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Robust Batch Cultivation Process for Recombinant Protein Production with *Escherichia coli* BL21 in a BIOSTAT® B Reactor

Application Note

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turning science into solutions

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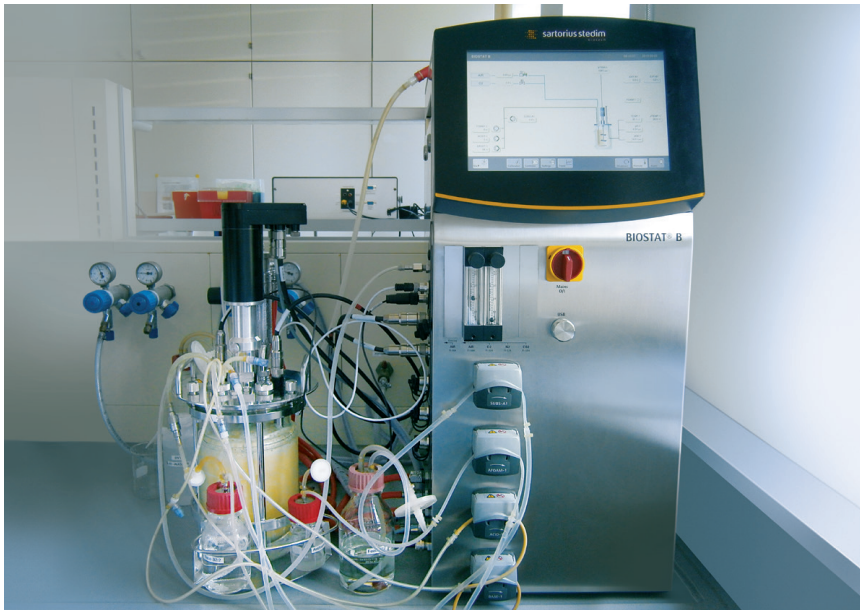
1. Introduction

Worldwide and since decades recombinant protein production is often done with wellknown apathogenic strains of *E. coli*, e.g. K12 derivatives like DH5, TG1, W3110 or members of the B family like B834, BL21, TKB1 (Casali 2003).

Up to now *E. coli* kept its importance as host strain due to the ease of transformation and genetically modification, its fast growing, and the fact that the strains are metabolically and genetically excellent characterized. For use in production processes a defined medium with exactly known components shows many advantages over so called complex media. With a defined medium and a fed-batch cultivation strategy it is possible to obtain very notable product concentrations in High Cell Density Cultivation processes. Nevertheless a two- or three component complex medium can help, not only during storage of the strain, cloning process or other preparation steps, but also in a robust, easy to set up batch production of the desired protein. LB or lysogeny broth is one of the most common media for cultivation of bacteria, consisting of just yeast extract, tryptone, and NaCl. The aim of our work was to develop a robust, extremely easy-to-use batch cultivation process as a tool to produce small to medium amounts of recombinant proteins in a small scale bioreactor. GFP (green fluorescent protein) was used as a reporter protein during this study but could be substituted by others proteins.



2. Material and Methods

2.1 Bacterial Strain

For the cultivations *Escherichia coli* BL21 (F^- ompT $hsdS_B(r_B^-m_B^-)$ gal dcm; Novagen, Merck, 69449-4) containing the plasmid pGLO (Bio-Rad, 166-0405EDU) was applied.

The expression of the encoded reporter gene GFP was induced by addition of L-arabinose to the culture.

2.2 Cultivation Media

2.2.1 Composition of Defined Medium

For seed culture preparation a defined mineral salt medium was used (Korz et al. 1995).

2.2.2 Composition of Complex Medium

For the following batch cultivation a complex media was used. It consists of 36 g l^{-1} glucose · 1 H_2O (Carl Roth, Germany) and 60 g l^{-1} yeast extract Flav-R-max (Ohly, Germany). To avoid Maillard reaction the glucose was solved in water (10% of total medium volume) and autoclaved separately. The pH was adjusted to 7.2 with 4 M NaOH.

2.3 Cultivation

2.3.1 Seed Culture Preparation

Before inoculating the bioreactor for batch cultivation, a high cell density cultivation with defined mineral salt medium was carried out until OD600 = 120. The culture was cooled to 20°C and glycerol was added to a final concentration of 15% (w/v) resulting in a cell density of OD600 ≈ 100. The seed culture was aliquoted in 45 ml portions in 50 ml centrifuge tubes and stored at –80°C until usage.

2.3.2 Batch Cultivation

The seed culture was thawed, centrifuged (5,500 × g, 15 min) and the obtained cell pellet resuspended in 50 ml of sterile complex media. This inoculum was transferred into the bioreactor. The batch cultivation was carried out in a 2 l double wall UniVessel® filled with 2 l complex medium (see chapter 2.2.2.) equipped with two 6-blade disks (Rushton turbine, impellers distance 74 mm), located 10 mm above the sparger. Temperature was controlled at

36 °C while no pH control was applied. The dissolved oxygen concentration DO was maintained at 30% saturation by increasing agitation speed (500 – 2,000 rpm) and O_2-enrichment if required. A constant air flow of 2 l min^{-1} was applied through a ring sparger. The cultivation conditions were maintained by the BIOSTAT® B controller.

Production of recombinant GFP was induced at OD600 = 19 ± 2 with 2, 6 or 10 g l^{-1} L-arabinose. Batches were finished when no further growth was detected.

2.4 Analytical Methods

After inoculation samples were taken in an interval of 30 min.

2.4.1 Offline Detection of Biomass Concentration

The optical density was determined at 600 nm (OD600). In addition, the cell dry mass was measured with an infrared moisture analyser MA 100 Q (Sartorius Stedim Biotech) by drying samples at 105°C. The drying process was aborted when mass variation went below 1 mg per 3 min. Wet biomass concentration was calculated from weight difference of empty 1.5 ml micro reaction tubes and filled ones with centrifuged samples (13,000 rpm, 3 min) after withdrawing the supernatant.

2.4.2 Quantitative GFP Determination by SDS-PAGE

Protein analysis was carried out with 18% Criterion™ TGX Stain-Free™ precast gels (Bio-Rad, 567-8074), 10 × TGS (Bio-Rad, 161-0772) and 4 × Laemmli sample buffer (Bio-Rad, 161-0747) in a Criterion™ electrophoresis cell. After electrophoresis the gel was stained with Coomassie (0.1% (w/v) Coomassie Brilliant Blue R250, 2.5% acetic acid, 20% ethanol) for 1 h on a rocking table and de-stained (5% acetic acid, 20% ethanol) until the background was transparent. For quantification of the gene product GFP standards (Vector Laboratories, MB-0752) with different concentrations were applied to the gel as well. The Imager Gel Doc™ EZ and the software Image Lab™ (both from Bio-Rad) were used for gel analysis.

3. Results

The time course of optical density, pH, pO_2%, and agitation speed is shown in Figure 1 for a cultivation with an inducer concentration of 2 g l^{-1} L-arabinose. The DO was controlled at 30% by increasing agitation speed and during a short period while the oxygen demand could not be satisfied by this measure by enrichment with pure oxygen (batch age 3.3 to 3.5 h). The pH decreased from the initial value of 6.9 at inoculation to 5.3 due to production of acetous metabolites and/or consumption of basic components by the cells which is, however, not considered detrimental for this strain.

After inoculation the cells grew exponentially with a specific growth rate of 1.1 h^{-1}.

Within 2 hours the induction cell density of OD600 = 19 ± 2 was reached and expression of the recombinant protein was induced by the addition of L-arabinose. Subsequently the cells grew with a specific growth rate of 0.5 h^{-1} for 1.5 h, then the specific growth rate declined and growth stopped at a final OD600 of approx. 56 after 7 h.

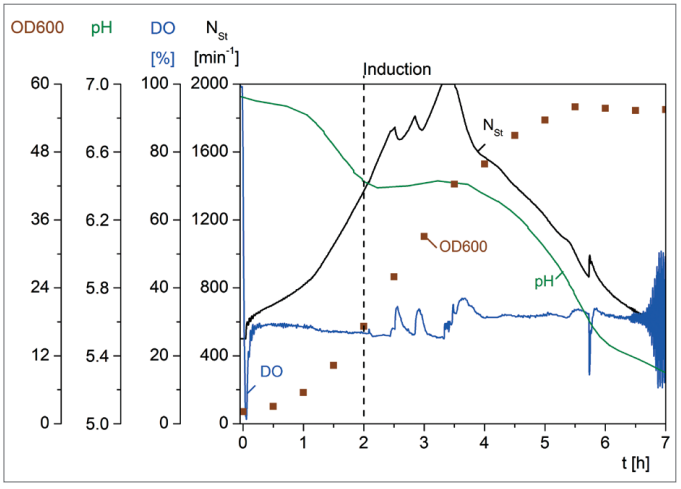


Figure 1: Batch cultivation of *E. coli* BL21 in glucose enriched complex medium. Time course of optical density, pH, DO and agitation speed are shown.

Cell dry mass concentration as well as wet biomass show a linear relationship with the optical density (data not shown).

Directly after induction one can observe the increase of the product concentration. Both the initial slope and the final concentration are dependent on the inductor concentration, Figure 2. The final product concentration rose within 2 to 3 hours past induction for the higher inductor concentrations of 6 and 10 g l^{-1} to a maximum of 0.6 to 0.8 g GFP l^{-1}, while the lowest investigated concentration of 2 g l^{-1} L-arabinose resulted in only 0.5 g GFP l^{-1}.

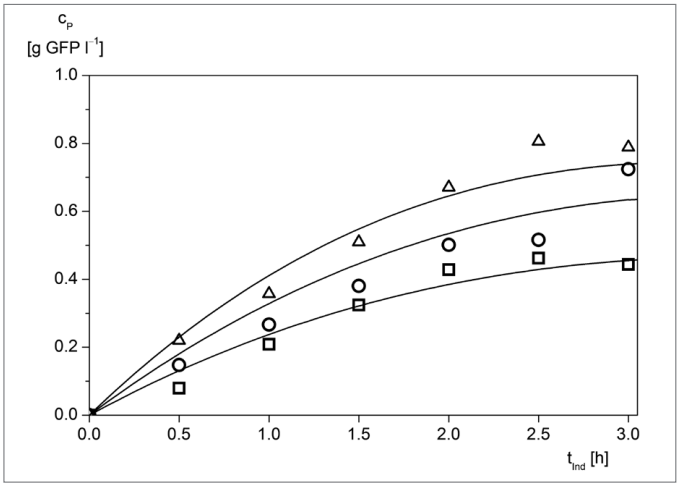


Figure 2: GFP concentration c_P after induction with 2 g l^{-1} □, 6 g l^{-1} ○, 10 g l^{-1} △ L-arabinose.

4. Summary

An extremely easy-to-use process for cultivation of *E. coli* cells and production of recombinant proteins in BIOSTAT® B type bioreactors was demonstrated. With just a two-component-medium and without any effort to control the pH a final cell density of OD600 ≈ 56 (BDM ≈ 20 g l^{-1}) was achieved. Direct inoculation with thawed cryocultures allows abandonment of individual and unequal seed cultures from shaking flasks and a process length of not more than a working day. The production of GFP was induced by addition of various concentrations of L-arabinose at OD600 ≈ 20. Maximum product concentration of 0.5 up to 0.8 g GFP l^{-1} was reached after two to three hours past induction. Our results confirm the strong dependency of the productivity of substantial concentration of the inducer L-arabinose as reported in the literature. It is supposed that further control of pH and feed will increase cell number and product concentration even within a simple media composition as described, which might be a topic of further investigation. As an aid for further downstream processing correlations between cell dry and cell wet mass concentrations and optical density has been verified.

5. Literature

Casali, N (2003). *Escherichia coli* Host Strains, in Casali N and Preston A (Editors) *E. coli* Plasmid Vectors – Methods and Applications, Methods in Molecular Biology, 235: 27-48

Korz DJ, Rinas U, Hellmuth K, Sanders EA, Deckwer W-D (1995). Simple fed-batch technique for high cell density cultivation of *Escherichia coli.* J Biotechnol 39: 59-65

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