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Chemostat studies of bacteriophage M13 infected *Escherichia coli* JM109 for continuous ssDNA production

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ABSTRACT

Steady state studies in a chemostat enable the control of microbial growth rate at defined reaction conditions. The effects of bacteriophage M13 infection on maximum growth rate of *Escherichia coli* JM109 were studied in parallel operated chemostats on a milliliter-scale to analyze the steady state kinetics of phage production. The bacteriophage infection led to a decrease in maximum specific growth rate of 15% from 0.74 h^{-1} to 0.63 h^{-1}. Under steady state conditions, a constant cell specific ssDNA formation rate of 0.15 ± 0.004 mg_{ssDNA} g_{CDW}^{-1} h^{-1} was observed, which was independent of the growth rate. Using the estimated kinetic parameters for *E. coli* infected with bacteriophage M13, the ssDNA concentration in the steady state could be predicted as function of the dilution rate and the glucose concentration in the substrate. Scalability of milliliter-scale data was approved by steady state studies on a liter-scale at a selected dilution rate. An ssDNA space-time yield of 5.7 mg L^{-1} h^{-1} was achieved with increased glucose concentration in the feed at a dilution rate of 0.3 h^{-1}, which is comparable to established fed-batch fermentation with bacteriophage M13 for ssDNA production.

1. Introduction

Continuous fermentation in an ideal stirred-tank bioreactor – simultaneously developed by Monod (1950) and Novick and Szilard (1950) – enables the study of microbial growth under defined and controlled reaction conditions at steady state (chemostat). The growth kinetics of various species have been investigated under comprehensive environmental conditions using chemostat studies (Bull, 2010; Hoskisson and Hobbs, 2005). The application of the chemostat facilitates the control of the cell specific growth rate by the dilution rate of the medium at a constant reaction volume. Therefore, chemostat studies are the ideal experimental setup to identify reproducible and convincing information of the cell population under defined, controlled, and constant physio-chemical conditions (Hoskisson and Hobbs, 2005).

The bacteriophage M13 is an F-specific, filamentous phage (Ff-phage), which selectively infects *Escherichia coli* (*E. coli*) carrying an F-pilus (Calendar, 2006; Marvin et al., 2014). The bacteriophage M13, with its filamentous structure (~900 nm length, 6.5 nm diameter) is composed of a circular ssDNA genome enveloped by five coat proteins. The other six encoded phage proteins are responsible for intracellular ssDNA amplification, regulation of M13 life cycle, assembly and extrusion of the phage particles into the extracellular space (Rakonjac et al., 2011). In opposite to lytic or temperent life cycle of viruses and phages, the filamentous bacteriophage M13 does not lyse the host cells. Furthermore, the host cells are able to divide after infection and to secrete phage progeny continuously. Beneath the application of bacteriophage M13 in phage display technology, it became a promising vehicle in nanobiotechnology (Sagona et al., 2016). Furthermore, the long and circular ssDNA genome is commonly used as scaffold in DNA origami technology, a bottom-up method to create DNA nanostructures in a self-assembling reaction (Linko and Dietz, 2013; Rothemund, 2006).

The infection of *E. coli* with bacteriophage M13 led to a decrease in maximum specific growth rate (Calendar, 2006; Salivar et al., 1964), which was solely investigated in batch fermentations. Due to the dynamic physico-chemical conditions in simple batch experiments (Hoskisson and Hobbs, 2005), the influence of phage infection on maximum specific growth rate is difficult or even impossible to interpret. Therefore, the continuous fermentation of *E. coli* JM109 infected with bacteriophage M13 will be investigated until steady state conditions are reached. Using the steady state data of biomass (dry cell mass), substrate (glucose) and product (ssDNA) concentration, the kinetic parameters can be estimated for the infected and uninfected growth of *E. coli* JM109. This enables the determination of the influence of phage infection on maximum growth rate under controlled and defined reaction conditions. Furthermore, the kinetics of phage production, whether it is growth rate associated or not, will be characterized in the

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Nomenclature

CDW	Cell dry weight, g L^{-1}
c_P	ssDNA concentration, mg L^{-1}
c_S	Substrate concentration, g L^{-1}
$c_{S,in}$	Substrate concentration in feed, g L^{-1}
c_x	Biomass concentration, g L^{-1}
D	Dilution rate, h^{-1}
DO	Dissolved oxygen, %
E. coli	*Escherichia coli*
K_S	Substrate affinity constant, g L^{-1}
k_P	Cell specific ssDNA formation rate, mg g^{-1} h^{-1}
μ	Growth rate, h^{-1}
μ_{max}	Maximum growth rate, h^{-1}
m_S	Maintenance coefficient, g g^{-1} h^{-1}
q_S	Substrate uptake rate, h^{-1}
ssDNA	Single stranded DNA
t	Time, h
τ	Residence time, h
$Y_{XS,\mu}$	Biomass yield coefficient, g g^{-1}

chemostat. Finally the question will be answered, whether a continuous fermentation of infected *E. coli* JM109 is an alternative to the established fed-batch processes for ssDNA production (Kick et al., 2015, 2017).

2. Materials and methods

2.1. Bacterial strain and bacteriophage M13

For the continuous fermentation *Escherichia coli* JM109 was used as host organism (Yanisch-Perron et al., 1985) and the bacteriophage M13 with a genome length of 8064 derived from M13mp18 (Douglas et al., 2009). To avoid the loss of the F-plasmid in *E. coli* JM109, the strain maintenance was carried out in defined medium without complex ingredients or prolin.

2.2. Media, preculture and inoculation

All continuous fermentations with *E. coli* on a milliliter- or liter-scale were performed with defined medium based on Riesenberg et al. (1991), described in detail recently (Kick et al., 2015). The preculture preparation for continuous fermentation on a milliliter- and liter- scale with 20 g L^{-1} glucose in the feed was divided into two steps. At first, 0.5 mL cryo culture was cultivated in 20 mL M9 minimal medium in a 250 mL shaking flasks without baffles on a rotary shaker (37 °C, 250 rpm), until stationary phase was reached. In the second step, 100 mL Riesenberg medium with 5 g L^{-1} glucose were inoculated with 1% of the stationary cells and cultivated for 14 h at 37 °C and 250 rpm in 1 L shaking flaks without baffles. The cells were used to inoculate the sterile medium in the reactors at an optical density (600 nm) of 1.0. For the continuous fermentation with 70 g L^{-1} glucose in the feed, a third inoculation step was introduced to achieve exponential growing cells for inoculation. Therefore, another 1 L shaking flask containing 100 mL Riesenberg medium with 5 g L^{-1} glucose was inoculated (5%) and incubated for 6–8 h (37 °C, 250 rpm) until an optical density of 2.0 was reached. The preparation of phage stock solution was performed as described in Sambrook et al. (2001).

2.3. Stirred-tank reactors, process monitoring and control

The continuous fermentations on a milliliter scale were performed in sterile single-use stirred-tank bioreactors with a working volume of 10 mL (bioREACTOR, 2mag AG, Munich, Germany). The magnetic inductive drive (bioREACTOR48, 2mag AG, Munich, Germany) was equipped with gas-inducing stirrers (Puskeiler et al., 2005; Weuster-Botz et al., 2005). 8 of the 48 parallel milliliter scale stirred-tank bioreactors were expanded for continuous operation as described in detail recently (Schmideder et al., 2015). The pH and DO concentration can be monitored by immobilized fluorimetric sensors (Kusterer et al., 2008; Janzen et al., 2015) at the bottom of the single-use bioreactor (MCR 8*2 v5, PreSens GmbH, Regensburg, Germany). In the milliliter scale stirred-tank reactors the pH was controlled with 12.5% (v/v) NH_4OH at pH 6.7 (set-point) either by a liquid handling systems in case of fermentations with uninfected *E. coli*, or by an additional peristaltic pump with 8 channels (MP8, DASGIP, Jülich, Germany) for the fermentations with infected *E. coli*. For sufficient oxygen supply, the speed of the gas-inducing impellers was set to 3000 rpm and the headspace of each milliliter reactor was purged with 0.1 L min^{-1} sterile air.

For the continuous fermentation on a liter scale an in-situ sterilizable stirred-tank bioreactor (KLF Advanced System 3.6 L, Bioengineering AG, Wald, Switzerland) with a working volume of 1.49 L was used. The pH was controlled at pH 6.7 with 12.5% NH_4OH at a temperature of 37 °C. Using two 6 plate Rushton turbines, a stirrer speed of 1400 rpm and a gas flow rate of 2 vvm, sufficient oxygen supply was achieved in the continuous fermentation with 20 g L^{-1} glucose. For the experiments with higher glucose concentration in the feed, the system pressure was increased up to 1.5 bar, additionally.

The protocols for the continuous fermentation on a milliliter- or liter-scale were the same at a feed glucose concentration of 20 g L^{-1}. The batch phase with 20 g L^{-1} glucose was initiated with an optical density of 1.0 and the glucose was consumed after 4–5 h. Glucose depletion was followed by the continuous fermentations at different dilution rates. The infection with bacteriophage M13 was already performed during exponential growth in the batch phase at a cell dry weight concentration of 5 g L^{-1} with a multiplicity of infection of 0.1 pfu cfu^{-1} (plaque forming unit, colony forming unit). For the continuous fermentation with a glucose concentration of 70 g L^{-1} in the feed, the fermentation was divided into three parts. After a batch phase with 25 g L^{-1} glucose, an exponential feeding phase was started with a preset growth rate of 0.3 h^{-1}. At a glucose mass flow of 21 g L^{-1} h^{-1}, the process was switched from fed-batch to continuous operation. The infection with bacteriophage M13 was initiated during fed-batch operation at a cell dry weight concentration of 25.2 ± 0.3 g L^{-1} with a multiplicity of infection of 0.003 pfu cfu^{-1}.

For the determination of the cell dry weight, the glucose and ssDNA concentration, 1.5 mL were collected at the outlet of the continuous operated milliliter scale stirred-tank reactors. The monitoring of the concentrations of dry cell mass and glucose was performed as described recently (Kick et al., 2017). For the ssDNA determination 1 mL cell broth was centrifuged for 10 min at 13,000 rcf (20 °C). The phages in the supernatant were precipitated by addition of 48 mg polyethylene glycol (PEG 8000) and 36 mg sodium chloride and incubated for 30 min (stock solution containing 240 g L^{-1} PEG 8000 and 180 g L^{-1} sodium chloride). After an additional centrifugation step (13.000 rcf, 10 min, 20 °C) the precipitated phages were resuspended in 100 µL TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.5) and were lysed using 200 µL lysis buffer (200 mM sodium hydroxide, 1% sodium dodecyl sulfate, 1 min) and 150 µL neutralization buffer (3 M potassium acetate, pH 5.5) on ice. After 15 min incubation the supernatant (10 min, 13,000 rcf, 20 °C) containing the genomic phage ssDNA was mixed with 450 µL chilled ethanol (96%, −20 °C). For DNA precipitation the samples were incubated on ice for further 30 min. The precipitated ssDNA (10 min, 13.000 rcf, 4 °C) was washed with ethanol (75%, −20 °C) for 10 min and centrifuged again. The supernatant was carefully discarded and the precipitated ssDNA was solved in 50–100 µL TE buffer. The determination of ssDNA concentration was performed using SYBR® Gold