Comparison of three microtitre plate-based methods for quantification of biofilm formation ability of bacteria contaminating food technologies

JANKA KOREŇOVÁ – JANKA LOPAŠOVSKÁ – TOMÁŠ KUCHTA

Summary
Crystal violet assay, ATP-luminescence assay using an "all-in-one" reagent and fluorescein diacetate assay, all in a microtitre-plate format, were compared at quantification of biofilm formation ability of selected bacteria contaminating food technologies. After optimization, all three assays produced reproducible results and facilitated rapid identification of strains with strong (*Staphylococcus saprophyticus*, *Pseudomonas aeruginosa*), moderate (*Staph. aureus*) and weak biofilm formation ability (*Escherichia coli*). However, the ATP-luminescence assay produced considerably high values for the *E. coli* strain with a weak biofilm formation ability which could not be reduced by extensive washing. For practical use, we recommend the crystal violet assay for cases when the biofilm needs to be quantified irrespectible from bacterial cell viability, and the fluorescein diacetate assay for cases when only the viable bacterial cells in the biofilm need to be quantified.

Keywords
food technology; biofilm; luminometry; fluorimetry; *Pseudomonas*; *Staphylococcus*

Bacterial contamination of food technological equipment, manipulation surfaces and production environment may cause contamination of food products and therefore should be avoided. A special attention should be paid to bacterial strains capable of forming biofilms since these may be very resistant to cleaning and sanitation. Recognition of the presence of biofilm-forming bacterial contaminants in a food processing facility implicates application of more effective cleaning and sanitation procedures to provide a sufficient hygienic status [1, 2].

Ability of bacteria to form biofilms is traditionally measured by microbiological methods. In this type of methods, a culture is allowed to adhere to the surface of a coupon made from a defined material, then harvested, dispersed to a single-celled suspension and enumerated by plate counting of colonies [3, 4]. However, such microbiological methods are labour- and time-consuming, require relatively large amounts of materials and solutions, and therefore are not suitable for high-throughput applications [5].

As alternative methods, several microtiter plate-based methods for quantification of biofilm formation ability of bacteria have been developed. These methods make use of a correlation between the ability of individual bacterial strains to form biofilms on any hydrophobic or partly hydrophobic surface, including the inner surface of microtitre plate wells usually moulded from polystyrene. For quantification of bacteria in the biofilm, the biomass or its specific components are stained and measured in a microtitre plate reader [6, 7].

In this study, we compare three microtitre plate-based methods for quantification of biofilm formation ability of bacteria. The first method employs crystal violet staining of the biomass and the measurement of absorbance in a spectrophotometer. This method is well-established in several laboratories and has been extensively optimized to achieve a sufficient level of reproducibility [6]. An advantage of the method is its low price due to the use of a cheap dye and a quite common equipment. Some drawbacks of the method have been identified, such as its tendency to overestimate the

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biofilm forming ability of strains producing extracellular polysaccharides and its inability to distinguish between live and dead bacterial cells [7].

The second method is based on quantification of ATP-luminescence. Several similar methods are available for rapid quantification of bacterial contaminants in food processing facilities. In fact, biofilm is not directly quantified by these methods, but rather ATP released from the cells, which provides information on bacterial cell viability [8, 9]. In this study, we use a new easy-to-use commercially-available "all in one" formulation that contains, in one solution, the luminescence-compatible cell lysis agent, luciferin and luciferase in an optimized reaction mixture [10]. The produced glow-type luminescence is measured by a luminometer.

In the third method, biofilm is quantified based on the conversion of fluorescein diacetate, which is non-fluorescent, to fluorescein, which is fluorescent and is measured by a fluorimeter. In this method, biofilm is again not directly quantified but rather the bacterial non-specific esterases which carry out the conversion of fluorescein diacetate to fluorescein. However, the quantity of these non-specific esterases correlates with the numbers of viable bacterial cells and this facilitates quantification of viable bacterial cells in the biofilm [7, 11].

In this article, we report on the comparison of the three microtitre plate-based methods for quantification of biofilm formation ability of selected bacteria contaminating food technologies.

MATERIALS AND METHODS

Bacterial strains

Pseudomonas aeruginosa 2/13, *Staphylococcus saprophyticus* subsp. *saprophyticus* 10/1, *Staphylococcus aureus* subsp. *aureus* 4/3 and *Escherichia coli* 3/2 were isolated from food processing facilities in Slovakia and identified by Czech Collection of Microorganisms, Brno, Czech Republic. These strains were selected to represent a scale of biofilm formation ability (*Ps. aeruginosa* 2/13 - strong with extracellular slime, *Staph. saprophyticus* subsp. *saprophyticus* 10/1 - strong, *Staph. aureus* subsp. *aureus* 4/3 - moderate, *Escherichia coli* 3/2 - weak). This selection was based on results of preliminary experiments using a microbiological method of biofilm formation ability determination with polystyrene and stainless steel coupons to form the biofilm on, vortexing with beads to remove the attached bacterial cells and plating for their quantification [12]. Strains were stored freeze-dried.

Biofilm formation

Strains were inoculated to tryptone soya agar (Merck, Darmstadt, Germany) and incubated for 20–24 h at 37 °C. One colony was transferred to 5 ml of tryptone soya broth (TSB; Merck) and incubated for 20–24 h at 37 °C. Then the culture was centrifuged at 10 000 *g* for 10 min and the sediment was resuspended in TSB to the absorbance of 0.5 at λ = 570 nm, that corresponded to 10^8 CFU.ml^{-1}, as calibrated in preliminary experiments. A volume of 100 μl of the culture was pipetted in a well of a 96-well polystyrene microtitre plate (colourless for experiments with spectrophotometric reading; Sarstedt, Nümbrecht, Germany; white for experiments with luminometric or fluorimetric reading; Nunc, Roskilde, Denmark) and incubated for 18–20 h at 37 °C.

Crystal violet assay

A modified version of a previously described method [13] was used. The biofilm formed in the well of the microtitre plate was washed a defined number of times with 200 μl of phosphate buffered saline solution (PBS) [14] and dried for 30 min at 37 °C in an inverted position. A volume of 50 μl of a 1% (w/v) solution of crystal violet (Merck) in ethanol was added and incubated for 15 min at 25 °C. The dye solution was aspirated away and the well was washed with 5 × 400 μl of distilled water. After removing water and drying for 10 min at 25 °C, 200 μl of the mixture ethanol : acetone (80 : 20, v/v) were added. The absorbance at 570 nm of the dye solutions was measured in ELx808IU microtitre plate spectrophotometer (BioTek, Winooski, Vermont, USA).

ATP-luminescence assay

The assay using BacTiter-Glo reagent (Promega, Madison, Wisconsin, USA) was carried out according to the instructions of the manufacturer [10]. The biofilm formed in the well of the microtitre plate was washed a defined number of times with 200 μl of 0.9% NaCl. Afterwards, 100 μl of 0.9% NaCl and 100 μl of BacTiter-Glo reconsituted reagent solution was added and incubated for 10 min at 25 °C. Luminescence was measured in a Genios microtitre plate reader (Tecan, Grödig bei Salzburg, Austria).

Fluorescein diacetate assay

A modified version of a previously described method [7] was used. The biofilm formed in the well of the microtitre plate was washed a defined number of times with 200 μl of a buffer containing, per liter, 20.9 g 3-(*N*-morpholino)propanesulfonic acid (MOPS; Sigma-Aldrich, Steinheim, Germany)

and 5.6 g NaCl. Afterwards, a volume of 100 μl of MOPS buffer and 100 μl of freshly prepared fluorescein diacetate working solution was added. Fluorescein diacetate working solution was prepared by 1 : 50 dilution in MOPS buffer of the stock solution containing 10 mg.ml^{-1} fluorescein diacetate (Sigma) in acetone; the stock solution was stored at –18 °C. The microtitre plate was incubated at 37 °C for 60 min in the dark. Fluorescence was measured in a Genios microtitre plate reader (Tecan) at an excitation wavelength λ_{ex} = 492 nm and an emission wavelength λ_{em} = 520 nm at 10 flashes per measurement, integration time 40 μs, in an optimal gain mode.

Data processing

Non-linear regression and other statistical calculations were performed using Prism 4 software (GraphPad, San Diego, California, USA).

RESULTS AND DISCUSSION

Optimization of the crystal violet assay

Number of washings of the biofilm formed in the well of a microtitre plate was optimized in an experiment with 1 to 5 washings. Results show that at least 2 washings were necessary to reduce the apparent excess of biomass detected by this assay with strong biofilm-forming strains *Staph. saprophyticus* 10/1 and *Ps. aeruginosa* 2/13. For the strain *E. coli* 3/2, which does not form biofilm, the output of this assay was correctly very low (Fig. 1). The number of two washings was selected for further experiments.

Optimization of the ATP-luminescence assay

Number of washings of the biofilm formed in the well of a microtitre plate was optimized in an experiment with 1 to 7 washings. Results show that at least 3 washings were necessary to reduce and stabilize the output for the strain *E. coli* 3/2, which does not form biofilm. The non-zero values obtained for this strain even after further washings suggest that the value of about 10 000 rlu (relative luminescence units) may be the baseline for this assay. ATP-luminescence values for strong and moderate biofilm-forming strains *Staph. saprophyticus* 10/1 and *Staph. aureus* 4/3, respectively, were gradually decreasing with further washing, while values for *Ps. aeruginosa* 2/13 remained stable (Fig. 2). The number of five washings was selected for further experiments.

Optimization of the fluorescein diacetate assay

Number of washings of the biofilm formed in the well of a microtitre plate was optimized in an experiment with 1 to 4 washings. Output values were decreasing only slightly for the strong biofilm-forming strain *Staph. saprophyticus* 10/1 and were decreasing somewhat faster for *Staph. aureus* 4/3 and *Ps. aeruginosa* 2/13. For the strain *E. coli* 3/2, which does not form biofilm, the output was low upon two or more washings (Fig. 3). The number of two washings was selected for further experiments.

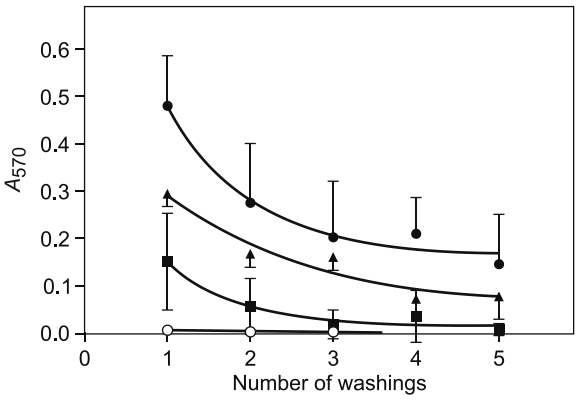

Fig. 1. Effect of the number of washings of wells containing biofilm on the output of the crystal violet assay.
Strains: *Staph. saprophyticus* 10/1 (filled circles), *Ps. aeruginosa* 2/13 (triangles), *Staph. aureus* 4/3 (squares) and *E. coli* 3/2 (open circles; values are very low).
Average values of six replicates ± standard deviation are presented; for the reason of a better clarity, error bars in only one direction are presented for the upper two curves.

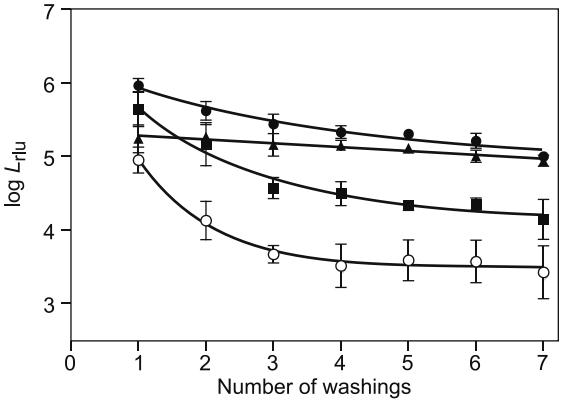

Fig. 2. Effect of the number of washings of wells containing biofilm on the output of the ATP-luminescence assay.
Strains: *Staph. saprophyticus* 10/1 (filled circles), *Ps. aeruginosa* 2/13 (triangles), *Staph. aureus* 4/3 (squares) and *E. coli* 3/2 (open circles).
Average values of six replicates ± standard deviation are presented.

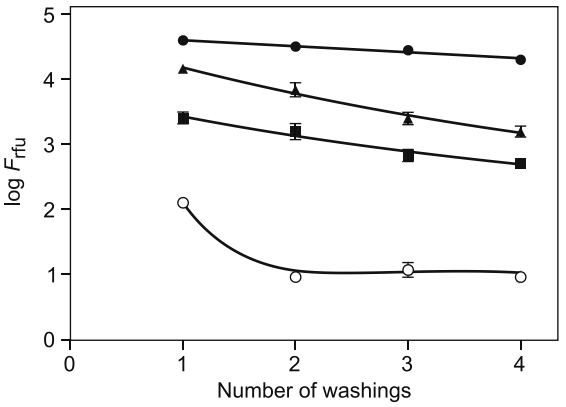

Fig. 3. Effect of the number of washings of wells containing biofilm on the output of the fluorescein diacetate assay.
Strains: *Staph. saprophyticus* 10/1 (filled circles), *Ps. aeruginosa* 2/13 (triangles), *Staph. aureus* 4/3 (squares) and *E. coli* 3/2 (open circles).
Average values of six replicates ± standard deviation are presented.

Comparison of the optimized assays

All the three assays were able to distinguish between strong, moderate and weak biofilm-forming bacterial strains. However, for the ATP-luminescence assay, considerably high luminescence values were obtained for the strain *E. coli* 3/2, which does not form biofilm (Tab. 1). This feature may lead to false positive results regarding the evaluation of biofilm forming ability of bacterial strains and makes this assay inferior to the other two. The reason of this phenomenon is not clear. Because ATP is a hydrophilic, unstable compound of a low molecular weight, it is improbable that its residues might persist bound to the microplate and cause the high background fluorescence. The phenomenon may be specific for the complex formulation used in this study.

Reproducibility of the crystal violet assay, when optimized and carefully carried out, may be considered acceptable for all strains including the extracellular polysaccharide-producing *Ps. aeruginosa*.

The fluorescein diacetate assay produced similar results, but somewhat higher values were obtained for both *Staphylococcus* strains. This raises the question of the distribution of non-specific esterase activity among strains of various species. However, the difference between strains with strong, moderate and weak biofilm formation ability was sufficient to recognize them. The fluorescein diacetate assay was better reproducible than the crystal violet assay.

CONCLUSIONS

All the three tested assays for quantification of biofilm formation ability of bacteria produced reproducible results and facilitated rapid identification of strains with strong, moderate and weak biofilm formation ability. However, the ATP-luminescence assay produced considerably high values for the strain with a weak biofilm formation ability. For practical, high-throughput use, we recommend the crystal violet assay for cases when the biofilm needs to be quantified irrespectible from bacterial cell viability, and the fluorescein diacetate assay for cases when only the viable bacterial cells in the biofilm need to be quantified.

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Tab. 1. Comparison of the optimized assays at quantification of the biofilm formation ability.

Strain	Crystal violet assay (A_{570})	ATP-luminescence assay (log L_{rlu})	Fluorescein diacetate assay (log F_{rfu})
Ps. aeruginosa	0.474 ± 0.183	5.32 ± 0.073	3.75 ± 0.185
Staph. saprophyticus	0.485 ± 0.118	5.48 ± 0.030	4.50 ± 0.052
Staph. aureus	0.047 ± 0.014	4.76 ± 0.067	3.29 ± 0.221
E. coli	0.004 ± 0.002	3.51 ± 0.293	1.34 ± 0.183

Values for blank (sterile medium) were always subtracted; average values from six replicate measurements ± standard deviation are presented.
A_{570} - absorbance at λ = 570 nm, L_{rlu} – luminescence in relative luminescence units, F_{rfu} – fluorescence in relative fluorescence units.

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