Fluorometric measurement of poly-$\beta$ hydroxybutyrate in Alcaligenes eutrophus by flow cytometry and spectrofluorometry

Abstract Two fluorometric assays for the determination of poly-$\beta$-hydroxybutyrate (PHB) inside intact cells are presented in this paper, a spectrofluorometric method and one based on a laser flow cytometer. The principles of these assays are given, and the results of these methods are compared with those of a gas chromatographic assay. All assays were used for the monitoring of batch cultivations of Alcaligenes eutrophus. The correlation between all methods was very good; however, the fluorometric analysis was the fastest, while the cytometric assay gave a direct insight into the PHB distribution over the population.

Introduction

Poly-$\beta$-hydroxybutyrate (PHB) is an energy and carbon reserve compound found in many bacteria. It is a chloroform-soluble polyester with approximately 25,000 $\beta$-hydroxybutyric units, and can be produced by many micro-organisms. For example, the bacterium Alcaligenes eutrophus yields up to 80% of the dry cell weight when fed with fructose (Bitar and Underhill 1990; Guske 1990; Schlegel 1985, 1990; Sonnleitner et al., 1979; Srienc et al. 1984; Steinbüchel 1989). PHB is a thermoplastic and biologically degradable polymer and is thus of high industrial interest (Steinbüchel 1991a, b).

As a carbon reserve, PHB can be reintegrated into the metabolism and can be consumed as a carbon source (Schlegel 1985; Steinbüchel 1991b). The cultivation medium for A. eutrophus contains fructose (Gottschalk et al. 1964; Peoples and Sinskey 1989; Sonnleitner et al., 1979), which is degraded via the Entner-Doudoroff pathway to pyruvate. The pyruvate is then converted to PHB through the Calvin cycle (Steinbüchel 1989; Steinbüchel and Schlegel 1989).

Until now, time-consuming methods have been used to analyse the level of PHB production. In these methods, the cells must first be dried. After drying, PHB must be extracted with chloroform and can then be determined gravimetrically, via infrared (IR) spectroscopy, or using gas chromatography (GC) (Braunegg et al. 1978). In the investigations presented here, the GC method was used as a reference for the measurement of the PHB content.

An easier method for quantitative PHB detection is the Nile Red fluorescence technique. Nile Red is a fluorescent lipid dye (Fowler and Greenspan 1985; Greenspan and Fowler 1985). This dye easily penetrates the cells in suspension and measurements can be performed after a short time. The fluorescence intensity of Nile-Red-stained cells results from the PHB concentration; non-PHB staining is low because the bacteria have almost no lipids (Schlegel 1985, 1990). The aim of our research was to investigate the relationship between the fluorescence data obtained from cytometric or spectrofluorometric measurements and the values obtained via conventional GC assays.

Materials and methods

Micro-organism and cultivation

A. eutrophus (DSM 531) was cultivated in a medium containing 10 g/l of fructose, 0.5 g/l K$_2$HPO$_4$, 1 g/l of NH$_4$Cl, 0.2 g/l of MgSO$_4$·7H$_2$O, 0.01 g/l of FeSO$_4$·7H$_2$O and 0.01 g/l CaCl$_2$·2H$_2$O per litre of distilled water (Schlegel 1985). The cultivation was
carried out in a 5-1 baffled erlenmeyer flask containing 21 medium. The environmental conditions were maintained at 37°C and pH 7.0. The preculture was grown in LB medium (casein peptone, yeast extract, and isotonic NaCl solution). Twenty millilitres of this preculture was used to inoculate the main culture.

Samples for all PHB assays were withdrawn from the cultivation every 3 h and centrifuged. After centrifugation, all cell samples were fixed in ice-cold ethanol solution (70%) for long-term storage.

Conventional analysis with a gas chromatograph

The methyl ester of β-hydroxybutyric acid was detected with a gas chromatograph (Perkin-Elmer, model Sigma 3B) equipped with a flame ionization detector (FID) and a methyl ester column. The parameters of the GC analysis were: injector temperature, 175°C; FID temperature, 250°C; initial oven temperature, 145°C for 7 min, heating at 39°C/min, final temperature, 240°C; injection volume 1 ml, flow rate 60 ml/min N2 and carrier gas pressure 350 kPa.

Ten millilitres of the cell suspension was used for PHB detection. The supernatant was decanted after centrifugation. The sediment was resuspended in water and was treated with an ultrasonic disintegrator for 10 min to obtain improved cell fragmentation. The sediment was frozen at -28°C and lyophilized. The dried samples were treated with 2 ml of an esterification mixture (50 mg benzoic acid as standard, 3 ml H2SO4, and 100 ml methanol) and 2 ml chloroform. The closed samples were heated to 100°C for 3.5 h. After cooling to room temperature, 1 ml H2O was added and the samples were shaken for 10 min. After the separation of phases, the denser phase, which contained the methyl ester, was analysed with the GC (Braunegg et al. 1978). In total, about 2 days were necessary to obtain GC data about the PHB content in the cells. These assay conditions cannot be regarded as sufficient for optimal process monitoring.

PHB staining with Nile Red

For all staining assays, a stock solution of Nile Red was prepared containing 1 mg/ml of Nile Red in acetone. Nile Red can be dissolved easily in lipophilic phases. Its solubility in water is poor and its fluorescence is quenched by water (Fowler and Greenspan 1985). Nile Red, which is non-toxic, can stain both fixed and unfixed cells; however the fluorescence intensity of fixed cells is higher. For staining, cells were resuspended in saline solution.

Previous investigations showed that a 10-ml aliquot of Nile Red stock solution can stain 1 ml cell suspension, up to an optical density (OD) of 5 with a PHB content up to 70% of cell dry weight. After 30 or 40 min, the cells are totally stained and the fluorescence maximum is reached. This assay can be used to detect PHB quantitatively.

Analysis with a laser flow cytometer

Flow cytometry is a technique to determine intracellular components and cell sizes. With this device, up to 5,000 cells can be analysed (Ormerod 1990; Shapiro 1985). In our investigations, a FACS Scan cytometer (Becton Dickinson) was used. The cells are oriented with their longest axis in the direction of the flow and aligned in a laminar flow stream by hydrodynamic focusing. A laser beam, which is perpendicular to the flow, is focussed on a spot a few micrometres in diameter inside the flow chamber. The cells cross the laser beam and the interaction between the beam and each cell provides a signal to the detector, which can be a photodiode or a photomultiplier. This technique allows the measurement of fluorescence or light scattering. In previous studies on the determination of PHB in A. eutrophus, light-scatter signals were used because PHB forms intracellular refractile bodies that alter the light-scattering properties (Srienc et al. 1984).

Before analysis in the cytometer, the cell samples were diluted so that the counting rate was about 1000 cells/s. An argon ion laser was used for excitation (488 nm) and a photomultiplier with a 600-nm filter was used for detection of the fluorescence. Cells were stained as described above. The fluorescence signals obtained from the photomultiplier are proportional to the component concentration.

Analysis with a spectrofluorometer

A spectrofluorometer is an instrument to record luminescence phenomena of fluorescence or phosphorescence samples at continuously variable wavelength. The intensity of the light beam incidened to a photomultiplier is the parameter to be measured. Emission scans, excitation scans and time courses of light intensity can be performed with this technique. In this paper, a LS-5 B spectrofluorometer (Perkin Elmer) was used.

The fluorescence excitation and emission spectra of A. eutrophus stained with Nile Red are given in Fig. 1. The maximum excitation wavelength is 543 nm, the emission maximum is 598 nm for stained PHB-containing A. eutrophus. Figure 2 shows the relationship between the OD of the cell suspension and the Nile Red fluorescence, after addition of a constant volume of the stock staining solution. In the range 0-5 OD units, the fluorescence intensity is linear. The data measured by the spectrofluorometer were kept at a constant OD of 0.3 (excitation wavelength 543 nm/emission wavelength 598 nm).

![Fig. 1 Fluorescence spectra of Alcaligenes eutrophus stained with Nile Red](image1)

![Fig. 2 Relationship between the optical density of a cell suspension and Nile Red fluorescence. (Cell suspensions were taken from the same sample and diluted)](image2)