Investigation into Aerosol Staining for Bio-aerosol Online Monitoring

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Dissertation Abstract

Investigation into Aerosol Staining for Bio-aerosol Online Monitoring

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Abstract

Bio-aerosols, or typically, airborne microbes as fungi which are known to have potential health risks, are emitted from various sources and can be transported in long distance as those associated with dust from China. In order to discuss the contribution of emission sources and related peak health risk as well as their transportation behaviors, the real-time monitoring not only on their concentration but also on detailed characteristics is important. However, such a tool is not available in the present except the UV optical particle counter (OPC), which can detect only the auto-fluorescence of particles. "Aerosol staining" of airborne microbes based on fluorescence staining techniques can be a tool providing such information in combination with detection technologies as flow-cytometry and UV OPC. In the present study, the mixing of aerosol microbes and mist of a fluorescent dye solution was discussed as a possible and the simplest method applicable to the aerosol staining process. As the first step, time dependency of microbe staining also in second order, which is important to avoid difficulties caused by a long retention time of mixed aerosol, was discussed for the staining of yeast (*S. cerevisiae*) by aqueous dye solutions, or, DNA staining florescence dye, or, DAPI (4',6-diamidino-2-phenylindole) and Auramine-O, a nonspecific fluorescent dye by using a spectrofluorometry. Next, the staining of yeast aerosol by mixing with the mist of two different florescence solutions of DAPI and Auramine-O was experimentally investigated. Through the first investigation on the time dependency of dye staining, DAPI and Auramine-O solution was confirmed to stain 50% of yeast less than 5 seconds after mixing at a dye concentration above 1 and 0.1 $\mu g\ ml^{-1}$ respectively. This result refers to that the aerosol staining by mixing of yeast aerosol and mist of conventional dye solution should work if the operational condition could be adjusted, properly. In the second investigation on the aerosol staining experiment taking into account ideal conditions obtained by the first investigation, the stained fraction of yeast by mist of two different dyes was evaluated using two different methods 1) spectrofluorometric analysis of a liquid sample of mixed aerosol collected by a liquid impinger and 2) light scattering analysis of auto-fluorescence of stained aerosol particles by a commercially viable particle counter. From both experiments, even by the aerosol staining using the simple mixing of yeast aerosol and dye mist, a fraction of yeast was confirmed to be stained, indicating the staining efficiency can be improved by applying another possible method as the electrical charging of dye particles and microbes to have more efficient contact between them. The present study could be the first step of a new methodology that could be called as "Aerosol staining" providing not only a benefit for bio-aerosol online monitoring but also various possibilities to detect characteristics of aerosol particles on line.

1. Introduction

The staining of bio-aerosol represents a tool that can be used to evaluate the detailed characteristics and concentration of such substances online by applying a fluorescence staining technique with an existing optical detection technologies, e.g. UV optical particle detection (UV-OPC) and flow cytometry (FCM). Stained bio-aerosols can be discriminated that overcomes an auto-fluorescence which requires a higher power of laser source. Such an online tool could be useful for examining emission sources and the transportation behavior of bio-aerosols. Mixing bio-aerosol and mist of fluorescent dye solution could be a possible "aerosol staining process", in which should be done rapidly to avoid difficulties such as increased deposition loss and complicated facilities caused by a long retention time of aerosol. For this purpose, the first step, time dependency of microbe staining in second order was discussed for the staining of yeast (*S. cerevisiae*) by aqueous dye solutions, or, DNA staining fluorescent dye, or, DAPI (4',6-diamidino-2-phenylindole) and Auramine-O, a nonspecific fluorescent dye by using a spectrofluorometry. Next, the staining of yeast aerosol by mixing with a mist of two different florescence solutions of DAPI and Auramine-O was experimentally investigated. In the second investigation on the aerosol staining experiment taking into account ideal conditions obtained by the first investigation, the stained fraction of yeast by mist of two different dyes was evaluated using two different methods 1) spectrofluorometric analysis of a liquid sample of mixed aerosol collected by a liquid impinger and 2) light scattering analysis of auto-fluorescence of stained aerosol particles by a commercial viable particle counter.

2. Time Dependency for the Staining Process of Microbes for Bio-aerosol Online Monitoring

In this study, time dependency of staining process was examined for a combination of yeast (*S. cerevisiae*) suspended in ultrapure water with DAPI and Auramine-O solution using a fluorescence spectrophotometer in conjunction with a dye concentration of 0.1-10 $\mu g\ ml^{-1}$ and a yeast concentration 1-100 $\mu g\ ml^{-1}$. The fluorescence intensity in each second and spectrum of a mixed solution was measured instantaneously and compared with that of dye or yeast solution.

2.1 Experimental

DAPI, a stable fluorescent dye, was prepared for a dye solution in ultrapure water without further purification. For Auramine-O is a non-specific fluorescent dye, to avoid contamination and photo quenching, fresh and filtrated solution of Auramine-O was prepared before each experiment by dissolved in ultrapure water and filtrated by 0.20 μm Millex-LG filter (Millipore Corp.). A dye solution was prepared in a 5 mm micro cuvette which was used since it was necessary to mix the solution inside the cuvette extensively. Dye was prepared in the micro cuvette and placed inside spectrofluorometer (Jasco FP8200, Jasco International Co., Ltd., Japan) before mixing with yeast solution.

Commercial baker's yeast granules (*S. cerevisiae*) was dried for ~20 hours at 105°C in an electric oven then gently ground to primary microbes (powder) by using a blender. Yeast powder was suspended in ultrapure water without further purification to prepare yeast solution. For a well-suspended mixture of yeast and dye solution and instantaneous measurement after mixing was needed, the yeast solution was injected directly using a micro syringe from outside spectrofluorometer through the injection hole above the closing cover of spectrofluorometer. The total volume of the solutions was adjusted to 1 ml. DAPI and Auramine-O at a final concentration of 0.1 and 1 $\mu g\ ml^{-1}$ were mixed with the yeast suspension which was

adjusted to a final concentration of 1-100 µg ml^{-1} to examine the consequences of yeast concentration. Yeast at a final concentration of 100 µg ml^{-1} was mixed with a dye solution at a final concentration of 0.1-10 µg ml^{-1} to study the consequence of dye concentration on the staining process (Table 2-1).

Table 2-1 Experimental conditions

Consequence of yeast concentration					
Dye concentration (µg ml^{-1})	Yeast concentration (µg ml^{-1})				
0.1	1	5	10	50	100
1	1	5	10	50	100
Consequence of dye concentration					
Yeast concentration (µg ml^{-1})	Dye concentration (µg ml^{-1})				
100	0.1	1	3	5	10

2.2 Results and Discussion

Fluorescence intensity of mixtures of DAPI 0.1 and 1 µg ml^{-1} with yeast solution at excitation and emission wavelength 360 nm and 456 nm respectively are shown in Fig. 2.1. The intensity of the mixtures increased instantaneously. The mixtures of 0.1 µg ml^{-1} DAPI needed longer time to reach equilibrium compared with the mixtures of 1 µg ml^{-1} DAPI. The proper amount of yeast (*S. cerevisiae*) for staining should be ≥10 µg ml^{-1} since the staining of live cells requires a plasma membrane that is permeable to fluorochrome that stoichiometrically stains DNA and depends on cell type which some of which take up fluorochrome at different rates (Darzynkiewicz, 2011).

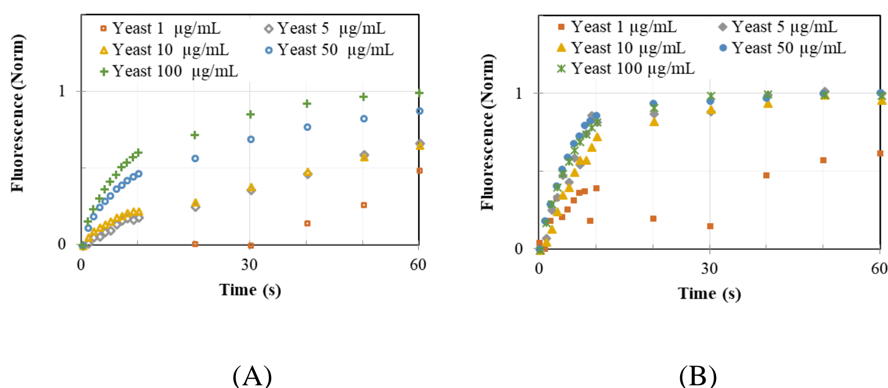


Fig. 2.1 Time dependency on fluorescence intensity change per 1 µg ml^{-1} yeast concentration (normalize). DAPI (A) 0.1 µg ml^{-1} and (B) 1 µg ml^{-1} concentration and yeast at a concentration of 1-100 µg ml^{-1}, excitation wavelength 360 nm and emission wavelength 456 nm

Fluorescence intensity of 0.1-10 µg ml^{-1} DAPI concentration mixed with 100 µg ml^{-1} yeast concentration increased instantaneously from that of DAPI solution. The mixtures of 0.1 µg ml^{-1} DAPI

required the longest time to reach equilibrium and the time decreased with increasing dye concentration as shown in Fig. 2.2.

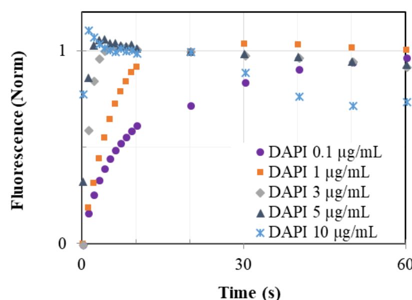
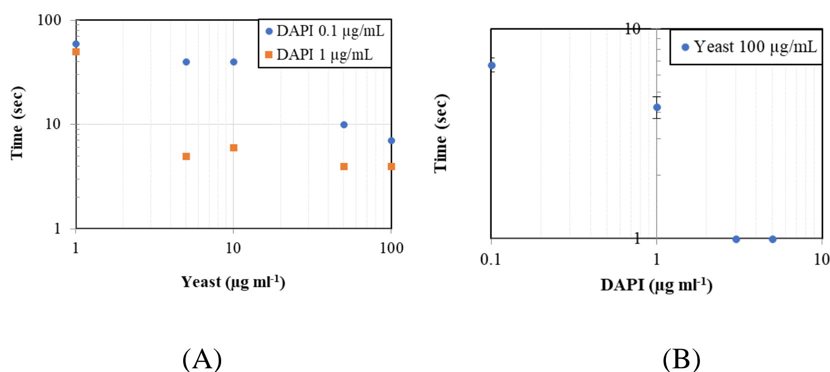


Fig. 2.2 Time dependency for fluorescence emission intensity change (normalize) of 0.1-10 µg ml^{-1} DAPI concentration and 100 µg ml^{-1} yeast concentration, excitation wavelength 360 nm and emission wavelength 456 nm



(A) (B)

Fig. 2.3 Time dependency for staining half of the charge amount of yeasts as (A) a consequence of yeast concentrations 1-100 µg ml^{-1}, (B) consequences of dye concentration for 0.1-10 µg ml^{-1}, excitation wavelength 360 nm and emission wavelength 456 nm

Fig. 2.3 shown time dependency at 50% of the maximum amount of stained yeast. These data indicated that half of the yeast fraction could be stained within 10 seconds using 0.1 and 1 µg ml^{-1} DAPI concentrations with a yeast concentration of ≥10 µg ml^{-1}. For 100 µg ml^{-1} yeast concentration, 50% of the maximum staining of yeast was achieved below 10 seconds by using 0.1-5 µg ml^{-1} DAPI concentrations. Thus, ≥10 µg ml^{-1} yeast with DAPI 3-10 µg ml^{-1} should be used in airborne microbe staining.

Fig. 2.4 shown the fluorescence intensity change per 1 µg ml^{-1} yeast of the mixtures of yeast with Auramine-O compared with that of yeast solution in ultrapure water. After injected yeast solution into Auramine-O 0.1 and 1 µg ml^{-1} solution the fluorescence intensity increased instantaneously. The mixtures of yeast with an Auramine-O concentration of 1 µg ml^{-1} resulted in greater stability than that of the mixtures of 0.1 µg ml^{-1} Auramine-O.

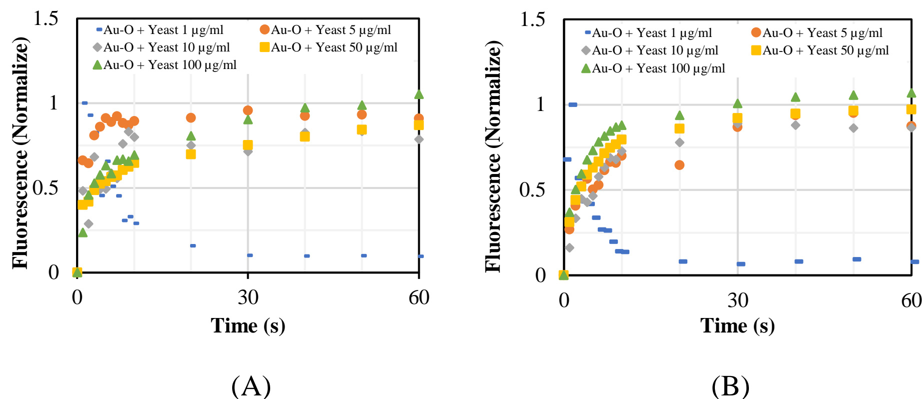


Fig. 2.4 Time dependency for fluorescence intensity change per 1 μg ml^{-1} yeast of the mixtures of Auramine-O (A) 0.1 μg ml^{-1} and (B). 1 μg ml^{-1} with yeast solution at the concentration of 1–100 μg ml^{-1}, excitation wavelength 435 nm and emission wavelength 510 nm

To study the consequence of Auramine-O concentration to staining time, yeast solution at the concentration of 100 μg ml^{-1} was mixed with 0.1-100 μg ml^{-1} of Auramine-O solution. Fig. 2.5 shown the instantaneous increasing of fluorescence intensity of the mixtures.

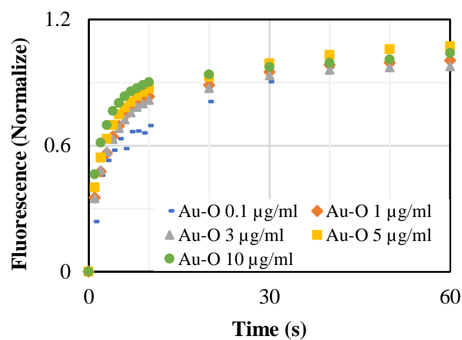


Fig. 2.5 Time dependency for fluorescence intensity change (normalize) of 0.1-10 μg ml^{-1} Auramine-O with yeast solution at the concentration of 100 μg ml^{-1}, excitation wavelength 435 nm and emission wavelength 510 nm

For time dependency at 50% of the maximum amount of stained yeast by Auramine-O shown in Fig. 2.6. The mixtures of 0.1 and 1 μg ml^{-1} Auramine-O with all concentrations of yeast required a staining time less than 10 seconds. For 100 μg ml^{-1} yeast concentration, 50% of the maximum staining of yeast was achieved by using 0.1-10 μg ml^{-1} Auramine-O concentrations at below 5 seconds.

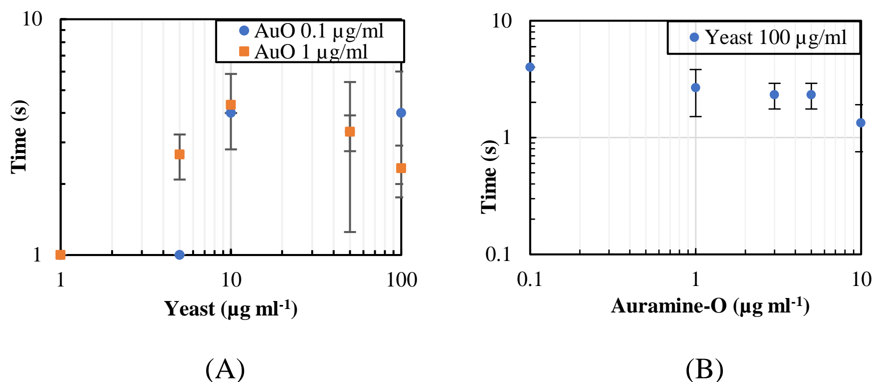


Fig. 2.6 Time dependency for staining half of the charge amount of yeasts as (A) a consequence of yeast concentrations 1-100 µg ml^{-1}, (B) consequences of dye concentration for 0.1-10 µg ml^{-1}, excitation wavelength 435 nm and emission wavelength 510 nm

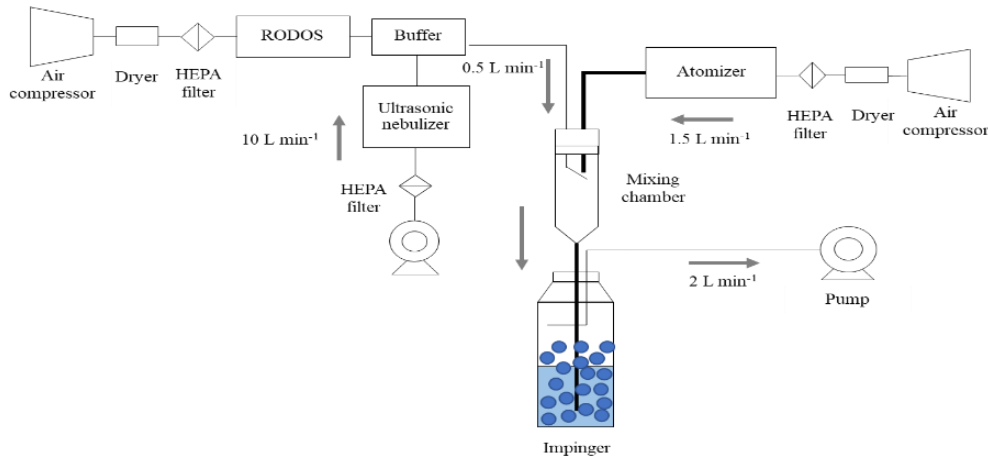
The results of the time dependency of yeast staining process by DAPI and Auramine-O indicated yeast *S. cerevisiae* was stained instantaneously by DAPI and Auramine-O solution at dye concentrations of 0.1-10 µg ml^{-1} and 50% of the maximum amount of stained yeast could be observed below 5 seconds when using the optimal microbe and dye ratios.

3. Bio-aerosol Staining by Fluorescent Dye Mist

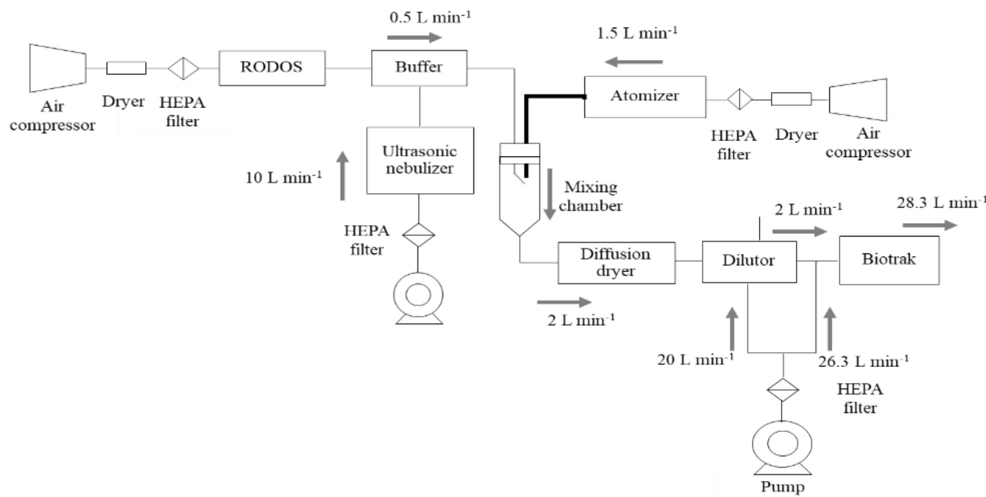
The staining of yeast aerosol by mixing with a mist of two different florescent dye solutions of DAPI and Auramine-O was experimentally investigated. The stained fraction of yeast by the mist of two different dyes was evaluated using two different methods 1) spectrofluorometric analysis of a liquid sample of mixed aerosol collected by a liquid impinger and 2) light scattering analysis of auto-fluorescence of stained aerosol particles by a commercial viable particle counter.

3.1 Experimental

The experimental set up for this study consisting of a microbe aerosol generator, an atomizer for dye solution, a mixing chamber of yeast and dye aerosols, where yeast are stained by mists of dye solution, and the evaluation of aerosol staining by spectrofluorometric analysis of a liquid sample of mixed aerosol collected by a liquid impinge (Fig. 3.1 (A)) and by light scattering analysis of auto-fluorescence of stained aerosol particles by a commercial viable particle counter (Fig. 3.1 (B)). The detailed methodology for each configuration is described separately in the following.



(A)



(B)

Fig. 3.1 Experimental set up (A) spectrofluorometric analysis (B) light scattering analysis

Test aerosol was generated by using a dry powder feeder for a laser diffraction particle size measuring system (RODOS, Sympatec Ltd., Germany). Yeast powder was dispersed by an ejector of the feeder using dried and cleaned air then yeast aerosol was partially supplied (0.5 L min^{-1}) to an aerosol mixing chamber after passing through a buffer chamber (25.2 L) for removal of large agglomerates and stable generation of test aerosol. Yeast aerosol had stability for more than 30 min for a continuous generation which enough for sample collection in this study.

Mist of dye solution was generated by an atomizer (1-Jet Atomizer 9302, TSI Inc., USA) using dried and cleaned air. The concentration of dye solution dosed in a reservoir of the atomizer was 3 μg ml^{-1}. Dye mist had stability for a continuous generation more than 30 min.

Aerosol mixing, test and dye mist aerosols were mixed in a tubular glass chamber at a total flow rate of 2 L min^{-1}. The chamber length was decided so as to give a residence time of 2 seconds, which is

enough to stain 50% of charged amount of yeasts 100 µg ml^{-1} by the DAPI solution at the lowest concentration (3 µg ml^{-1}) in this study, or, less than 1 second (Piriyakarnsakul et al. 2018). The aerosols were mixed at the top of mixing chamber which was configured to mix aerosols at the end of dye mist supplying tube. For a better mixing, the mixed aerosols were funneled at chamber bottom. The temperature of the laboratory was 22±2 ℃ when the aerosol mixings were carried on. Two different relative humidity was adjusted before mixing chamber, or, 31±4 and 65±2% RH.

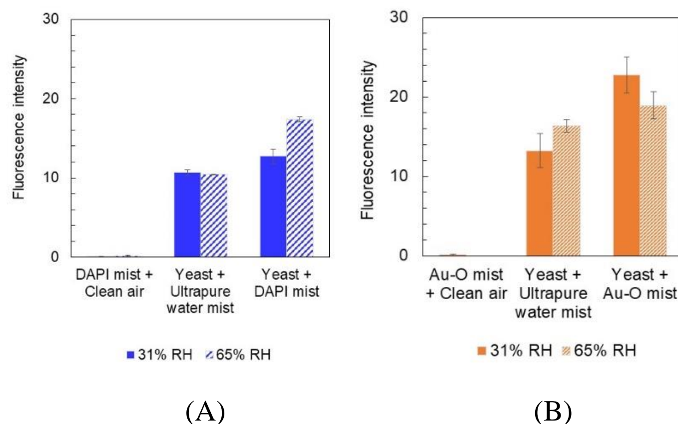
Collection of mixed aerosols for spectrofluorometric analysis, an impinger filled with ultrapure water of 25 mL was connected to the bottom of mixing chamber to collect stained yeasts as a liquid sample. To ensure an efficient collection of yeasts, glass bead of an average diameter 2-3 mm were filled in the impinger up to a depth of 60 mm, which is similar to a unit used for microbe collection in a previous report (Yamaguchi et al. 2012, 2014). The collection efficiency of the impinger of particles in the range of 2-10 µm, corresponding to fraction, primary and coagulates of yeasts, was confirmed to exceeded 89%. The mixed aerosol was collected by the impinger at a flow rate of 2 L min^{-1} for 2 min, below which the staining of yeast by DAPI and Auramine-O in impinger was conformed to be negligible.

Spectrofluorometric analysis, the fluorescence emission intensity of collected solution which transferred from impinger was analyzed by spectrofluorometer with a 5 mm light path synthetic silica micro cuvette. The excitation and emission wavelength of DAPI at 360 nm and 456 nm respectively, were used for the measurement of mixed aerosols in DAPI staining. Excitation and emission wavelength of 435 and 510 nm, respectively were used to analyze for Auramine-O staining. Each collected solution was measured at least three replications.

Biotrak® 9510-BD, TSI, USA, a commercial real-time viable particle counter, was used to detect an auto-fluorescence of mixed aerosols. The mixed aerosols from the end of mixing chamber at a flow rate of 2 L min^{-1} passed through a diffusion dryer and dilutor before flowed into Biotrak®. The total (T) and viable (V) particles were measured and evaluation as the ratio of viable to total particle (V/T). Each mixed aerosol was measured at least 10 cycles (1 cycle was 1 min) after a pause of 15 seconds.

3.2 Results and discussion

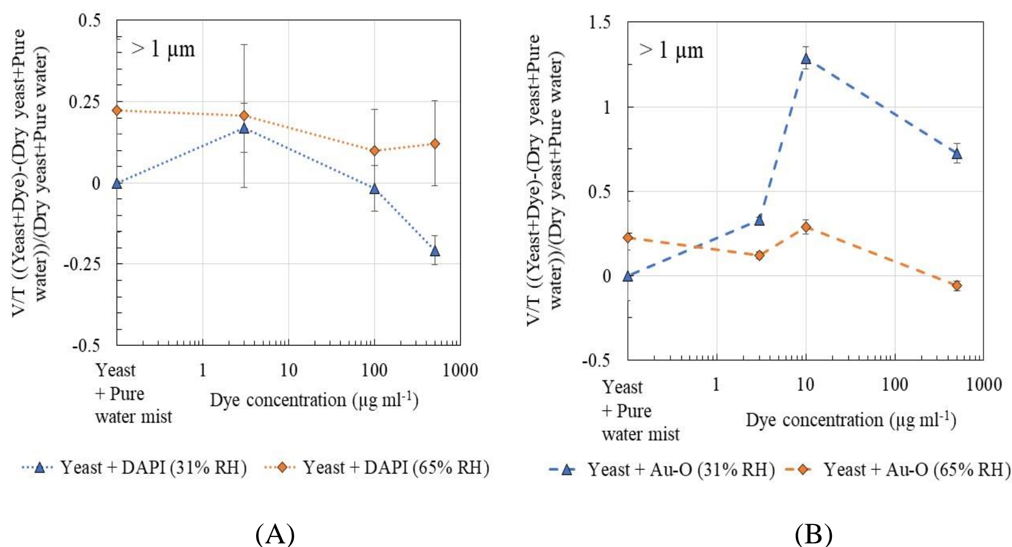
Fig. 3.2 shown the increasing of fluorescence intensity of mixed aerosols of yeast with DAPI mist compared with the mixed aerosols of yeast with ultrapure water mist indicated that yeast *S. cerevisiae* could be stained in the aerosol state by DAPI mist at a concentration of DAPI 3 µg ml^{-1} likewise the mixed aerosols of yeast with Auramine-O mist. Humidity had the different effects to the aerosol staining process of two different dyes. High relative humidity advantaged for DAPI staining, contrasting Auramine-O staining which humidifying caused rather low increasing of fluorescence intensity.



(A) (B)

Fig. 3.2 Fluorescence intensity of aerosol staining by (A) DAPI and (B) Auramine-O mist solution

Fig. 3.3 shows the increasing of V/T ratio of mixed aerosols of yeast with dye mist compared with that of mixed aerosols of yeast with ultrapure water mist indicating that yeast aerosol could be stained by DAPI and Auramine-O mist by the simplest mixing method. These results were consistent with the results of the spectrofluorometric analysis. The type of dye mist, the concentration of dye mist solution, relative humidity and yeast aerosol particle size affected the aerosol staining. The effect of humidity to aerosol staining by DAPI and Auramine-O showed the consistence with the results of the spectrofluorometric analysis.



(A) (B)

Fig. 3.3 V/T ratio of mixed aerosols with (A) DAPI and (B) Auramine-O mist in particle size >1 μm

4. Conclusion

"Aerosol staining" was investigated based on the fluorescence staining technique in aqueous solution. For aerosol staining, time dependency of microbe staining in second order is important to avoid difficulties caused by a long retention time of mixed aerosol, thus the staining of yeast (*S. cerevisiae*) by aqueous dye solutions, or, DNA staining fluorescent dye, or, DAPI and Auramine-O, a nonspecific fluorescent dye by using a spectrofluorometry was discussed. Through this investigation on the time dependency of dye staining, yeast could be stained by DAPI, and Auramine-O solution instantaneously resulted in the enhancement of fluorescence intensity. DAPI and Auramine-O were confirmed to stain 50% of yeast less than 5 seconds after mixing in an optimal condition. By the time dependency investigation, the stained fraction of yeast by dye mist using the simple mixing of yeast aerosol with two different dyes was evaluated. From both spectrofluorometric analysis of a liquid sample of mixed aerosol collected by a liquid impinger and the light scattering analysis of auto-fluorescence of stained aerosol particles by a commercial viable particle counter shown that even by the aerosol staining using the simple mixing of yeast aerosol and dye mist, a fraction of yeast was confirmed to be stained. Indicating that the staining efficiency can be improved by applying other possible method as the electrical charging of dye particles and microbes to have more efficient contacts between them. The present study could be the first step of a new methodology that could be called as "Aerosol staining" providing not only a benefit for bio-aerosol online monitoring but also various possibilities to detect characteristics of aerosol particles online.

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平成３０年８月７日

学位論文審査報告書（甲）

１．学位論文題目（外国語の場合は和訳を付けること。）

Investigation into Aerosol Staining for Bio-aerosol Online Monitoring

（気中浮遊微生物オンライン計測のためのエアロゾル染色法に関する検討）

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(2) 氏　名（ふりがな）　Suthida PIRIYAKARNSAKUL（スティダー　ピリヤガーンサクル）

３．審査結果の要旨（600～650字）

提出された論文に対し，書面および口述審査を実施し，以下のように判断した。

本論文は，空気中に浮遊する微生物の種類・特性をオンライン評価するための核心技術となる「エアロゾル染色」の実現可能性とその特性に検討を加えたものである。本論文ではまず，オンライン・エアロゾル測定に必須となる微生物迅速蛍光染色の可能性を確認するため，模擬微生物としてイースト菌を用いて代表的蛍光染色物質（DAPI, Auramine-O）の短時間染色特性を様々な条件で確認し，適切な条件下では５秒以下で50%以上を染色可能なことを明らかにした。次に，イースト菌エアロゾルと蛍光染色液ミストの混合エアロゾルを，１）捕集後に蛍光分光計測，および２）市販紫外線蛍光散乱測定器で検知微生物濃度をオンライン測定する２通りの方法でエアロゾル染色特性に検討を加え，適切な蛍光染色液濃度と混合後保持時間を設定すれば，気中状態でDNA染色が可能であること（DAPI），微生物蛍光散乱強度を増幅できること等を明らかにした。また，相対湿度を上げて蛍光染色液ミスト濃度を増やすことで染色効率を改善できる場合があることを示した。

以上のとおり，本論文で提案された「エアロゾル染色技術」は，気中微生物特性のオンライン評価を実現する上で重要なブレイクスルーを達成し，同技術の今後の展開の方向性にも明確な指針を示すとともに，微生物迅速染色特性に関する様々な新しい知見も与えている。一連の成果は１編の英語論文にまとめられており，国内外の学会・ワークショップでの成果発表回数も多く，英語の語学力も十分と認められる。以上より，博士（学術）の学位を授与するに値すると判断する。

４．審査結果　(1) 判　　定（いずれかに〇印）　（合　格）・　不合格

(2) 授与学位　博　士（　学術　）