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SHORT COMMUNICATION

Vital Staining of Bacteria by Sunset Yellow Pigment

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Abstract

In this study, we describe a method for discriminating pathogenic bacteria with a dye. First, we determined that among several colours tested, the sunset yellow pigment easily coloured *Escherichia coli* bacteria yellow. Next, we demonstrated that *E. coli* O157:H7, *Shigella flexneri* O301, *Staphylococcus aureus* and *Bacillus subtilis* could all be well marked by sunset yellow pigment. Finally, we performed bacterial viability assays and found there was no effect on bacterial growth when in co-culture with sunset yellow. Our results suggest that sunset yellow is suitable pigment to dye microorganisms.

Key words: bio-safety, pathogenic bacteria detection, sunset yellow, vital staining

In the global effort to cure many human diseases, a large number of pathogenic bacteria is cultured for different types of experiments (Jünger *et al*., 2012). However, numerous accidents have been reported when laboratories mishandled deadly germs (Cohen *et al*., 2004; Tucker, 2003). Moreover, leaks of contaminated waste, spilled bacterial or viral cultures, and defective airtight seals remain common occurrences in many laboratory settings. Accordingly, to improve the level of biological safety, an indicator that can detect spilled pathogenic bacteria is needed. There are numerous methods to stain bacteria in order to characterize and discriminate between them (Salleh *et al*., 2012); however, most dyes have been found to be toxic and inhibit bacterial growth (Salleh *et al*., 2011). To date, only few studies have investigated non-poisonous methods of dyeing cells in culture (Pérez-Díaz and McFeeters, 2009), therefore, a more effective staining method is needed.

Here, we present a convenient and safe method of microbial staining with the sunset yellow food dye. Using this food dye, we were able to trace the source of the leak or pathogenic microorganism without affecting bacterial growth, which we believe is necessary to allow for further characterization.

First, we investigated the ability of several pigments, such as coccinellin, erythrosine and FD&C Yellow No. 6 (sunset yellow, $C_{16}H_{10}N_2Na_2O_7S_2$), to dye bacteria, and found the pigment sunset yellow to be the most potent dye. Furthermore, previous studies have shown that sunset yellow is a synthetic dye and a small molecule that can easily dissolve in water. Accordingly, these characteristics allow sunset yellow to affix to bacteria when the dye is added to the nutrient medium. The ability of sunset yellow to colour bacteria offers a new way to indicate the trail of microorganisms, allowing researchers to physically monitor their spread. Accordingly, based on these characteristics, we believe this dye may become a good candidate for colouring bacteria and monitoring their spread.

Escherichia coli strain DH5α, *E. coli* O157:H7, *Shigella flexneri* O301, *Staphylococcus aureus* and *Bacillus subtilis* were used in this study. *E. coli* was grown in Luria Bertani (LB) medium aerobically at 37°C, while other strains were grown in LB medium in both liquid broth and on agar plates. We utilized coccinellin, erythrosine and FD&C Yellow No. 6 (sunset yellow, $C_{16}H_{10}N_2Na_2O_7S_2$) dyes in this study. A coloured LB culture media with the addition of sunset yellow was used

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to culture *E. coli* strain DH5α, thereby colouring the bacteria (De Mey *et al*., 2008). The pigment doses used in all experiments ranged from 0.05 g/ml to 5 g/ml. All cultures were put into 5 ml eppendorf tubes and centrifuged at 10.000 × *g* for 5 min to allow for bacterial collection (Ngwa *et al*., 2013). Sunset yellow plating medium was used to grow *E. coli* at 37°C for 24–48 h. To test the ability of sunset yellow to dye pathogenic bacteria, *E. coli* O157:H7 and *S. flexneri* O301 were cultured in media containing sunset yellow. Again, different dilutions of sunset yellow pigment were used to culture *S. aureus* and *B. subtilis*.

Colony count assays were performed to determine the effect of sunset yellow on bacterial viability. *E. coli* DH5α cells were serially diluted (10^{-5}, 10^{-6} or 10^{-7}). Subsequently, 50 µl of diluted *E. coli* DH5α cells were added to 5 ml of a 43°C solution of 1% agarose in 100 mM NaCl, 10 mM sodium phosphate and 1% trypticase soy broth. Next, the entire 5-ml sample was poured over a conventional trypticase soy agar underlay. After incubating overnight, the colonies in the overlay were counted (Ferreira *et al*., 2010). Using the results of the plate counts, a growth curve was created to determine the incubation time and amount of *E. coli* required for the coloured culture.

Alternatively, turbidity readings of a 10-hour *E. coli* DH5α culture were taken every hour and PD was measured at a wavelength of 600 nm (Choi *et al*., 2010). Different inoculum sizes were set to 1% and 0.1% of the bulk volume. A growth curve was plotted using the results of the changes in OD values during the 10-hour growth. All the data were statistically evaluated with SPSS/13.0 software (SPSS Inc., Chicago, IL). Values of $P < 0.05$ were considered statistically significant. All the results are expressed as the mean ± standard deviation (SD).

During the *E. coli* culture, we could easily observe the turbidity of the media in each tube after 5 hours. We noted that sunset yellow was easily able to colour the bacteria yellow (Fig. 1A). The collected precipitate showed the coloured culture and the pigment successfully turned the bacteria yellow (Fig. 1B). More importantly, if some pathogenic bacteria had spilled out during the process, we would have been able to find them and promptly decontaminate the area. After culturing with the dye, we were able to observe the shape, size and traits of the bacteria (Fig. 1C).

When the sunset yellow plating medium was used, the *E. coli* bacterial colonies were coloured after growth at 37°C for 24–48 h. These methods were similar to those used by Zimmermann *et al*. (1978). Those colonies were adhered on the surface of the filter paper and were coloured yellow when compared with the control group colonies, which possessed sunset yellow staining that was not well adhered on the surface of the bacterial cells. More importantly, it could colour these cells. Thus, the prediction regarding the sunset yellow colouring mechanism proved to be correct. In Fig. 1D, tubes 1 and 3 are the cultures of O157:H7 and *S. flexneri* O301, respectively, in LB culture medium. It was apparent that O157:H7 in tube 2 and *S. flexneri* O301 in tube 4 were well labelled. The same phenomenon appeared in the groups of *S. aureus* and *B. subtilis* in Fig. 1E and 1F, respectively. In this way, the spillage of pathogenic bacteria could easily be detected. The pathogenic bacterial colonies of *S. aureus* and *S. flexneri* O301 in Fig. 1G show the clearly coloured cells. Compared with the uncoloured bacterial cells in Fig. 1A and B, *S. flexneri* O301 germinated and was marked well, as shown in images (Fig. 1C and D). The same results were found for *E. coli* O157:H7, as shown in images (Fig. 1E and F). Pathogenic bacteria are only a small portion of all bacterial families; however, the vital staining method is universal.

We performed colony counting and determined that sunset yellow dye did not inhibit bacterial growth. *E. coli* was cultured for 12–16 hours in coloured LB before being spread onto solid LB. Sunset yellow was shown to be an ideal dye that had little influence on bacterial growth (Fig. 2A). *E. coli* strain DH5α was cultured overnight and then diluted (10^{-5}, 10^{-6}, 10^{-7} or 10^{-8}). One hundred microliters of the diluted sample was then plated on solid LB with and without sunset yellow. These solid LB cultures were placed into a 37°C incubator and cultured overnight (Adler *et al*., 2011). Bacteria cultured in these coloured media were then sub-cultured in media without dye. Next, we chose the 5^{th} culture generation and applied the same methods of examination. As shown in Fig. 2B, those bacteria grew well and exhibited little difference compared with the control groups. Fig. 2C and D showed that at different bacterial loads (1% or 0.1%), the bacteria could grow in both normal LB and media containing sunset yellow LB. The *E. coli* cultured with sunset yellow added to the LB appeared to grow slightly slower than *E. coli* in normal LB between 1 and 4 hours. The growth was identical at 4 to 10 hours (Conrad *et al*., 2010; Cox *et al*., 2000). These results showed that there was no difference in bacterial growth and we concluded that sunset yellow pigment had no effect on bacterial growth.

Previously, rats were given FD&C Yellow No. 6 by gavage at 0, 60, 100, 200, 400, 600 or 1000 mg/kg body weight/day on days 0–19 of gestation. At the doses given, FD&C Yellow No. 6 was neither toxic nor teratogenic (Poul *et al*., 2009; Sasaki *et al*., 2002). The stability of this dye had been shown by Nevado *et al*. (1998). This method of staining with sunset yellow was a comprehensive, effective, stable, straightforward and safe way to stain some types of microorganisms. Although several studies have provided unequivocal evidence that

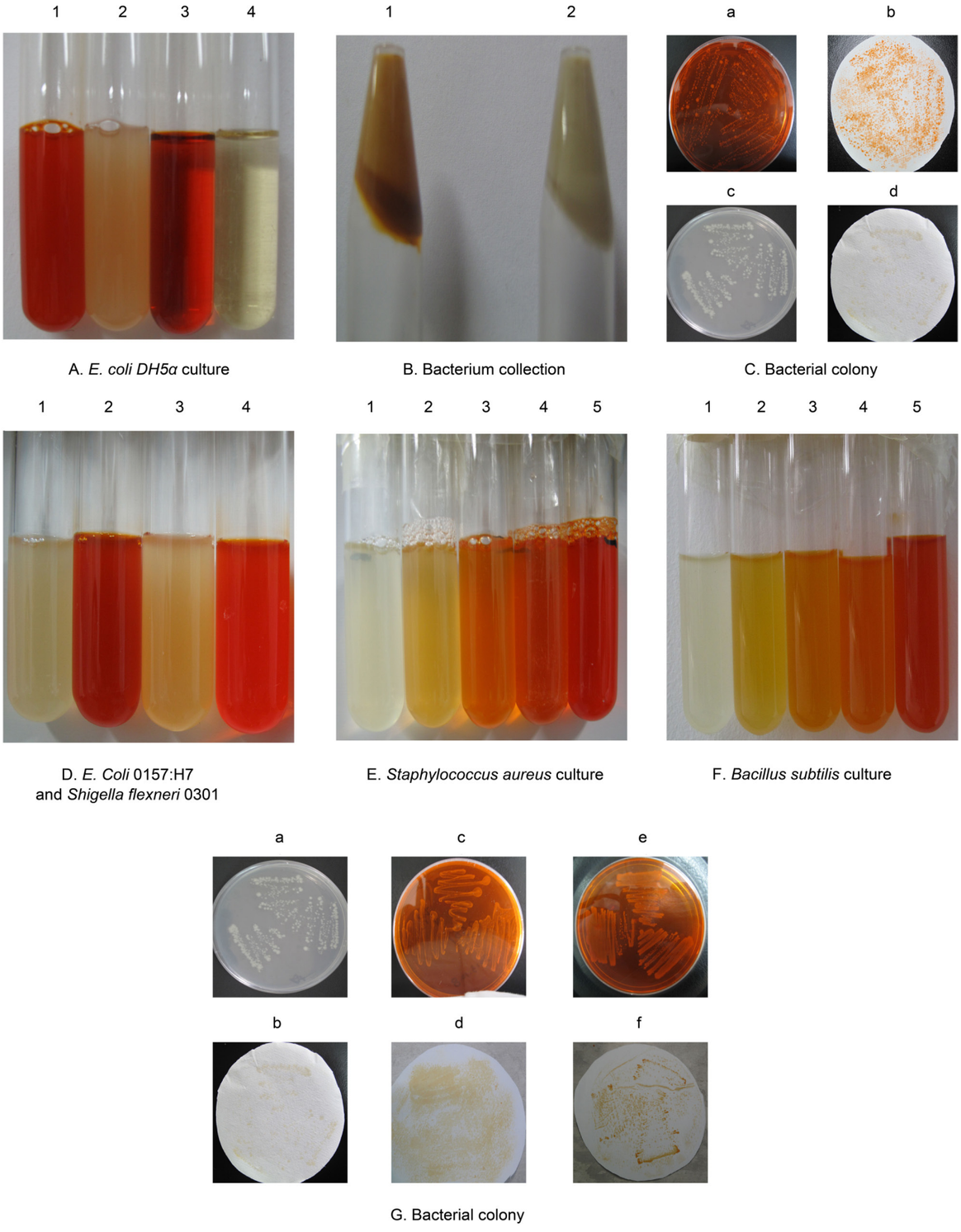

Fig. 1. The direct observation of *E. coli* DH5α, *E. coli* O157:H7, and *S. flexneri* O301 that had been stained with sunset yellow.

(A) The LB that was used to culture DH5α contained the sunset yellow dye (tube 1) or without pigment (tube 2). And tubes 3 and 4 contained sterile test media. (B) The precipitates were collected from 80 ml of culture. Tube 1 contained the precipitate collected from the sunset yellow LB, and tube 2 contained the precipitate collected from the normal LB. (C) The *E. coli* colonies grown in normal LB plating medium are shown in c and d, and those grown in sunset yellow plating medium are shown in a and b. (D) Sunset yellow LB was used to culture *E. coli* O157:H7 and *S. flexneri* O301. Different densities of sunset yellow LB were used to cultivate *S. aureus* (E) and *B. subtilis* (F). (G) The pathogenic bacterial colonies in d and f were *S. aureus* and *S. flexneri* O301, respectively, while the control group in Figure a was *S. flexneri* O301.

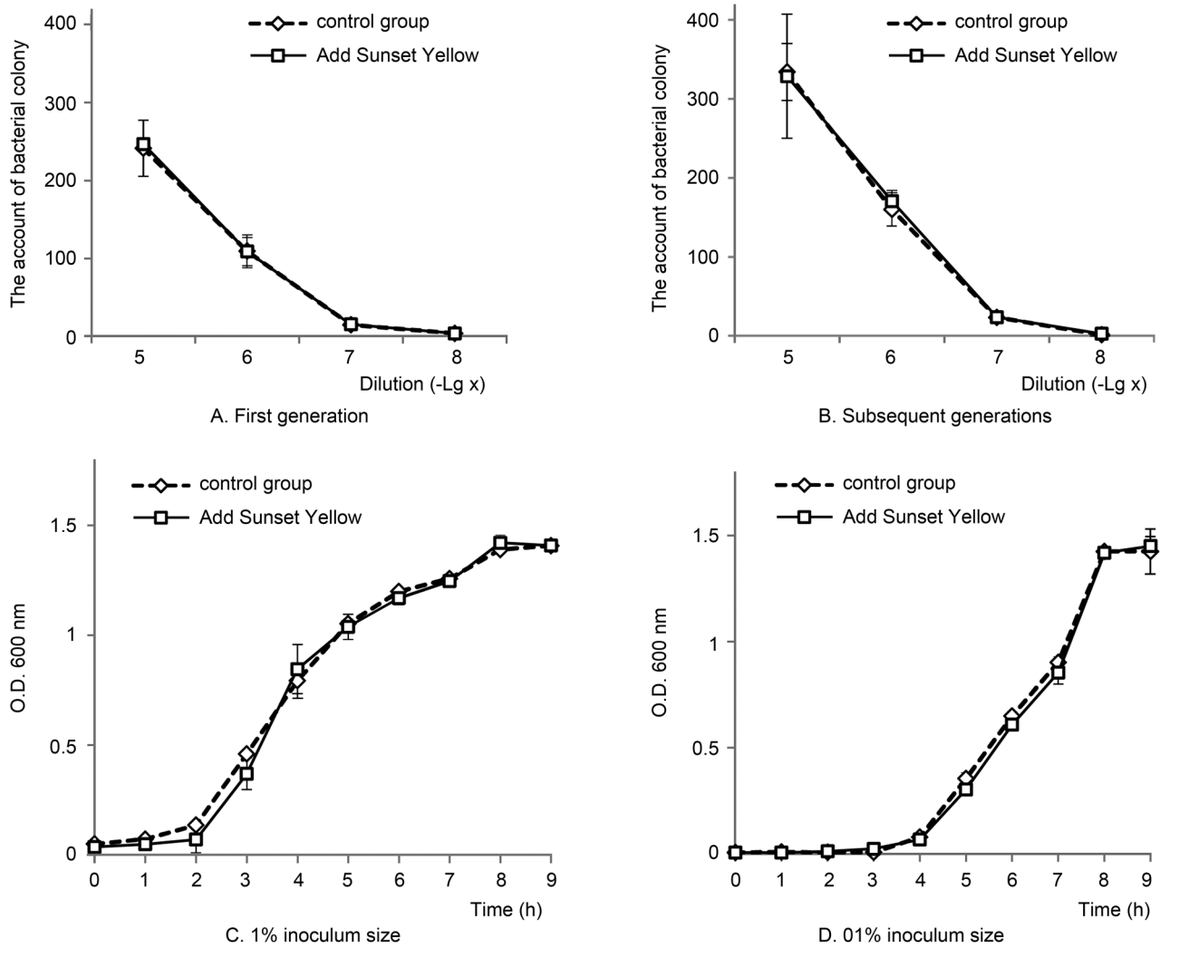

Fig. 2. Effects on the growth of *E. coli*.

(A) The counts of bacterial colonies at different dilutions after their first generation. These 4 dilutions demonstrate that there was no influence on *E. coli* DH5α on the number of colonies formed from generation to generation using new LB from beginning to end. These 4 dilutions show the growth rates of the experimental and control groups were nearly the same. (C) The one-step growth curve of *E. coli* DH5α. (D) The one-step growth curve of re-beginning to *E. coli* DH5α (0.1% inoculum size). From these results, we determined that there was no effect on the growth of *E. coli*.

bacteria isolated from humans, such as *E. coli*, *Shigella* sp., *S. aureus* and *Bacillus* sp., have the ability to cleave the azo linkage of sunset yellow to produce aromatic amines, which induce urinary bladder cancer, the most commonly used Sunset Yellow may not produce such adverse cytotoxic, mutagenic, or carcinogenic effects (Chung *et al.*, 2008).

In summary, in this study, we described a novel method of using a coloured dye to stain several types of pathogenic bacteria in culture. Importantly, the culture media, with added sunset yellow pigment, allowed for bacterial growth while simultaneously colouring all progeny. Therefore, we propose that this new coloured-LB culture system might provide a new method for labelling bacteria, especially pathogenic bacteria. The hope of this research is that after treatment with the sunset yellow pigment, any spillage or leak of pathogenic bacteria from culture will become visible and will be easy to monitor. The use of the sunset yellow dye as an indicator of bacterial distribution is both time- and cost-effective. Moreover, this method can be used for a much broader application in the dying of other types of microbes.

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