking for 30 s, the tip of the pen was snapped, letting the luciferase-fluorescein reaction system flow down. The mixture was shaken rapidly for 5 s, and then relocated into HY-LiTE® 2 ATP Rapid Detection System to measure the RLU. Each test was carried out twice, and the mean value of the two test results was taken as the final result.

Synergistic/antagonistic test of BAB with other lysing agents

Previous studies have shown that both TCA and CTAB can effectively lyse bacteria to obtain their ATP in an intact manner. In addition, Hibitane and BAB have a significant synergistic effect on the lysis of *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* [1]. Hence, this paper attempts to explore the synergistic/antagonistic effects of BAB with each of the three agents (i.e., TCA, CTAB and Hibitane) on the lysis of the five characteristic polluting fungi in jet fuels.

Table 1 lists the components of the different lysing agents. Then, the mixed fungi were lysed through the steps in (1), (2) and (3), respectively, and subjected to RLU measurement. Each test was carried out twice, and the mean value of the two test results was taken as the final result.

Table 1. The components of different lysing agents

No	Components
1	0.05% BAB
2	0.05% CTAB
3	0.05% TCA
4	0.05% Hibitane
5	0.05% (BAB+CTAB)
6	0.05% (BAB+TCA)
7	0.05% (BAB+Hibitane)

Plotting of ATP standard curves

The ATP standard solutions with different concentration gradients (1E-11, 1E-10, 1E-9 and 1E-8 mol/L) were divided into two groups: one group only contains ATP standard and the other contains ATP standard, 0.05% BAB and 0.025% Hibitane. The two groups were subjected to fluorescence detection by the ATP test pen. 25 μL of the ATP standard solution was taken for each detection. Each test was carried out three times, and the mean value of the three test results was taken as the final result.

Results and analysis

Fluorescence values of six fungi suspensions

Through the rejuvenation, the suspensions of the five characteristic polluting fungi and the suspension of the mixed fungi were obtained at different concentrations. To reflect the degree of microbial contamination, the ATP concentrations of the six suspensions were quantified by the HY-LiTE JET A1 Fuel Test method recommended by the International Air Transport Association (IATA). The test results are presented in Table 2.

Table 2. The RLUs of the six fungi suspensions

Fungi	RLU
Cladosporium	32000
Penicillium restrictum	2300
Aspergillus penicillioides	38000
Alternaria	6900
Chaetomium globosum	4800
Mixed fungi	19000

BAB and BAC lyses of characteristics fungi

BAB and BAC lyses of single fungi suspensions

The cationic surfactants BAB and BAC are quaternary ammonium salts. The lysis function comes from their sterilization effects. According to previous studies [10, 17, 18], single-chain quaternary ammonium salts like BAB and BAC, which are positively charged in water, can align closely in the same direction on the surface of microorganisms, forming surface micelles. These salts also gradually permeate into the lipid and protein layers of the cell membrane, and thus change the permeability of the cell membrane. As a result, the intracellular dissolved matters (e.g., ATP) will flow out of the cell and the enzymes (e.g., ATPase) will be deactivated, putting an end to the microorganism. This subsection aims to select a quaternary ammonium salt that can effectively lyse the characteristic polluting fungi in jet fuels.

Figs. 1 and 2 show RLUs after the BAB and BAC lyses of the five characteristic polluting fungi, respectively. It can be seen that the RLUs were high in the suspensions of all five characteristic fungi, when the BAB and BAC concentrations were between 0.02% and 0.15%, indicating a good lysis effect. When the BAB and BAC concentrations surpassed 0.15%, the RLUs was reduced rapidly with the increase in the concentration of the lysing agents. The rapid decline may be the result of the sensitivity of luciferase to BAB and BAC. The excess BAB and BAC greatly suppressed and even eliminated the activity of luciferase. The suppression effect is particularly obvious at the lysing agent concentration of 0.3%, when the RLUs of the five bacteria was 70-90% lower than those at the concentration of 0.02%.

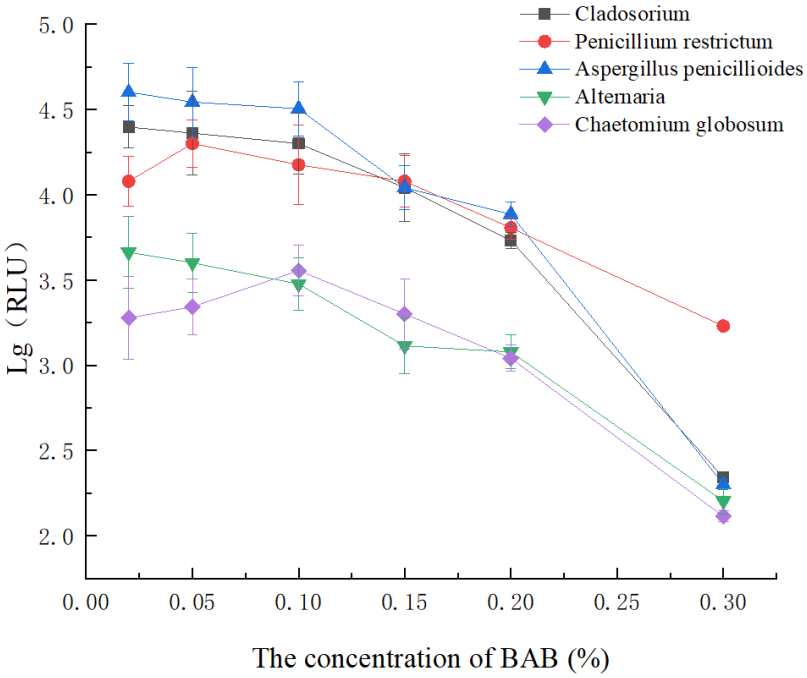

Fig. 1 Lysis effects on the five characteristic fungi at different BAB concentrations

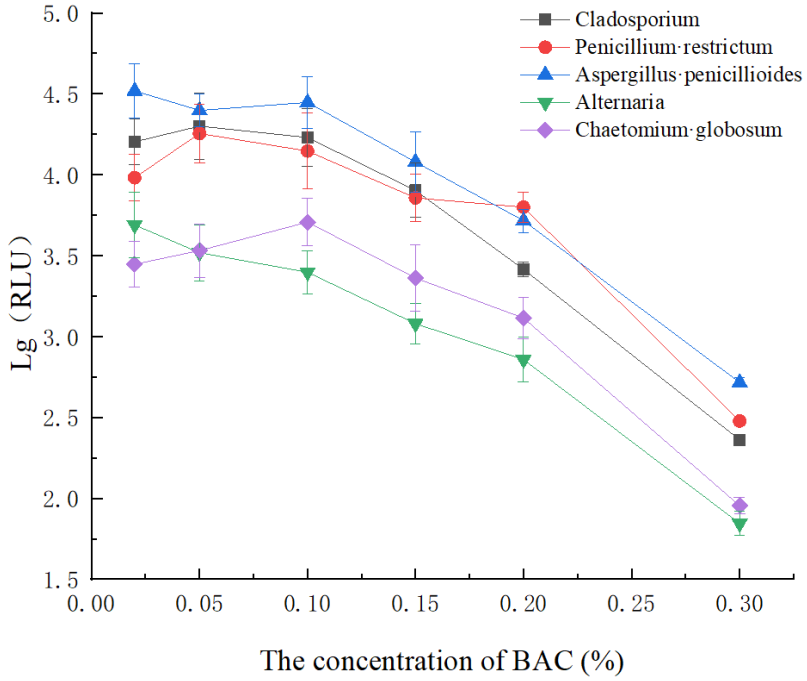

Fig. 2 Lysis effects on the five characteristic fungi at different BAC concentrations

Table 3 provides the preferred type and concentration of lysing agent of the five characteristic fungi. As shown in the table, the five characteristic fungi differ in the preferred type and concentration of lysing agent, owing to their differences in the cell membrane structure. The varied structures of the cell membrane affect the penetration of the BAB and BAC, such that the membrane permeability changes differently and the intracellular dissolved matters flow differently among the microorganisms. The preferred type and concentration of lysing agent is follows: 0.02% BAB for *Cladosporium* and *Aspergillus penicillioides*, 0.05% BAB for *Penicillium restrictum*; 0.1% BAC for *Chaetomium globasum*; 0.02% BAB or 0.02% BAC for *Alternaria*. Hence, the BAB and BAC concentrations should fall in 0.02-0.1% for the lysis of the mixed fungi.

Table 3. Preferred type and concentration of lysing agent of the five characteristic fungi

Type of fungi suspension	**Preferred type of lysing agent**	**Preferred lysing agent concentration, [%]**
Cladosporium	BAB	0.02
Penicillium restrictum	BAB	0.05
Aspergillus penicillioides	BAB	0.02
Alternaria	BAB/BAC	0.02
Chaetomium globasum	BAC	0.10

BAB and BAC lyses of mixed fungi suspension

The five characteristic fungi were mixed in equal proportions, forming the mixed fungi suspension. The high-throughput sequencing shows that the five fungi account for over 80% of all polluting microorganisms of the jet fuels. This subsection examines the lysis effect of BAB and BAC on the mixture of the five fungi. The purpose is to discover the lysing agent that applies to multiple polluting microorganisms, shedding theoretical new light on subsequent microbial detection in contaminated jet fuels.

Fig. 3 displays the RLUs after the BAB and BAC lyses of the mixed fungi suspension. Obviously, the BAB achieved the optimal lysing effect at the concentration of 0.05%, while the BAC did so at 0.06%. The BAB outperformed the BAC in the overall lysing effect and the maximum RLU. The different lysing effects and RLUs between the two lysing agents come from the different lengths of the alkyl chains, which affect the surface binding ability of the two agents. As a result, the permeability of the cell membrane changed differently under the two agents. Through this group of tests, the optimal lysing agent for the polluting microorganisms in jet fuels was determined as 0.05% BAB.

Effect tests on the combination between BAB and other lysing agents

The CTAB and TCA have been proved to have good lysis effects on bacteria. However, there is no report on their lysis effects on the characteristic fungi of jet fuels, not to mention their synergistic/antagonistic effects with BAB. In addition, Hibitine is known for its good synergy with the BAB in the lysis of bacteria. Considering the above, this subsection investigates the synergistic effects of different lysing agents on the characteristic fungi of jet fuels.

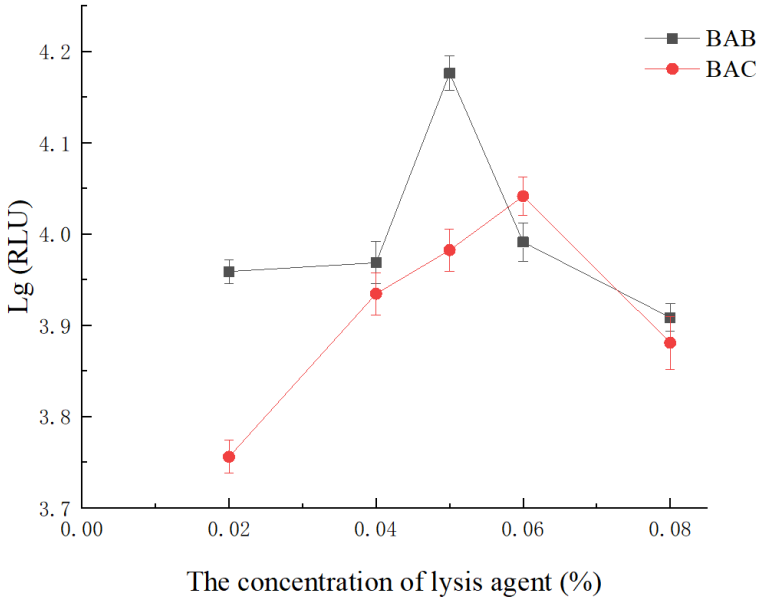

Fig. 3 Lysis effects on the mixed fungi at different BAB and BAC concentrations

As shown in Fig. 4, the lysing agents were ranked as BAB > Hibitine > CTAB > TCA by the lysing effect on the mixed fungi. The CTAB and TCA exhibited good lysis effect on bacteria, especially gram-positive bacteria, yielding high RLUs. However, the two agents failed to achieve a desirable lysing effect on the characteristic fungi of jet fuels. The possible reason may be the different cell structures between bacteria and fungi: the cell membrane of bacteria mainly consists of peptidoglycan, while that of fungi is mostly chitin. Obviously, the equal proportion mixture between Hibitine and BAB had a better lysing effect than that of the two single agents combined, which enhanced the accuracy and stability of fluorescence detection. Of course, the specific synergistic mechanism needs further study.

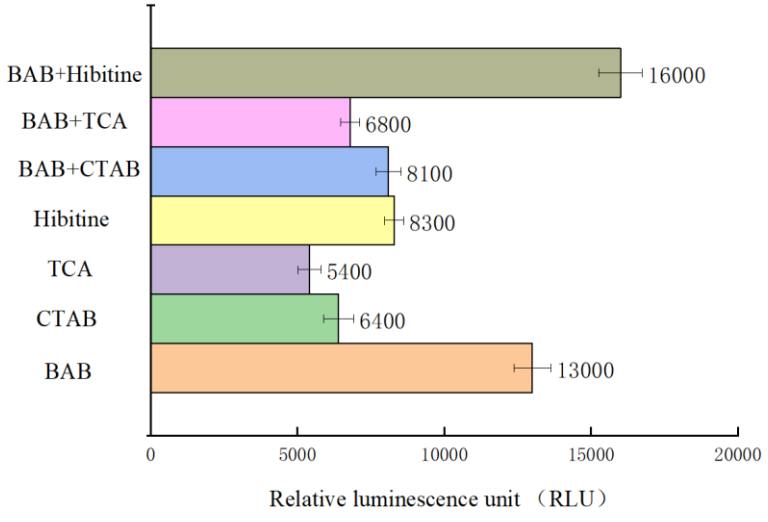

Fig. 4 Synergistic effects of multiple lysing agents

Fig. 5 shows the effects of Hibitine on the BAB lysing of the mixed fungi. It can also be seen that the best lysis effect appeared after 0.025% Hibitine was added to 0.05% BAB. This means the 0.05% BAB + 0.025% Hibitine solution is a desirable lysing agent for the polluting microorganisms in jet fuels.

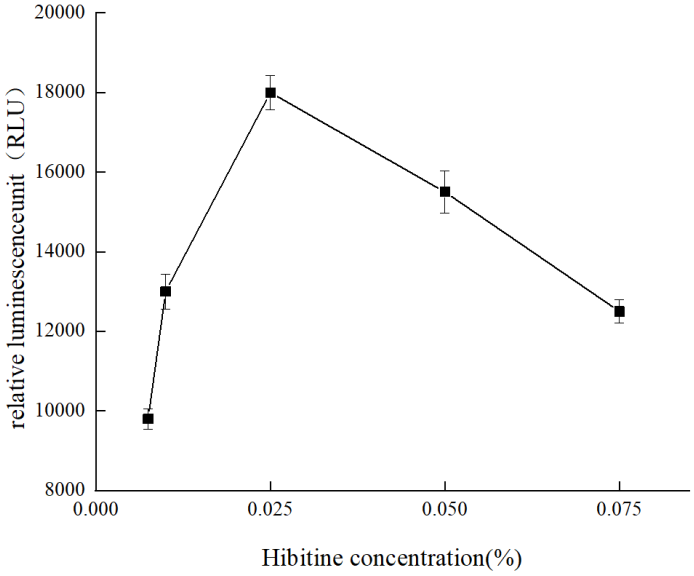

Fig. 5 The effects of Hibitine on the BAB lysing of the mixed bacteria

Drawing of ATP standard curves

Most microbial lysing agents are known for their varied suppression effects on luciferase. This is an important reason for the low sensitivity of ATP biofluorescence detection in microbial contamination of jet fuel. To mitigate and eliminate the suppression effects, the author set up a luciferase-fluorescein reaction system together with Hefei Peakedness Biological Technology Co., Ltd., and adjusted the blending ratios of various luciferase protectants, such as bovine serum albumin (BSA), dithiothreitol (DTT), glutathione (GSH) and diethylaminoethyl dextran (DEAE-Dx). The effects of these protectants were discussed against the ATP standard curves (Fig. 6).

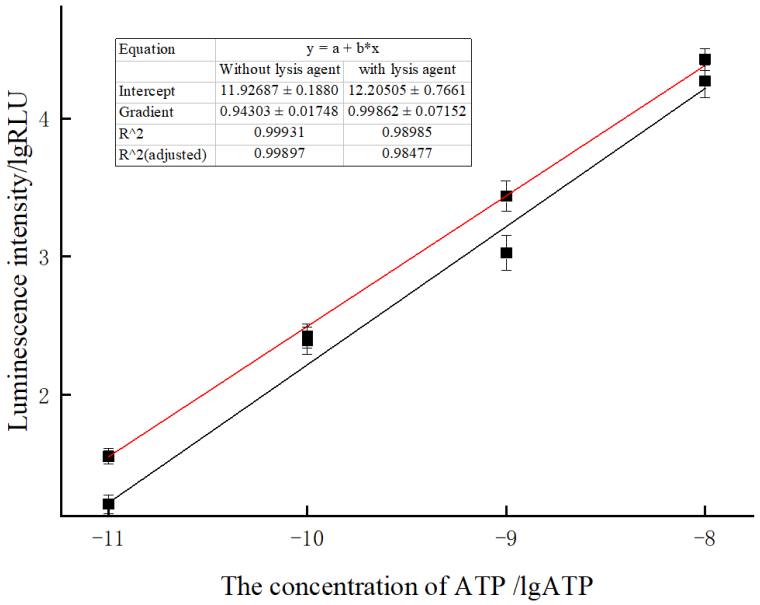

Fig. 6 ATP standard curves: the red line is the ATP standard curve without lysing agent; the black line is the ATP standard curve with lysing agent.

Fig. 6 shows that the luciferase-luciferin reaction system maintained good enzymatic activity with or without lysing agent. Within a certain range, the ATP concentration had a good linear

relationship with the RLU. The ATP concentration in the reaction substrate can be obtained by comparing the RLUs of subsequent experiments with the curves. The linear correlation coefficient R^2 was 0.9989 for the ATP standard curve without lysing agent and 0.9847 for that with lysing agent. Thus, the luciferase-fluorescein reaction system has a good adaptability to lysing agents. In addition, the RLUs with or without lysing agents were subjected to a two-sample T-test on Origin. No significant difference was observed between the two sets of data ($p = 0.9768 > 0.05$). Therefore, the luciferase-fluorescein reaction system can effectively reduce the negative impacts of lysing agents on luciferase activity.

Conclusions

This paper investigates the lysing effects of two quaternary ammonium salts, BAB and BAC, on the five characteristic polluting fungi of jet fuels. The two cationic surfactants were found to have good lysing effects on all five polluting fungi. The BAB was selected as the main component of the lysing agent, as it could apply to more types of polluting microorganisms of jet fuels. Through repeated tests, the optimal BAB concentration was determined as 0.05%, under which a high RLU can be achieved for both single fungi and mixed fungi.

In addition, the CTAB and TCA failed to achieve the same lysing effect on fungi as that on bacteria. The author also discovered that the BAB lysing effect on the characteristic polluting fungi can be enhanced by adding 0.025% Hibitine, and thus initialized the components of the lysing agent for the characteristic polluting microorganisms of jet fuels.

After that, the luciferase-luciferin reaction system was optimized such that the ATP standard curves with or without lysing agent had the same linear correlation. Compared with the current detection methods for microorganisms of jet fuels (e.g. plate culture method and HY-LiTE JET A1 Fuel Test), the proposed ATP biofluorescence method boasts advantages in detection time (30 s vs. 3 days of the plate culture method), detection cost (each HY-LiTE JET A1 fuel test costs RMB 380 yuan), and detection accuracy.

The optimized luciferase-luciferin reaction system, coupled with the mixture of BAB and Hibitine, outshines the other lysing detection methods in terms of lysing effect, operation simplicity, cost and impact on luciferase activity. However, the luciferase activity is also affected by lysis time, pH, temperature and metal ions (Cu^{2+}, Mg^{2+} and Ca^{2+}) during the microbial lysis. These influencing factors will be examined in future research, with the aim to acquire stable and reliable fluorescent signals, and to ensure the accuracy and stability of the test results.

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