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Short communication

A novel method for high-throughput data collection in predictive microbiology: Optical density monitoring of colony growth as a function of time

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ABSTRACT

Recently, the focus of predictive food microbiology has shifted towards more mechanistically-inspired modelling. Together with this trend, the need for methods that allow rapid data collection at the (intra)cellular level, as well as the intermediate subpopulation/colony level, has emerged. Although several experimental techniques are currently available to study colony dynamics in/on solid media, their widespread implementation as high-throughput methods remains a challenge.

In this research, a novel method is presented to study colony growth based on optical density measurements performed in microtiter plates. An area scan procedure was applied to monitor individual *Escherichia coli* colonies in 48-well plates at 30 °C. Based on a fixed threshold value to separate the object (colony) from the background, the colony area was determined as a function of time.

With this technique, expansion of the colony in radial direction could be monitored. Practical limitations (i.e., maximum achievable resolution and colony size) of the proposed method were investigated. A comparison was made with existing methods at the level of hardware requirements, data acquisition and data processing. Overall, the novel optical density method proved to be a flexible, high-throughput tool for monitoring (the mechanisms of) microbial colony growth in solid(like) systems.

1. Introduction

Ever since the end of the 19th century, characterisation of microbial development in/on agar in petri dishes has been a typical and basic laboratory method applied in microbiology. In contrast to liquid systems where microbial growth occurs planktonically, microorganisms in/on a (semi)solid surface organise within colonies. Both in academic research and industry (e.g., for quality control), computer colony counters are frequently used as basic laboratory equipment to determine the viable count, i.e., the number of colony-forming units per mL or g of a sample (for an overview of devices, see Puchkov (2010)). In recent years, it has become apparent that much more information and fundamental knowledge can be gathered from studying individual microbial colonies and their interactions. With the advent of automated image analysis techniques, enabling faster and more objective measurement of colony characteristics, new methodologies have been explored. For instance, in diagnostic microbiology, the appearance of colonies (i.e., colour, texture, shape, type of surface and edges) aids in the detection and identification of microorganisms (e.g., Banada et al., 2007; den Hertog et al., 2010). Depending on the research area and/or application of interest, the specific colony parameter of interest, e.g., number, colour, size, shape, morphology, will differ. In the context of food microbiology, colony growth is an important feature of solid(like) food products that are contaminated with a pathogen or spoilage microorganism.

In solid(like) systems, diffusion limitation (nutrients, metabolites) acts as a constraint on microbial growth. According to Malakar et al. (2002), this constraint only becomes important when colonies are allowed to reach certain sizes (corresponding to $> 10^5$ cells/colony). At inoculation densities above a certain level ($\geq$100 CFU/mL), colonies do not grow to these sizes and growth approaches that of broth cultures. However, at lower inoculation densities, which are more realistic in terms of actual food contamination, this approximation no longer holds true. When it comes to modelling the microbial behaviour in real food systems, it has been illustrated that models developed from broth experiments do not suffice and generally overestimate the growth rate/growth potential (Wilson et al., 2002). Moreover, the fact that most models do not take into account the more solid(like) character of food products is considered as one of the major shortcomings of predictive microbiology (Ross et al., 2000).

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Traditionally, predictive models focused on the overall microbial population by means of *macroscopic* models. In the last decennium, a quest for more mechanistically-inspired predictive models has emerged (e.g., Van Impe et al., 2009), implying model development at the *mesoscopic* (i.e., subpopulations within a population, colonies) and even the *microscopic* (i.e., (intra)cellular) level (see, e.g., Ferrer et al. (2008) for a definition of these levels). Together with these trends, the need for methodologies that enable rapid data collection for the characterisation of individual cells or colonies has emerged. As the traditional plate count technique is costly and labour-intensive, the combination of microscopy and image analysis has been gaining interest. For instance, at the single cell level, Elfwing et al. (2004) determined the individual doubling time of a cell, defined as the time needed to double the pixel size, for a large number of individual bacterial cells growing in a liquid environment by using a flow chamber in which the mother cells were spontaneously attached to a transparent solid surface. Similar methodologies have been followed by other authors to investigate characteristics of individual cells (Niven et al., 2006) and small populations of a few cells (Danias et al., 2011) growing on a solid substrate. Guillier et al. (2006) performed automated image analysis on bacterial colony growth as a means to study individual lag time distributions of immobilised cells. Monitoring of individual colony dynamics by means of microscopy has also been used to describe the overall population behaviour (Skandamis et al., 2007; Theys et al., 2009).

Although several (microscopy-based) methods are available to study colony dynamics in/on solid media, their widespread use in predictive food microbiology remains a challenge. Compared to liquid systems, data collection on/in solid(like) systems comes with several impracticalities (e.g., with regard to inoculation and sampling). In the existing experimental setups, carefully controlled conditions (e.g., temperature) are often difficult to achieve and specific recipients may be required (e.g., the gel cassette system, designed at the Institute of Food Research, Norwich, UK). As a result, the flexibility of these systems and their use as high-throughput techniques are limited (e.g., only a small number of experimental conditions can be investigated simultaneously). Another disadvantage when combining microscopy with image analysis techniques is the complexity of the data processing procedure, i.e., several steps, each of which introducing a certain error, are needed to translate the digital image into numeric data.

In this study, a novel method for quantifying individual colony dynamics is presented and its potential as a high-throughput alternative to the existing methods is explored. In previous work, optical density (OD) measurements were used to determine the growth/no growth boundary of *Zygosaccharomyces bailii* in gelled media (Mertens et al., 2011). Data collection was performed using the area scan method, in which OD values were measured at nine different positions in a microtiter plate well. In the present study, the area scan method is exploited at a much higher resolution to characterise individual colony dynamics of *Escherichia coli*. Hereto, a microplate reader is used that enables OD measurement at a large number of measuring points per well (i.e., 124×124 grid, corresponding to $>15{,}000$ points per well). To illustrate its potential, this novel measurement method is applied to investigate the evolution of colony area as a function of time.

2. Materials and methods

2.1. Bacterial strain and inoculum preparation

E. coli K12 MG1655 (CGSC #6300) was obtained from the *E. coli* Genetic Stock Center (Yale University) and stored in Brain Heart Infusion broth (BHI) (Oxoid) supplemented with 25% glycerol (Acros Organics) at −80 °C. For the preparation of the inoculum, a loopful of the stock culture was transferred in 20 mL of BHI broth and subsequently incubated at 37 °C for 9 h. Next, 200 μL of the cell suspension was transferred to 20 mL of fresh BHI broth and incubated at 37 °C for 15 h.

2.2. Growth medium

The growth medium consisted of BHI supplemented with 5% (w/v) agar (Technical Agar No. 3, Oxoid). After autoclaving, the hot medium was distributed into 48-well microtiter plates (Greiner Bio-One) with 350 μL medium/well. After solidification of the medium, the microtiter plates were sealed with parafilm. The microtiter plates were prepared at least three days before use.

2.3. Inoculation procedure

100 μL of the second preculture (9 log (CFU/mL)) was six times decimally diluted in liquid BHI. Then, 150 μL of the last dilution was transferred to 850 μL BHI. This corresponded to a cell density of approx. 2.2 log (CFU/mL). Next, every well of the microtiter plate was inoculated with 10 μL of the bacterial suspension, resulting in approx. 1 to 2 individual cells per well. In order to obtain surface colonies, the spot inoculation method was applied, i.e., the 10 μL inoculum was pipetted as a single drop onto the agar surface. The microtiter plate was placed in an incubator at 30 °C without the lid. After 30 min, the liquid inoculum had dried completely onto the agar surface, and the microtiter plate was placed in a temperature-controlled microplate reader at 30 °C.

2.4. Data processing

E. coli growth was assessed by measuring the OD at a wavelength of 595 nm using a FilterMax™ F5 microplate reader with Multi-Mode Analysis software (Molecular Devices). Instead of the traditional single-point measurement, the 'area scan' method was applied, i.e., OD was measured at multiple positions in a well. In total, an area scan of 124×124 points (resulting in a total of 15,376 OD values) was performed per well (= spatial resolution) at each sampling time. In the FilterMax™ system, the scan is carried out row by row. It took approx. 20 min to complete one row (corresponding to 8 separate wells) of a 48-well microplate. This resulted in a time window of approximately 2 h for the total plate (= temporal resolution).

Data processing was performed in MatLab Version 7.9 (The MathWorks, Inc.). For each sampling time and position, the OD at time zero (blank) (Fig. 1a) was subtracted from the raw OD value (Fig. 1b), resulting in a processed data set as depicted in Fig. 1c. Next, a threshold level was defined to separate the object (bacterial colony) from the background. The threshold level was determined as the lowest OD value for which microbial colonies could be distinguished from artefacts (e.g., colour changes of the medium during time). Based on preliminary tests, the threshold OD was set at 0.010, which is within the specifications reported by the manufacturer of the microplate reader (i.e., accuracy $>$ 99%, reproducibility $>$ 99.5%). It should be noted that this threshold value is valid for the specific BHI-agar (5% (w/v) agar) combination studied here. Other combinations of microbiological media and gelling agents, or other concentrations of agar, may require different threshold values. As a result, a matrix of 0 (background) and 1 (colony) values was obtained, which is illustrated in Fig. 1d. Important to note is that Fig. 1a, b, c & d are merely an illustration of the different data processing steps, rather than the actual output of the method which is numeric. Taking the distance between adjacent measuring positions into account, individual colony characteristics were determined. The colony area A [mm^2] was calculated based on the fact that each measurement point represents the centre of