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Two-colour fluorescence fluorimetric analysis for direct quantification of bacteria and its application in monitoring bacterial growth in cellulose degradation systems

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ABSTRACT

Monitoring bacterial growth is an important technique required for many applications such as testing bacteria against compounds (e.g. drugs), evaluating bacterial composition in the environment (e.g. sewage and wastewater or food suspensions) and testing engineered bacteria for various functions (e.g. cellulose degradation). T?=1,^FigItem(1) ^ReloadFigure=Yesraditionally, rapid estimation of bacterial growth is performed using spectrophotometric measurement at 600 nm (OD600) but this estimation does not differentiate live and dead cells or other debris. Colony counting enumerates live cells but the process is laborious and not suitable for large numbers of samples. Enumeration of live bacteria by flow cytometry is a more suitable rapid method with the use of dual staining with SYBR I Green nucleic acid gel stain and Propidium Iodide (SYBR-I/PI). Flow cytometry equipment and maintenance costs however are relatively high and this technique is unavailable in many laboratories that may require a rapid method for evaluating bacteria growth. We therefore sought to adapt and evaluate the SYBR-I/PI technique of enumerating live bacterial cells for a cheaper platform, a fluorimeter. The fluorimetry adapted SYBR-I/PI enumeration of bacteria in turbid growth media had direct correlations with OD600 ($p > 0.001$). To enable comparison of fluorescence results across labs and instruments, a fluorescence intensity standard unit, the equivalent fluorescent DNA (EFD) was proposed, evaluated and found useful. The technique was further evaluated for its usefulness in enumerating bacteria in turbid media containing insoluble particles. Reproducible results were obtained which OD600 could not give. An alternative method based on the assessment of total protein using the Pierce Coomassie Plus (Bradford) Assay was also evaluated and compared. In all, the SYBR-I/PI method was found to be the quickest and most reliable. The protocol is potentially useful for high-throughput applications such as monitoring of growth of live bacterial cells in 96-well microplates and in assessing in vivo activity of cellulose degrading enzyme systems.

1. Introduction

Monitoring bacterial growth is essential for assessing many microbial applications. Determination of bacterial cell numbers can be done by direct and indirect methods. The commonest and oldest methods for this are turbidimetric measurements (optical density at 600 nm, OD600) and viable (plate/colony) counts (Breed and Dotterrer, 1916, Koch, 1970). Turbidimetric measurements are indirect methods which are fast and usually preferred when a large number of cultures are to be counted. The readings obtained from these measurements are a representation of the cell numbers (Koch, 1970). However to obtain definitive numbers, the readings must be correlated initially with cell number determined by other means (e.g. plate counts). Plate counts on the other hand give a direct measure of viable cells within the sample. Direct enumeration by microscopy using Petroff-Hausser counting chambers can also be performed (Treuer and Haydel, 2011). The aforementioned methods are however not universally applicable due to various limitations. Turbidimetric methods are unreliable for direct enumeration of bacteria cells in media containing insoluble substances such as food and environmental samples. Unavailability of suitable culture media and low concentrations of viable bacteria are major limitations to plate counting. To get around these challenges, different approaches based on the use of fluorochromes have been devised to investigate microbial viability and density (Barbesti et al., 2000, Caron and Badley, 1995, Diaper et al., 1992, Foladori et al., 2010, Kaprelyants and Kell, 1992, Porter et al., 1996, Tamburini et al., 2014).

Fluorochromes used in staining and enumerating bacteria cells by flow cytometry are based on membrane integrity, DNA binding and energy transfer between the fluorochromes (Barbesti et al., 2000,

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Gregori et al., 2001, Humphreys et al., 1994, Sgorbati et al., 1996). Barbesti et al. (2000) demonstrated that when DNA is simultaneously stained by SYBR-I (membrane permeant) and PI (non-membrane permeant) there is a decrease in the fluorescence of SYBR-I and an increase in the fluorescence of PI. This is due to a strong energy transfer between the two fluorochromes which facilitates discrimination between living and dead bacteria. This transfer is due to the extremely high quantum yield of DNA bound SYBR-I complex (~0.8, Molecular Probes Inc., USA) and the overlapping of its emission spectrum (Fig. 1) with the absorption spectrum of PI. As a result, the fluorescence of SYBR-I is 'quenched' by PI when stained with both. 'Dead cells' are regarded as cells with compromised membranes. The compromised membrane integrity allows both PI and SYBR-I to permeate the cells at which point such cells will fluoresce red (PI) when excited. 'Live cells' on the other hand allow only SYBR-I to permeate and when excited, fluoresce green.

Fluorescence has been used in quantitation for a long time. Although the technique has improved over time, a major challenge it faces is standardization and references for fluorescent measurements. A special issue on "Quantitative Fluorescence Cytometry: An Emerging Consensus" published by the journal *Cytometry* identified some of these challenges (Lenkei et al., 1998a, 1998b). Among them were (1) inter-laboratory comparisons (Purvis and Stelzer, 1998, Waxdal et al., 1998, Zenger et al., 1998), (2) instrumentation (Purvis and Stelzer, 1998, Wood, 1998, Wood and Hoffman, 1998), and (3) reagent and calibration standards (Gratama et al., 1998, Lenkei et al., 1998a, 1998b, Purvis and Stelzer, 1998, Schwartz et al., 1998, Shapiro et al., 1998, Wood and Hoffman, 1998, Zhang et al., 1998). A common response to these challenges has been the development of a fluorescence intensity standard (FIS), the MESF (molecules of equivalent soluble fluorochrome) for use in flow cytometry (Gaigalas et al., 2001, Schwartz et al., 2004, Schwartz et al., 2002, Wang et al., 2002). The MESF is based on a comparison between the number of fluorophores in two solutions, where one solution is a standard with known values. The standard is often a suspension of labelled microbeads. Although all these standardizations contribute to making the use of flow cytometry a better platform for enumerating live bacteria than OD600 and colony counting, there are some challenges that do not favour its wide use. First, the flow cytometers are relatively expensive and not available in many laboratories monitoring and enumerating bacterial growth. Secondly, the use and interpretation of flow cytometric data require special training and expertise. Furthermore, flow cytometers require regular servicing which is not available particularly in most developing regions such as sub-Saharan Africa. There is therefore a need to adapt the SYBR-I/PI principle of differentiating and enumerating bacteria for a simple and cheaper platform such as fluorimetry. As mentioned above, a major challenge for fluorescence measurement is the inability to make comparable fluorescence intensity measurements across laboratories and between different instruments. In response to this, a FIS based on DNA stained with SYBR-I and PI was also developed. This standard, like the MESF used in flow cytometry, is based on equivalency between the intensity of fluorophores in two solutions, one standard (known concentration(s) of DNA) and the other the unknown sample. An alternative method for semi-quantification of bacteria using the total protein content of the sample was also evaluated and compared with the two-colour fluorescence method.

As the world's fossil fuel reserves deplete, there is a growing need to develop sustainable fuels (Creutzig et al., 2015, Fulton et al., 2015, Nuffield Council on Bioethics, 2011). Solar electric, hydropower (hydroelectric, tidal and ocean thermal power) and geothermal appear are safe sustainable sources of energy. However, these sources are not practically useful in the long-distance transport sector, thus emphasizing the need for liquid fuels. The only sustainable source of liquid fuels appears to be plant derived biofuels. The United States (US) and the European Union (EU) have individually set targets to expand the production and use of biofuels. The Energy Independence and Security Act (EISA) of 2007 establishes that blending of renewable fuels (biomass diesel and cellulosic biofuels) into transportation fuels in the US should increase from 9 billion gallons in 2008 to 36 billion gallons by 2022 (Van Dyk and Pletschke, 2012). Similarly, the EU has also projected biofuel supply in transportation fuels to reach 25% by 2030 (Himmel et al., 2007, US EPA, 2013). Despite the potential of plant derived biofuels to meet these targets, large scale applications are currently problematic due to the difficulties in converting plant biomass (mostly made of cellulose, hemicellulose, lignin, pectin, and protein) into bioalcoholic derivatives and biodiesel. Naturally occurring cellulose degrading microbes use a battery of multiple catalytic enzymes to hydrolyse cellulose. We have described applications of synthetic biology that expand the technical capabilities of engineering efficient cellulose degrading enzyme systems (Duedu and French, 2016, French et al., 2015, Lakhundi et al., 2017) making it potentially easier to develop an ideal biofuel producing microorganism (IBPM) (French, 2009). Characterization and fine tuning of microbial cellulose degrading systems requires reliable methods for monitoring growth of cells on cellulose as a main carbon source. Substrates used for such experiments (e.g. avicel, paper and pre-treated plant materials) are generally insoluble. This makes the use of turbidimetric methods (e.g. OD600), which are fast and suitable for screening large numbers of samples, unreliable. Colony counting, on the other hand, is an arduous process. Dual staining of samples with SYBR-I and PI has been demonstrated as a useful method for quantifying bacteria in environmental and food samples using flow cytometry. The application of this method for fluorimetry has not been reported. Here, SYBR-I/PI dual staining was used to evaluate the cell content of cultures containing microcrystalline cellulose (avicel).

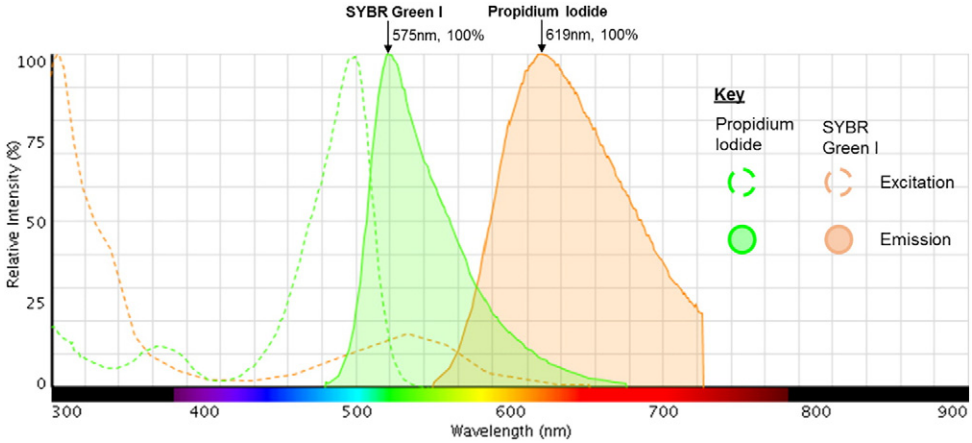

Fig. 1. Excitation and emission spectra of SYBR Green I and propidium iodide from Fluorescence SpectraViewer, Life Technologies.