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Note

A simple imaging method for biomass determination

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Abstract

An inexpensive and fast method based on images taken during growth of bacterial cells on multi-well plates was developed for biomass quantification. A correlation of 85% between the results obtained by image analysis and optical density measurements was obtained. This simple method allows the assessment of growth with highly aggregated cell cultures and the rapid screening of a large number of carbon sources.

Keywords: Growth quantification; Substrate screening; Image analysis; Cell clustering; Toxic substrates

There are numerous methods currently available for the determination of biomass. Among these, plate counting and direct counting procedures using microscopic methods are widely used. The differences between the results obtained with these two types of methods depend on the number of dead and/or non-viable cells, i.e., on the number of cells which are not able to form colonies, and also on both the selectivity of the growth medium and the incubation conditions used for the agar plates. In the majority of the papers published in the last two decades, fluorescent methods were the preferred technique due to their high sensitivity (Poglazova et al., 1996). Epifluorescence microscopy is generally acknowledged to be one of the most adequate methods for the quantification of microorganisms in all habitats (Fry, 1990; Kepner and Pratt, 1994). However, this technique has also several drawbacks, such as the inability to carry out further studies on the microorganisms used in the observations. The direct epifluorescence filter technique, in which the microorganisms are stained with dyes such as acridine orange has been accepted as a quantitative method for determining the number of bacteria in aquatic environment (Heldal et al., 1994). However, the visual counting of bacteria on filters is laborious and time-consuming. To overcome this, automated methods were developed. The most promising were

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cyto- and spectrofluorimetric methods (Paul and Myers, 1982; Poglazova et al., 1984; Poglazova et al., 1996), especially flow cytometry (Tyndall et al., 1985; Donelly and Gaigent, 1986; Alcon et al., 2004; Laplace-Builhé et al., 1993).

Quantification of specific populations is achieved by molecular techniques, such as fluorescent antibody and in situ hybridization techniques, which allow direct detection and in situ identification of microorganisms (important to assess the existence of subpopulations in biofilms). In this case, the reliability of the techniques depend on the physiological state and on the general properties of a particular strain. Besides being expensive methods, for having automated image analysis, high-quality images must be acquired with the inherent costs of the necessary image-acquisition hard and software.

The ability of a bacterial strain to degrade a certain substrate, e.g., hydrocarbon or alcohol, can and has been successfully used for bioremediation and biocatalytic purposes. However, the majority of these substrates are toxic and affect the cellular membrane (de Carvalho et al., in press). This can lead to modifications at the cellular membrane level and thus in cell hydrophobicity, altering the cell adhesion properties. Often clusters of cells are formed and consequently biomass growth cannot be assessed by turbidity measurements, since the clusters absorb and scatter light differently as compared to their free cell counterparts.

In the present study, we describe a simple and fast method to assess cell growth in the presence of alcohols and hydrocarbons. As shown below, cell growth could be monitored by taking photographs of the plates where cells were growing and the corresponding growth rates could be calculated by image analysis. The microorganism chosen was *Rhodococcus erythropolis* DCL14, which has been found able to degrade a large number of hydrocarbons and alcohols and even fuel oil. When this strain was cultured in shaken flasks, cell clustering was observed in the presence of the majority of these carbon sources (de Carvalho and da Fonseca, in press).

In the present work, growth was carried out in plates with ninety-six 300-μl wells, containing 150 μl of mineral medium (Wiegant and de Bont, 1980), and in plates with twenty-four 2.75-ml wells, containing 400 μl of mineral medium. Three wells were used per carbon source concentration. The initial carbon source concentrations tested were 0.125% and 0.25% (v/v). The organic solvents used as sole carbon and energy sources were ethanol (99.8%), butanol (>99.5%), propanol (>99.5%), *n*-dodecanol, cyclohexane (>99.5%) and toluene (>99.5%) from Merck; *n*-octane (>99%) purchased from Merck-Schuchardt; methanol (>99.8%), *n*-hexane (>99%) and *iso*-octane (>99.5%) from Riedel-de Haën; *n*-undecane (99%), *n*-tetradecane (99%) and *n*-hexadecane (99%) purchased from Sigma; cyclohexanol (99%) and *n*-dodecane (>99%) from Aldrich; pentane (99%) purchased from Fluka; *n*-heptane (95%) from Lab-Scan; and *n*-nonane (99%) from Acros. Growth was carried out at 28 °C and 200 rpm in a Heidolph Inkubator 1000 and the optical density was measured, without further delays to avoid cell aggregation, at 600 nm with a Spectra Max 340 PC from Molecular Devices. When cell clusters were observed, cells could be separated before carrying out optical density measurements by increasing agitation to 600 rpm for 30 s.

The plates were photographed seven times during the growth time course, after being allowed to settle for 30 s, using a COHU camera with a Cosmicar TV 12.5–75 mm zoom lens. The lens aperture was set to 1.8 to have as little depth-of-field as possible and the zoom was fixed at 60 mm to decrease image distortion. All images were grabbed at the same magnification in the Red–Green–Blue system. The acquisition software was Matrox Inspector 2.1. Four images of each plate were taken at each time: the corners of the plate were photograph separately and the wells that were common to more than one picture were used as controls during image analysis. Fig. 1a,b shows examples of the photographs grabbed during the experiments.

The plates were agitated in a rotary shaker incubator, as previously mentioned. Due to the high hydrophobicity of *R. erythropolis* cells (de Carvalho et al., 2000; de Carvalho and da Fonseca, 2004), they were mainly positioned at the surface or inside the solvent hydrophobic droplets (de Carvalho and da Fonseca, 2003). These, in turn, were mainly located at the surface of the liquid phase since they have densities lower than water. Furthermore, on the surface, the access to oxygen was also easier. Thus, cell growth could be observed, under the naked eye, mainly at the surface of the aqueous phase. When