Flow-Cytometric Detection of Changes in the Physiological State of *E. coli* Expressing a Heterologous Membrane Protein During Carbon-Limited Fedbatch Cultivation

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Received 19 January 2005; accepted 12 April 2005

Published online 2 September 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/bit.20575

Abstract: The key to optimizing productivity during industrial fermentations is the ability to rapidly monitor and interpret the physiological state of single microbial cells in a population and to recognize and characterize different sub-populations. Here, a flow cytometry-based method for the reproducible detection of changes in membrane function and/or structure of recombinant *E. coli* JM101 (pSPZ3) expressing xylene monooxygenase (XMO), was developed. XMO expression led to compromised but not permeabilized cell membranes. This was deduced from the fact that recombinant cells only stained with ethidium bromide (EB) and not with propidium iodide (PI). During the glucose-limited fedbatch cultivation, an increase from 25% to 95% of EB-stained cells was observed, occurring between 2 and 5 h after induction. Control experiments confirmed that this increase was due to the recombinant protein production and not caused by any possible effects of varying substrate availability, high cell density, plasmid replication or the presence of the inducing agent. We hypothesize that the integration of the recombinant protein into the cell membrane physically disrupted the functionality of the efflux pumps, thus resulting in EB-staining of the recombinant cells. This method enabled us to detect changes in the physiological state of single cells 2–4 h before other indications of partial cell damage, such as unbalanced growth, acetate accumulation and an increased CO₂ production rate, were observed. This method therefore shows promise with respect to the further development of an early-warning system to prevent sudden productivity decreases in processes with recombinant *E. coli* expressing heterologous membrane proteins.

Keywords: ethidium bromide; fedbatch cultivation; *E. coli*; flow cytometry; physiological state; propidium iodide; recombinant membrane protein; xylene monooxygenase

INTRODUCTION

During fedbatch processes with recombinant microorganisms, the following factors often lead to physiologically and structurally compromised cells: over-expression of a foreign protein, high cell density as well as varying substrate availability both changing in progression of the feed-profile and/or caused by variations in the micro-environment in a non-ideal bioreactor (Andersson et al., 1996; Onyeaka et al., 2003; Schmidt et al., 1999; Soriano et al., 2002; Yoon et al., 2003). Such changes in recombinant microorganisms are supposed to be the reason for a decrease in productivity during biotechnological processes (Christiansen et al., 2003). Therefore, detailed process monitoring, specifically focussing on the actual physiological state of the recombinant microbial population, is the key to understanding, standardizing and maximizing the overall process productivity (Hewitt et al., 1999; Kacmar et al., 2004). Depending on the nature of the process data determined, different levels of process understanding ranging from “black-box” information to highly detailed information on the individual cells or even cell structures can be acquired. Conventional monitoring methods mostly provide population averaged values (i.e., concentrations of metabolites and biomass, dissolved O₂ concentration, CO₂ production rate, etc.), while single-cell analysis is usually limited to the determination of colony-forming units (Harms et al., 2002). The latter method is, however, tedious and the results become available with a time delay of 1 or more days. Moreover, recent information on the occurrence of an “active but non-culturale” (ABNC) state in many microorganisms including *E. coli* underlines the requirement for alternative methods for the detection of “active” cells (Barer and Harwood, 1999; Kell et al., 1998; Nyström, 2001). Indeed, “active cell state” is not only limited to cellular division, but in fact includes a wide array of other characteristics such as respiration, enzyme activity, and both the structural integrity and the physiological functioning of the cell wall (Joux and Lebaron, 2000).

Since flow cytometry provides rapid and statistically reliable information on cells in aqueous solutions (Shapiro, 2005).
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Pure cultures of 

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Bacterial Strains 

MATERIALS AND METHODS 

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method development and not the process per se was the focus 

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our investigations, and therefore the complex industrial 

process exploiting this particular recombinant strain (i.e., an 

organic and aquatic phase process; Bühler et al., 2003) 

was simplified to an experimental model system in which 

interpretable changes could be introduced more easily. 

MATERIALS AND METHODS 

Bacterial Strains 

Pure cultures of E. coli JM101 and recombinant E. coli JM101 (pSPZ3) (described in Panke et al., 1999) were obtained from A. Schmid (ETH Zürich, Switzerland). The cultures were stored in 20% glycerol at -80°C. With respect to the expression of heterologous protein, three types of cultures were distinguished: 

1. the XMO-expressing recombinant strain (E. coli JM101 (pSPZ3), with plasmid and with induction); 

2. the non-induced recombinant strain (E. coli JM101 (pSPZ3), with plasmid but without induction); and 

3. the unmodified strain (E. coli JM101, without plasmid and without induction). 

Culture Medium 

The mineral medium contained per liter (final concentration): glucose, 7 g; KH₂PO₄, 13.3 g; (NH₄)₂HPO₄ (Aldrich Chemical Company, Milwaukee, WI), 4.0 g; citric acid anhydrite, 1.7 g; MgSO₄·7H₂O, 0.62 g; 1 mL of a 1% (w/v) thiamine hydrochloride solution (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 0.1 mL antifoam polypropylene glycol P2000 and 5 mL of a trace elements solution (adapted from Panke et al., 1999), containing 1M hydrochloric acid and per liter: FeSO₄·7H₂O, 8.87 g; CaCl₂·2H₂O, 4.70 g; MnCl₂·4H₂O, 1.50 g; ZnSO₄·7H₂O (Riedel-de Haen GmbH), 1.87 g; H₃BO₃, 0.3 g; Na₂MoO₄·2H₂O, 0.25 g; CuCl₂·2H₂O, 0.15 g and Na₂EDTA·2H₂O, 1.08 g. Except when stated otherwise, all chemicals were from Fluka (Buchs, Switzerland) Chemie AG. The components were dissolved in deionized water to 90% of the final volume, and the inoculum made up the remaining 10% of the volume. The pH of the mineral medium was adjusted to 6.8 with NH₃·aq, 25% (v/v), and subsequently to 7.4 with 5M NaOH. Glucose and MgSO₄·7H₂O were sterilized separately, trace elements solution, thiamine hydrochloride solution and kanamycin were separately sterilized by filtration (0.22 μm, Milllex GP, Millipore, Carrigtwohill, Ireland) and added after heat sterilization (120°C, 20 min) to cooled-down mineral medium. The Luria- Bertani (LB) medium contained per liter: tryptic digest of casein (Biolife, Milan, Italy), 10 g; bacto yeast extract (Biolife, Milan, Italy), 5 g; NaCl, 10 g. The pH was adjusted to 7 with 5M NaOH and the solution was sterilized (120°C, 20 min). When the recombinant strain E. coli JM101 (pSPZ3) was cultured, the medium was supplemented with 0.05 g kanamycin per liter final concentration. 

Cultivation 

Shake-flask batch cultures (200 rpm) were grown overnight in LB medium at 30°C prior to use. Fedbatch cultivation was carried out in a 3.5 L glass reactor (MBR Bio Reactor AG, Wetzikon, Switzerland; PCS AG, Wetzikon, Switzerland) with a working volume of 2 L at a constant temperature of 30°C and a pH of 7.4 ± 0.2. The pH was controlled with either NH₃·aq, 6.25% (v/v) or H₃PO₄, 5% (v/v). The dissolved oxygen concentration (pO₂) in the reactor was kept higher than 20% air saturation with an agitation rate of 1,000 rpm and an airflow of >4 L/min. If required, the air was enriched with pure oxygen, but only to a concentration of less than 30% O₂ to prevent toxic effects. The inoculum for the initial batch phase (10%) was prepared by adding 5% of an overnight LB culture to the above described mineral medium and growing it for 8 h in Erlenmeyer flasks on a rotary shaker at 200 rpm and 30°C. After the initial batch cultivation in the reactor and before starting the fedbatch cultivation, an additional 4 mL of a 1% (w/v) thiamine hydrochloride solution, 4 mL trace elements solution (composition see above) and 0.1 mL antifoam (polypropylene glycol P2000) were directly added per liter of culture. The feed solution contained per liter a final concentration of 803 g glucose monohydrate and 19.6 g MgSO₄·7H₂O, both dissolved in deionized water, and the pH was adjusted to 3 with 1M hydrochloric acid. The exponential feed profile (Eq. 1) for fedbatch cultivation was started with a feed rate of 6 g/h feed solution described above and an exponential increase by a factor of 0.15/h.

\[
F(t) = 6 \cdot e^{0.15t}
\] (1) 

If required, XMO expression in E. coli JM101 (pSPZ3) was
induced after 2 h fedbatch cultivation by adding 0.02% dicyclopopylketone (DCPK; Bühl er et al., 2000).

**Fluorescent Staining and Flow Cytometry**

Fedbatch samples were diluted with filtered (0.22 μm, Millex GP, Millipore) supernatant from the same sample to a concentration of (1–5) · 10⁷ cells/mL. Supernatant was specifically used to avoid any impact of dilution on the physiological characteristics of the cells. Aliquots of 500 μL of the diluted sample were stained with either a combination of 0.5 μL EB (red fluorescing, 25 mM, Fluka Chemie AG) and 0.75 μL SYTO® 9 (green fluorescing, 3.34 mM, Molecular Probes, Leiden, The Netherlands), or with the combination of 0.75 μL propidium iodide (PI) (red fluorescing, 20 mM, Molecular Probes) and 0.75 μL SYTO® 9. The EB staining concentration was applied according to Hewitt et al. (1999) whereas PI and SYTO® 9 concentrations were used according to the manufacturer’s instructions (www.probes.com). The stained samples were incubated in the dark at 30°C for 10–30 min before measurement. After incubation samples were diluted in filtered (0.22 μm, Millex GP, Millipore) commercial mineral water (EVIAN, Evian, France), so that the concentration for flow cytometry (FCM) analysis was less than (1–5) · 10⁵ cells/mL. FCM was performed using a PASIII flow cytometer (Partec, Münster, Germany) equipped with a 25 mW argon ion laser, emitting at a fixed wavelength of 488 nm. Green fluorescence (SYTO® 9) was collected in the FL1 channel (520 ± 20 nm), and red fluorescence (EB, PI) was collected in the FL3 channel (>610 nm). For all measurements FL1 was triggered and signals were collected in a 3 decade logarithmic scale. Data were analyzed using Flowmax® software (Partec). Gates were constructed on the combined red and green fluorescence dot-plots in order to differentiate among the various sub-populations. Bacteria that stained with SYTO® 9 but excluded EB or PI were located within gate R1 (see Fig. 1). Gate R1 includes not only green fluorescence (FL1) but also red fluorescence (FL3) because the emission spectra of SYTO® 9 is tailing into the red emission range (www.probes.com). Cells that also stained with EB or PI in addition to SYTO® 9 showed a decreased green fluorescence combined with increased red fluorescence and were located within gate R2. The percentage of the EB- and PI-stained cells was subsequently calculated from the gated area (R2).

![Figure 1](https://www.interscience.wiley.com)
relative to the total number determined by SYTO® 9-staining only. Staining results were controlled with fluorescence microscopy, using an inverted microscope (Olympus IX 51) equipped with the appropriate filters.

**Fluorescent Staining Controls**

Untreated, depolarized, and permeabilized E. coli JM101 cells were used as controls for EB and PI staining. For this, E. coli JM101 cells with an initial optical density of 0.1 measured at 450 nm (OD<sub>450 nm</sub>, 1 cm cuvette; Uvikon 930 Spectrophotometer) were grown in Erlenmeyer flasks in the same mineral medium as described above for 4 h on a rotary shaker at 200 rpm and 30°C (μ<sub>max</sub> = 0.5/h). For flow cytometry, the culture was diluted with filtered supernatant to a concentration of (1–5)·10<sup>7</sup> cells/mL. Such cells represented “untreated” cells. Furthermore, 500 μL of the same diluted cell suspension was incubated with 1.33 μL carbonyl cyanide m-chlorophenyl hydrazine (CCCP 15 mM) at 30°C for 30 min to depolarize the cells previous to staining (Lambert and Le Pecq, 1984). In order to permeabilize the cells, the diluted cell suspension was incubated for 3 min at 75°C. Starved cells in the stationary phase were produced by extending the time of batch culture described above to 30 h. XMO-expressing E. coli JM101 (pSPZ3) cells were grown in Erlenmeyer flasks to an initial OD<sub>450 nm</sub> of 0.2 under the same conditions as described above. After 2.5 h of batch cultivation XMO production was induced with 0.02% DCPK. Cells were harvested 6.5 h after induction under non-substrate-limited conditions (4.4 g/L glucose, 0.65 g/L acetate). Dilution, staining and flow cytometry was done as described above.

**Enzyme Activity Assay**

Whole-cell assays to determine XMO activity was adapted from Bühler et al. (2000). Cells were harvested by centrifugation (17,700g, 4°C, 3 min) and resuspended to a cell dry weight (CDW) of 0.5 g/L in 50 mM potassium phosphate buffer, pH 7.4, containing 1% (w/v) glucose. The numerical value of CDW used in the assay was determined indirectly by OD<sub>450 nm</sub> measurement (CDW [g/L] = 0.21·OD<sub>450 nm</sub>). Dried cells of the same mineral medium were isolated by HPLC on a YMC ODS-AS column (pore size, 10 nm; particle size, 3 μm; inner diameter, 50 × 3 mm; YMC, Kyoto, Japan) with a mobile phase gradient of (0 min) 80% H<sub>2</sub>PO<sub>4</sub> (0.1% w/v), 20% acetonitrile (supragradient grade, Scharlau, Nr:AC0331, Barcelona, Spain) at a flow rate of 0.7 mL/min and (8 min) 20% H<sub>2</sub>PO<sub>4</sub> (0.1% w/v), 80% acetonitrile at a flow rate of 0.8 mL/min. UV-detection of metabolites was done at 210 nm. One unit (U) defined as the activity that produces 1 μmol of total products (3,4-dimethylbenzalcohol, 3,4-dimethylbenzaldehyde, 3,4-dimethylbenzoic acid) in 1 min. Specific activity was expressed as activity per g CDW (U/(g CDW)).

**Additional Analytical Methods**

CDW during fedbatch cultivation was determined using 1–2 mL of sample and pre-dried, calibrated filters (0.22 μm, Millex GP, Millipore). The number of colony-forming units (CFU) was determined by serial dilution of the cell suspension in Dulbecco’s phosphate buffered saline (DPBS), using the pour-plate method with TSA-agar (Biolfite, Milan, Italy) and subsequent incubation at 37°C for 24–48 h. Glucose and acetate concentrations (detection limit at 0.01 g/L in the supernatant) were measured by HPLC, using an Aminex HPX-87H column (particle size, 9 μm; inner diameter, 300 × 7.7 mm; BioRad #125–0140, Hercules, CA) with a mobile phase of 3 mM H<sub>2</sub>SO<sub>4</sub> at a flow rate of 0.6 mL/min and a refractometer detector. The CO<sub>2</sub> and O<sub>2</sub> concentrations in the exhaust gas, which were used to calculate the specific carbon-dioxide production rate, were determined by EPGAS (Biospectra AG, Schlieren, CH).

**RESULTS**

**Control Experiments for the Suitability and Reliability of the Method**

The control experiments, performed with E. coli JM101 cells in different extreme physiological states, demonstrated the appropriateness of staining with EB to detect different levels of compromised cell membranes. Flow cytometry (FCM) data (Fig. 1) showed that exponentially growing (μ<sub>max</sub> = 0.5/h) untreated E. coli cells stained with SYTO® 9, but neither with PI nor with EB. The fluorescence emission spectra of SYTO® 9 enabled the detection of cells on both the green channel (520 nm) and the red channel (>610 nm), thus resulting in the specific pattern on the dot-plots (see “Materials and Methods” for details). Exponentially growing cells which were permeabilized by means of a heat treatment (75°C, 3 min) did stain with both PI and EB (Gate R2). Cells that were severely depolarized with 40 μM CCCP stained only with EB and not with PI. Further FCM controls relevant for the particular fedbatch cultivation process were carried out (data not shown), the results of which are summarized as follows:

1. Nutrient starvation of E. coli JM101 cells (30 h, 30°C) did not result in EB- or PI-staining of the cells;
(2) XMO-expression of E. coli JM101 (pSPZ3) under substrate-excess batch conditions (i.e., not substrate-limited) led to EB-staining of 76% of the cells 6.5 h after they were induced;

(3) Fedbatch cultivations with unmodified E. coli JM101 and the addition of the induction agent (0.02% DCPK) confirmed that this chemical inducer does not affect either EB- or PI-staining.

Changes in Growth and Substrate Utilization

The expression of XMO in recombinant E. coli JM101 (pSPZ3) altered the growth and substrate utilization pattern as compared to non-induced recombinant E. coli JM101 (pSPZ3) and unmodified E. coli JM101 (Fig. 2). Note that the exponential feed profile, which determined a specific growth rate of 0.15/h, was identical in all three cultivations. No residual glucose (>0.01 g/L) was detected in any of them. Figure 2A shows data for unmodified E. coli JM101 during first 10 h of fedbatch cultivation. CDW, total cell counts and CFU increased according to the feed profile throughout the cultivation. The correlation between CDW and total counts confirmed that balanced growth occurred. A fedbatch cultivation of the non-induced recombinant strain displayed similar results (data not shown). In Figure 2B the data for the XMO-expressing recombinant strain with induction after 2 h are depicted. CDW increased exponentially but the increase was lower than that observed for the non-induced recombinant or the unmodified strain, respectively. Moreover, the data for the XMO-expressing strain showed that the increase in CDW was not reflected by a parallel increase in the total counts or CFUs (Fig. 2B). This unbalanced growth became more apparent in the second half of the fedbatch cultivation. Table I shows the specific growth rates (μ) based on total counts achieved by three different cultures. The non-induced recombinant strain and the unmodified strain both exhibited specific growth rates within the expected range. The same holds for the XMO-expressing strain during the first 6 h of cultivation ((0.16 ± 0.06)/h), but subsequently the specific growth rate dropped significantly ((0.02 ± 0.06)/h). Based on this finding, two different cultivation periods can be recognized for the XMO-expressing strain, namely 0–6 h and 6–10 h. For the latter cultivation period, both the biomass yield (Y_X/S) and the final CDW concentration were lower for the XMO-expressing strain in comparison to either the non-induced recombinant strain or the unmodified strain (Table I). The specific carbon dioxide production rates (CPR; mmol CO₂/(g (biomass) × h)) for the three fedbatch cultivations are compared in Figure 3. The CPR data for the non-induced recombinant and the unmodified strains displayed no significant difference, while the XMO-expressing recombinant strain produced significantly more CO₂ per hour and gram of cells (i.e., a higher specific CPR). These results indicate that the XMO-expressing strain was converting more carbon to CO₂ than to biomass, hence was affected in the balance of assimilation to dissimilation. Plasmid maintenance of the non-induced recombinant strain caused no significant change in the CO₂ production. A carbon balance confirmed that during the fedbatch process (0–10 h) with the XMO-expressing strain 59% of the total glucose-carbon was converted into CO₂ whereas the non-induced recombinant strain and the unmodified strain only converted between 46% and 47% of the total carbon into CO₂ (Table II) The variations of specific CPRs were the main cause for the differences in biomass yield and final CDW concentration observed (Table I). The reduced biomass yield in the second cultivation period (6–10 h) of the XMO-expressing strain also accounts for the difference between observed and expected specific growth rate (0.15/h) based on CDW (Table I). Furthermore, XMO expression resulted in an altered carbon metabolism, indicated not only by a higher specific CPR, but also by eventual acetate accumulation. No acetate accumulation was observed at the end of fedbatch cultivations of the non-induced recombinant strain or the unmodified strain (Fig. 4A and B). During cultivation of the XMO-expressing strain acetate accumulation was first detected after 8 h and it increased to a final concentration of 1 g/L. However, the main change in carbon balance of the XMO-expressing strain was caused by the higher specific CPR and not by the acetate accumulation.

Figure 2. Comparison of methods to quantify the growth during fedbatch cultivation. A: unmodified E. coli JM101 and (B) XMO-expressing E. coli JM101 (pSPZ3). Total counts were determined by flow cytometry of SYTO®-9-stained cells. The broken line at 6 h indicates the time point when a significant change in the specific growth rate based on CDW and total counts was identified (i.e., 95% confidence intervals of the specific growth rates do not overlap).
Early Detection of Physiological Changes by Flow Cytometry

With FCM it was possible to detect the detrimental effect of XMO expression on membrane functioning of *E. coli* 2–4 h earlier than with conventional methods. The population of XMO-expressing *E. coli* cells sampled during fedbatch cultivation showed an increase from 25% to 95% in the percentage of positively EB-stained cells, occurring between 4 and 7 h (Fig. 4C). No such effect was seen for the non-induced recombinant strain or the unmodified strain (Fig. 4D). The dynamic shift in EB-staining of XMO-expressing cells can also be clearly seen in FCM dot plots from which the percentages of EB-stained cells were calculated (Fig. 5). Sub-populations that became clearly stained with EB during fedbatch cultivation were shifting from the region defined by gate R1 and the intermediate region to the region defined by gate R2. XMO expression led to compromised but not to permeabilized cells as the percentage of PI-stained cells remained lower than 5% throughout the cultivation period (Figs. 4C and 5). EB-staining indicated the effect on membrane functioning 2 h before the specific growth rate changed (Fig. 2B) and 4 h before acetate accumulation was visible (Fig. 4A). Within this time period the maximum specific heterologous XMO activity was reached (Fig. 4A). It has to be pointed out that these results were reproducible in parallel fedbatch cultivations, both in terms of the time period of occurrence and the magnitude of change. Using the same exponential feed profile but inducing XMO after 6 h instead of 2 h, resulted in the same delay until cells stained with EB. This rules out any effect of cell density or cell age on the staining properties of the cells.

**DISCUSSION**

Flow Cytometric Detection of Physiological Changes Prior to Acetate Accumulation

Adverse physiological and structural changes affecting recombinant bacteria during industrial cultivation have major repercussions for the productivity of a biotechnological process. The precise causes of these changes are not

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**Table I.** Comparison of specific growth rates, biomass yields, and end concentrations of CDW for fedbatch cultivations using XMO-expressing recombinant, non-induced recombinant and unmodified *E. coli*.

<table>
<thead>
<tr>
<th>Strain type (process phase)</th>
<th>$\mu$ [1/h]$^b$</th>
<th>$Y_{X/S}$ [g/g]$^c$</th>
<th>CDW [g/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>XMO-expressing recombinant*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–6 h fedbatch</td>
<td>$0.16 \pm 0.06^d$</td>
<td>$0.16 \pm 0.01^d$</td>
<td>$0.46 \pm 0.03$</td>
</tr>
<tr>
<td>6–10 h fedbatch</td>
<td>$0.02 \pm 0.06^d$</td>
<td>$0.12 \pm 0.01^d$</td>
<td>$0.36 \pm 0.02$</td>
</tr>
<tr>
<td>Non-induced recombinant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–10 h fedbatch</td>
<td>$0.20 \pm 0.04^d$</td>
<td>$0.17 \pm 0.01^d$</td>
<td>$0.47 \pm 0.02$</td>
</tr>
<tr>
<td>Unmodified</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–10 h fedbatch</td>
<td>$0.15 \pm 0.02^c$</td>
<td>$0.15 \pm 0.01^d$</td>
<td>$0.43 \pm 0.04$</td>
</tr>
</tbody>
</table>

For fedbatch cultivations with XMO-expressing recombinant *E. coli*, two different phases from 0 to 6 h and from 6 to 10 h were distinguished. Values are significantly different if stated 95% confidence levels do not overlap.

*Induction after 2 h.

$^b$Specific growth rate calculated using linear regression $\mu(t) = \frac{\ln(x_t) - \ln(x)}{t - t_i}$.

$^c$Calculated from total counts values obtained by SYTO$^b$ 9-staining.

$^d$Calculated from CDW values.

$^e$Biomass yield calculated using linear regression $Y_{X/S}(t) = \frac{x_t - x_{t_i}}{Y_{X/S}}$.

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**Table II.** Basic carbon balance for the three different fedbatch cultivations, expressed as percentage $C_{out}$ of total $C_{in}$.

<table>
<thead>
<tr>
<th></th>
<th>Recombinant XMO-expressing <em>E. coli</em></th>
<th>Recombinant non-induced <em>E. coli</em></th>
<th>Unmodified <em>E. coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomass</td>
<td>41.5</td>
<td>57.1</td>
<td>58.4</td>
</tr>
<tr>
<td>Glucose</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Acetate</td>
<td>2.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>58.6</td>
<td>46.4</td>
<td>46.4</td>
</tr>
</tbody>
</table>

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Figure 3. Influence of XMO expression (—, black) and replication of heterologous plasmid (—, dark gray) on the specific CO$_2$ production rate (CPR) compared to the behavior of unmodified strain (—, light gray). The CO$_2$ and O$_2$ concentrations in the exhaust gas needed to determine CPR were measured during glucose-limited fedbatch cultivation with identical exponential feed profile (Eq. 1). The broken line at 6 h indicates the time point where a significant change in the specific growth rate and the biomass yield was identified (i.e., 95% confidence intervals do not overlap).
always clearly understood and conventional detection methods often lack the required specificity and/or are too arduous to be of practical relevance for online process control. A non-specific method typically used to determine the state of E. coli cultures is the analysis of acetate concentration. In processes such as the one studied in this study (typically involving glucose as the carbon source), acetate accumulation is an unwanted effect and can be a sign of process malfunction (Jensen and Carlsen, 1990; Luli and Strohl, 1990; Sandén et al., 2003). Acetate accumulation can result for a number of different reasons, such as excess carbon leading to higher specific glucose uptake rates than the respiratory capacity of the tricarboxylic cycle can handle (Holms, 1996), a changed carbon metabolism due to recombinant protein production (George et al., 1992), or a decreasing respiratory capacity of the cells during a fedbatch process (Konstantinov et al., 1990). However, this reliable general process indicator (i.e., acetate accumulation) does not provide information on specific cellular changes and can usually only be detected once the changes have occurred. In this study we have seen that fedbatch cultivation of XMO-expressing recombinant E. coli JM101 (pSPZ3) was accompanied by unbalanced growth (Fig. 2B), an increased specific CPR (Fig. 3) and eventual acetate accumulation (Fig. 4A). None of these adverse responses were detected during fedbatch cultivation of the non-induced recombinant bacteria, nor for that of the unmodified strain (Figs. 2A, 3, and 4B). Importantly, flow cytometric analysis with EB-staining allowed us to detect significant and quantifiable physiological changes of the entire XMO-expressing cell population 4 h before acetate accumulation started (Fig. 4A and C). The flow cytometry method therefore potentially provides a most valuable “on-line” process monitoring tool.

Membrane Function or Structure Targeting Dyes Compared to Culturability

XMO likely consist of two sub-units; one that is soluble (XylA), and one which is membrane bound (XylM) (Suzuki et al., 1991). Since at least one component of XMO is located on the inner membrane of recombinant cells, the cytoplasmic membrane is a likely target to be affected by XMO-expression (Panke et al., 1999; Shaw and Harayama, 1995). Membrane function or structure targeting dyes such as EB, propidium iodide and bis-(1,3-dibutylbarbituric acid)trimethine oxonol [DiBAC4(3)] have been used successfully for assessing the bacterial physiological state in fedbatch reactors (Christiansen et al., 2003; Hewitt et al., 1999; Hohenblum et al., 2003). In this study we have demonstrated EB-staining to be an excellent indicator of the particular cellular physiological changes linked to XMO-expressing bacteria in the process studied. PI-staining displayed no increase throughout the process, indicating that XMO expression did not lead to permeabilized membranes. Using DiBAC4(3) we found too much variability in the staining results, in particular with bacteria grown at different growth rates and in different physiological states. This precludes the use of the dye with confidence in this fedbatch process. Control experiments demonstrated the essential differences between PI and EB (Fig. 1). The large PI molecule (668 g/mol) reveals extreme states of cell wall damage (permeabilization) and for this reason is considered to be a reliable “dead” cell indicator (Nebe-von Caron et al., 1998). Conversely, the small EB molecule (394 g/mol) can enter an intact cell, but is pumped out under normal physiological conditions (Midgley, 1987; Paulsen et al., 1996). However, if the efflux pumping activity of the cells is compromised, for

Figure 4. Comparison of acetate accumulation, EB and PI staining of XMO-expressing recombinant E. coli JM101 (pSPZ3) (black; A, C), non-induced recombinant E. coli JM101 (pSPZ3) (gray; B, D), and unmodified E. coli JM101 (open symbols; B, D) during glucose-limited cultivation in fedbatch using an identical exponential feed profile. The broken lines at 4 and 6 h indicate the time where an increasing number of XMO-expressing E. coli JM101 (pSPZ3) cells stained with EB, as well as the time when the accumulation of acetate started.
example by the absence of a transmembrane proton gradient, these cells will retain EB (Midgley, 1987). Hence EB appears to be more sensitive than PI to minor alterations in the cytoplasmic membrane, and seems to be also a less reliable indicator of “dead” cells (Nebe-von Caron et al., 1998).

In this particular study, the number of neither PI- nor EB-stained cells correlated well with changes in culturability as determined by plating. Figure 2B shows a 40%–60% reduction in culturability of the XMO-expressing cells after the first 9 h of cultivation. At this point, no cells were stained significantly by PI, while more than 90% of cells were susceptible to EB. It is thus argued that while definite changes occurred in the membrane of XMO-expressing bacteria during fedbatch cultivations, these alterations did not necessarily imply a complete loss of culturability, and certainly did not imply permeabilization of the cell membrane.

From a methodological point of view it is important to state that we have used the green fluorescing total DNA stain SYTO<sup>1</sup>, together with both PI and EB. This offered dual advantages. Firstly, SYTO<sup>1</sup> staining could be used to quantify total cells. Secondly, because the emission spectrum of this dye reaches into the red channel (above 610 nm), it was also employed to enhance detection of PI- and EB-staining. This allowed detection of minor staining differences on the combined dot plot data (Fig. 5) long before they were noticeable if only single staining was employed.

### Interpretation of EB Staining

The fact that EB stained the XMO-expressing cells, but PI did not, allows interpretation of the actual changes that occurred at cellular level. One possibility is that increased energy requirements as a consequence of the foreign plasmid replication and protein expression caused an energy deficiency, resulting in malfunctioning of the efflux pumps and other membrane processes (Schmidt et al., 1999). Soriano et al. (2002) related an increase in cell size and ceasing cell division of a recombinant *E. coli* to the deviation of the general metabolic fluxes from all cellular processes towards plasmid maintenance and foreign protein synthesis. However, in this study we have seen no changes in the dissimilation rate and other growth characteristics during fedbatch cultivation of the non-induced recombinant strain (Fig. 3). Moreover, batch experiments with the XMO-expressing strain under excessive substrate conditions displayed an EB-staining behavior similar to that in fedbatch cultivations, suggesting energy deficiency may not have been the cause of the staining in this particular case. We propose an alternative hypothesis for EB-staining of XMO-expressing cells, namely that integration of the recombinant product (XMO) as an integral protein of the inner membrane of the organism, physically disrupted the functionality of the efflux pumps, which are assumed to consist of components in the inner membrane, the outer membrane and the periplasmic space (Nikaido, 2001; Zgurskaya and Nikaido, 2000). This hypothesis derives credibility from the fact that EB-staining occurred in the same time frame when maximum specific XMO activity was detected (5 h after induction, 7 h after fedbatch start; Fig. 4A). Moreover, in the XMO-expressing cells almost no further increase in cell number was seen after 6 h of fedbatch cultivation while cell dry weight continued...
to increase (Fig. 2B). This suggests that the integrated heterologous protein did not only affect the activity of the efflux pumps but also the functionality of the entire membrane, which resulted in retardation of cell division and an increase in cell size (Fig. 2B). In fact, the data presented in this study largely corroborate similar previous findings for the expression of alkane hydroxylase, of which the membrane bound component (AlkB) is similar to XylM of XMO (Suzuki et al., 1991). Witholt and co-workers have shown that upon expression of AlkB, a reduction occurs in the maximum specific growth rate of the recombinant bacteria, which was accompanied by a decrease in cellular division (recognized by filamentous growth) (Chen et al., 1996). In addition, they have shown that expression of AlkB leads to significant changes in the fatty acid composition of the cellular membranes, and incorporation of the protein in the membrane up to 15% of total cellular protein (Chen et al., 1996; Nieboer et al., 1997). Based on these results, it was proposed that the incorporation of the foreign protein in the membranes of the recombinant cells were a cause of change in the physiology of the expressing cells (Chen et al., 1996). Inducing the cells with 0.02% DCPK after 6 h instead of 2 h led to the same delay before the EB-staining occurred, which means that it is not the culture age that determined EB-staining, but the time period during which the cells were induced. Hence it can be deduced that a delayed induction at a higher biomass concentration, might lead to better overall productivity, though further investigations with regards to the effect of this approach on the organic phase product formation are still required (Bühler et al., 2000).

CONCLUSIONS

Flow cytometry is an easy and rapid analysis method that can provide additional information about particular physiological changes to single cells. During the particular fedbatch process in which recombinant XMO was expressed, it was established that:

1. The expression of the heterologous membrane protein affected cell physiology when compared to the non-expressing strain;
2. Physiological changes of single cells were detected earlier with flow cytometric analysis of EB-stained cells than with acetate accumulation, unbalanced growth or increased CPR;
3. Throughout the different cultivations, it was found that the time-course of the EB-staining pattern was reproducible.

For all the reasons above, the flow cytometry method which was investigated shows considerable promise with respect to the further development of an early-warning monitoring system to prevent sudden productivity decreases in processes with recombinant bacteria.

The authors kindly thank Andreas Schmid and Bruno Bühler for providing the recombinant E. coli JM101 (pSPZ3) strain and the essential information on the particular recombinant production process, Markus Kneubühl for the HPLC analysis, as well as Martin Rieger and Maggi Lussi Bell for draft reading.

References


