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Expression Tuning in *E. coli*: Discussion of state-of-the art technologies and multivariate investigation of the pBAD mixed feed system

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MASTER THESIS

Expression Tuning in *E. coli*: Discussion of state-of-the art technologies and multivariate investigation of the pBAD mixed feed system

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Abstract

Background: Tuning of transcription is a powerful process technological tool for efficient recombinant protein production in *E. coli*. Many challenges such as product toxicity, formation of inclusion bodies, cell death and metabolic burden are associated with non-suitable (too high or too low) levels of recombinant protein expression. Tunable expression systems allow adjusting the recombinant protein expression using process technological means. This enables to exploit the cell's metabolic capacities to a maximum.

Aims: In this thesis, tunable recombinant protein expression in *E. coli* is reviewed thoroughly from a biological and process technological point of view. In a consequent step expression tuning is applied to produce vascular endothelial growth factor-A_{165} (VEGF-A_{165}), a pharmacologically relevant key player in angiogenesis, in correctly folded and active form in *E. coli* periplasm. Therefore the well-established system for expression tuning, the *E. coli* pBAD mixed feed platform, is investigated for the development of an upstream production process.

Material and Methods: An *E. coli* C41 strain with intact L-arabinose metabolism was used in a mixed feed environment with D-glucose as main substrate and L-arabinose as inducing substrate. Following Quality by Design (QbD) principles, the three most promising critical process parameters (CPPs) namely the specific growth rate, specific inducer uptake rate and temperature were investigated in a design of experiments. A 23 factorial design (3 factors at 2 levels) for GIII and 22 factorial design in the case of DsbA signal sequence were conducted, each with a set of 3 center points.

Results: So far expression tuning was only addressed in a few studies. For the first time these studies were reviewed with respect to latest findings on induction kinetics and mechanistics. According to the current level of knowledge some promoter system were successfully for expression tuning, in some cases analytical evidence on single cell level is still pending and some attempts did only influence protein expression on population level.

For the first time, a promising mixed feed system was applied for tunable protein expression in the periplasm of E. coli. Beside of the observation of quality and quantity dependencies on the investigated CPPs it was demonstrated that the product transcription rate could indirectly be included in an experimental design by the successful use of a tunable promoter system in E. coli

Conclusion: In summary, the use of a tunable expression system was successfully applied in the development of an upstream process for the production of VEGF-A_{165} in the periplasm of *E. coli.* We anticipate that expression tuning is able to tackle further issues caused by inappropriate transcription levels and therefore is not only a major benefit for process development, but can pave the way for continuous production of biopharmaceuticals by the issues of constant product quality and culture long term stability

CONTENT

1 Background and Motivation

With a market value of $140 billion for 2013 the biopharmaceuticals market share of the overall pharmaceutical market is expected to rise to 20% in 2017 (from 18% in 2012). 54 biopharmaceutical products were approved between 2010 and 2014 in the United States and in European Union, adding up to a total of 212 approved biopharmaceuticals. With the increasing demand of more complex proteins the proportion of mammalian-based expression systems is increasing. However microbial production hosts still prevail. With 29 % percent of the 54 approved products being produced in *E. coli*, this microbial organism is still the single most common non-mammalian production host (Walsh 2014). With its fast growth kinetics, well-established genetics and fermentation technology this gram-negative bacterium is a cheap and reliable host for recombinant protein production. However more complex products push *E. coli* to its limits. Problems caused by incorrect folding or the lack of post translational modifications result in a shift towards alternative production hosts (Berlec and Strukelj 2013). Several drawbacks like incorrect folding are caused by to high specific protein production rates and can be tackled by lowering the expression rate to acceptable levels. Hence efforts are made to overcome these drawbacks by adjusting the recombinant product formation rate by genetic and process technological means. Tunable recombinant protein expression realized by adjusting the recombinant gene transcription rate on cellular level is a promising strategy in order to adjust protein expression to levels where the recombinant product is produced in soluble and active form.

2 Roadmap and structure of this thesis

This thesis deals with expression tuning in a theoretical and experimental way. It consists of two parts.

The first part of this thesis, a manuscript ready for submission, provides an extensive review of tunable recombinant protein expression in *E. coli*. In a first step a concise and comprehensive definition of expression tuning is proposed. Various *E. coli* promoter systems are thoroughly reviewed regarding induction mechanisms and kinetics with respect to expression tuning. Furthermore process technological methods to achieve expression tuning are discussed. Finally a roadmap to achieve expression tuning is proposed and its applicability and benefits for more efficient bioprocessing are discussed.

The second part of the thesis was conducted in the course of an industrial project and deals with the development of an upstream production process of VEGF-A_{165} in the periplasm of *E.coli.* Within this framework expression tuning was applied and used as a novel degree of freedom in process development. Therefore the thoroughly investigated *E. coli* pBAD mixed feed expression platform was for the first time used for recombinant protein production in the periplasm of *E. coli (Sagmeister et al. 2013a; Sagmeister et al. 2013b).*

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Sagmeister P, Schimek C, Meitz A, Herwig C, Spadiut O. 2013b. Tunable recombinant protein expression with E. coli in a mixed-feed environment. Applied Microbiology and Biotechnology.

Walsh G. 2014. Biopharmaceutical benchmarks 2014. Nature Biotechnology 32(10):992–1000.

3 Manuscript considered for peer-reviewed publication enclosed in this thesis

3.1 Master thesis: Part I – review

Tunable recombinant protein expression in *E. coli*

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Manuscript ready for submission.

Individual Authorship contributions: Lukas Marschall performed the literature research and drafted the manuscript. Patrick Sagmeister structured and drafted the manuscript. Christoph Herwig conceived the study. All authors read and approved the final manuscript.

Tuning of transcription is a powerful tool for process development and control. In combination with a suitable control strategy it is possible to separately control the specific growth rate and the transcription rate on cellular level, gaining

Berlec A, Strukelj B. 2013. Current state and recent advances in biopharmaceutical production in Escherichia coli, yeasts and mammalian cells. Journal of industrial microbiology & biotechnology 40(3-4):257–274.

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4 PART I

Tunable recombinant protein expression in *E. coli*

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5 Abstract

Tuning of transcription is a powerful process technological tool for efficient recombinant protein production in *E. coli*. Many challenges such as product toxicity, formation of inclusion bodies, cell death and metabolic burden are associated with non-suitable (too high or too low) levels of recombinant protein expression. Tunable expression systems allow adjusting the recombinant protein expression using process technological means. This enables to exploit the cell's metabolic capacities to a maximum. Introducing a novel degree of freedom in process control tuning of transcription might act as an enabling tool for continuous processing by providing culture stability and long-term production periods.

Within this article, we review genetic- and process technological aspects of tunable expression systems in *E. coli*, providing a roadmap for the industrial exploitation of the reviewed technologies. Furthermore, we thoroughly review reported tunable promoter systems and discuss methodical approaches with respect to the newest findings regarding induction mechanisms and kinetics. We attempt to differentiate the term "expression tuning" from its inflationary use by providing a concise definition and highlight interesting fields of application for this versatile new technology.

Dependent on the type of inducer (metabolisable or non-metabolisable) different process strategies are required in order to achieve tuning. In order to acquire tuning of protein production as a new degree of freedom for process control two main strategies have been identified: continuous supply of non-metabolisable inducer or mixed-feed system with a metabolisable inducer as second carbon source.

The technological maturity and clear process-technological benefits of the reviewed technologies anticipate a fast industrial implementation and will further advance the applicability of *E. coli* as principal microbial production platform for the production of recombinant products.

Keywords: all-or-none-induction, *E.coli*, promoter, transcription, tunable

6 Introduction

The relevance of the gram negative bacterium *Escherichia coli* (*E. coli*) for the basic biotechnological research as well as for industrial exploitation is outstanding. *E. coli* served as the primary model organism within the development of modern biotechnology. As a consequence, researchers today have access to a broad spectrum of genetic tools and cultivation techniques, enabling simple and predictable genetic manipulation and cultivation on inexpensive media to high cell densities. As regards industrial exploitation, *E. coli* emerged as the primary production workhorse for the biotechnological production of primary and secondary metabolites as well as recombinant proteins. This is reflected by the fact that 29% of all biopharmaceutical products approved as biopharmaceuticals between 2010 and July 2014 are produced in *E. coli* (Walsh 2014).

Overall productivity and product quality obtained from *E. coli* processes is determined by the complex interplay of processing mode, product to be produced as well as the expression system applied.

Main challenges of recombinant protein production in *E. coli* are associated with non-suitable (too high or too low) level of recombinant expression:

1) First, high-level expression of recombinant products can lead to the formation of unfolded or partially folded insoluble protein aggregates known as inclusion bodies which show no catalytic function or activity (Baig et al. 2014; Hartley and Kane 1991).
2) Second, high level expression and the presence of foreign plasmids drain the hosts' metabolic resources (Bentley et al. 2009; Bienick et al. 2014; Glick 1995; Mairhofer et al. 2013), which results in reduced productivity. Here it is frequently observed that a reduction of the protein expression level leads to increased end product titers, since the cells can be maintained in a productive state for a longer time (Sagmeister et al. 2014; Sagmeister et al. 2013c) (Fig. 1).

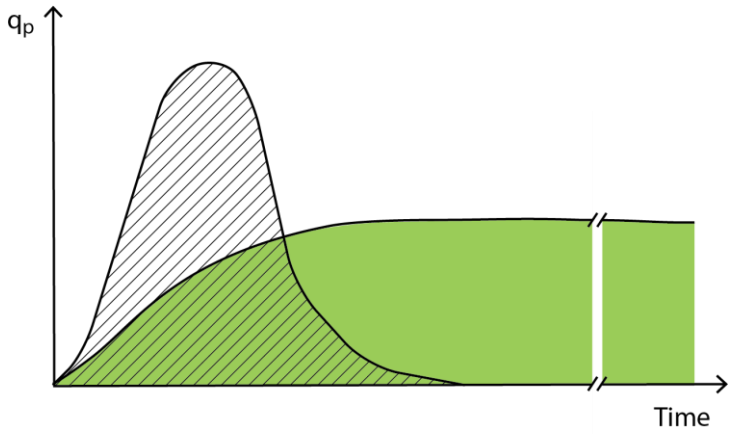

Fig. 1: By reducing the protein expression level the cells can be maintained in a productive state for a longer time, which results in higher end product titers (q_p – specific cellular productivity).

3) Third, the production of many recombinant products, especially proteins containing disulfide bridges, demands translocation between compartments of the *E. coli* cell factory (Baneyx and Mujacic 2004). Here, too high levels of recombinant protein expression can lead to the blocking of translocation pathways.

The level of recombinant protein expression is affected by the strength of the expression system which involves the strength of the promoter used, the plasmid copy number (Bienick et al. 2014) as well as process technological parameters such as temperature and the specific growth rate (Rodríguez-Carmona et al. 2012; Singh et al. 2012). “Expression tuning”, also referred to as “fine-tuning of protein production” and “modulation of expression”, intends to solve the aforementioned challenges by providing a technological framework to adjust recombinant protein expression to a level which is optimal for protein folding, protein translocation and long-term productivity. Hence, tuning allows to exploit the cell factory to a maximum. Furthermore, expression tuning could find applications in fundamental research such as enzyme control analysis (Jensen, 1993). In this review article we aim at giving a concise and comprehensive overview of current state of the art methods and technologies for expression tuning. First, we discuss the contemporary

scientific conception of “expression tuning” and aim at the proposition of a sound and comprehensive definition (Section 7). Secondly, we review and discuss methods for expression tuning. *E. coli* promoter systems which enable expression tuning are reviewed in section 10. Special attention is drawn on features relevant to enable expression tuning on cellular level. Furthermore, their integration to process technological methods to achieve expression tuning is discussed (Section 11). Thirdly, we propose a roadmap for the development of industrial tunable expression systems (Section 12). Finally, in an outlook chapter, we discuss the applicability and benefits of tunable expression systems for more efficient bioprocessing and the acceleration of bioprocess development (Section 14).

7 Defining tunable recombinant protein expression

Although many authors refer to “tunable recombinant protein expression” for various purposes, to our knowledge a clear and uniform definition is still missing in literature. Some authors refer to “tuning” of expression or gene dosage as adjusting the plasmid copy number (Camps 2010; Xu et al. 2013), which can also unintentionally be submitted to change in course of the bioprocess (Teich et al. 1998). Another definition of tuning of expression refers to the modulation of promoter strength by construction of a set of promoters of different strengths through promoter engineering (Alper et al. 2005; Brewster et al. 2012; Dehli et al. 2012; Mey et al. 2007). In these cases the actual tuning is achieved through genetic engineering. Furthermore, the adjustment of the expression level via simple process parameters such as temperature, medium composition is sometimes referred to as expression tuning as well (Correa and Oppezzo 2011). “Tuning” is often referred to as adjusting the production of recombinant proteins on cellular level, whereby tuning solely on population level (e.g. the formation of subpopulations) is clearly excluded from the definition (Khlebnikov et al. 2002; Lee and Keasling 2005; Striedner et al. 2003). However, this definition only considers the tuning with respect to specific titer and not the specific cellular productivity (q_p), which is

varying along the production period (Sagmeister et al. 2014). When speaking of tuning of q_p one has to bear in mind that this definition does not consider the location of the bottlenecks for recombinant protein production, whereby either transcription, translation, translocation or folding of recombinant proteins can be bottlenecking (Baneyx and Mujacic 2004; Brinkmann et al. 1989; Harris and Kilby 2014; Tegel et al. 2011; Wagner et al. 2007). Bearing in mind that the effective specific cellular productivity (q_{p_eff}) is actually composed of production of recombinant product (q_p) and the degradation of product ($q_{degregation}$), we propose following definition for expression tuning:

Expression tuning is referred to as the purposeful adjustment of the recombinant gene transcription rate on cellular level.

$$q_p = \min\left(q_{transcription}, q_{translation}, (q_{translocation},) q_{folding}\right) \quad \textbf{Eq. 1}$$

$$q_{p\_eff} = q_p - q_{degegation} \quad \textbf{Eq. 2}$$

8 Formation of subpopulations of producing and non-producing cells (bistable behavior) – an impediment to expression tuning

Following the presented definition of expression tuning, it is of utmost importance to understand, consider and make it impossible that observed tuning on population level might be attributed to the formation of subpopulations of producing and non-producing cells. If this is the case, tuning of the expression levels is not achieved, which is also called “bistable behaviour”, refering to the formation of two subpopulations. Homogeneous expression on cellular level is also often referred to as “graded response”. Here, knowledge on the mechanisms that lead to these “all or none induction phenomena” are reviewed.

Novick and Weiner (Novick and Weiner 1957) identified for the lac operon that apparent tuning on population level may be the result of the formation of subpopulations of fully induced and non-induced cells (all-or-none induction, bistability). This was confirmed by Maloney and Rotman (Maloney and Rotman 1973). They observed that the inducer amount only influenced the number of induced cell rather than the transcription rate on cellular level and proposed a model describing this phenomenon (Novick and Weiner 1957). 40 years later Siegele and Hu reported this behavior for the arabinose utilization pathway (Siegele and Hu 1997). Further studies using mechanistic modeling and single cell analytics investigated autocatalytic systems and the switching kinetics of inducible systems (Carrier and Keasling 1999; Fritz et al. 2014; Megerle et al. 2008; Ozbudak et al. 2004). Afroz et al. investigated and compared eight metabolic pathways of *E. coli* with respect to all-or-none behavior and created a deterministic model (Afroz et al. 2014b). Their model related the type of response (graded or bistable) to the strength of positive feedback (inducible inducer transport) and negative feedback (inducible inducer catabolism). Bistable responses were expected for low negative feedback and high positive feedback and graded responses for high negative feedback and low positive feedback. The extent of bistability of different pathways is believed to be influenced by cooperativity in the expression of pathway transporters (Afroz et al. 2014b).

9 General strategies to achieve tuning on cellular level

Based on the insights in the mechanistic that lead to unwanted bistable behaviour (formation of subpopulations of producing and non-producing cells), several strategies based on targeted engineering of metabolic pathways have been reported.

Several authors perform knockout of genes for inducer transport to omit bistable responses (Afroz et al. 2014b; Khlebnikov et al. 2000; Marbach and Bettenbrock 2012). The knockout of transport genes breaks the positive feedback of the inducer on the production of its own transport proteins and eliminates the bistable behaviour (Afroz et al. 2014a; Fritz et al. 2014;

Khlebnikov et al. 2000). Using inducer transport knock-out strains, induction can be achieved by induction with gratuitous inducers (Marbach and Bettenbrock 2012) or by induction of the natural inducer. The natural inducer is transported into the cell by a separate copy of the transporter gene under control of a different promoter (Afroz et al. 2014a; Khlebnikov et al. 2000).

Another reported general strategy is the transcription of an additional gene (Wagner et al. 2008). The widely-used T7 expression system (Studier and Moffatt 1986) was modified by addition of a vector harbouring T7 lysozyme under control of the *rhaBAD* promoter. Modulation of T7 lysozyme expression, a natural inhibitor of T7 polymerase, by varying the rhamnose concentration converted the all-or-none response to a uniform response (Schlegel et al. 2012; Wagner et al. 2008).

Furthermore, we recently showed for the araBAD operon that the use of metabolisable inducer in a mixed-feed environment results in a graded response (Sagmeister et al. 2013c). Using this strategy a recombinant protein expression can be tuned by simply adjusting the uptake rate of the inducing substrate.

10 Promoter Systems for tunable recombinant protein expression

In the following section, promoter systems that are reported to feature tunability are reviewed. For each system, a short overview of general characteristics is given. Subsequently, its functionality is reviewed and discussed with respect to its tunabilty. The systems discussed in this section are outlined in Table 1.

10.1 plac system

10.1.1 General characteristics of the plac system

The p_{lac} promoter can either be induced by its natural inducer lactose (allolactose) or by gratuitous inducers like isopropyl β-D-1-thiogalactopyranoside (IPTG) and thiomethyl-β-D-

galactoside (TMG) (Herzenberg 1959). Like lactose TMG and IPTG are both recognised by lactose permease (LacY) and can therefore enter the cell membrane either by active or diffusive transport or a combination of both (Fernández-Castané et al. 2012; Hansen et al. 1998; Maloney and Rotman 1973; Marbach and Bettenbrock 2012). The intracellular inducer concentration can also decrease in course of the process: Lactose can be metabolized by *E. coli* using its inherent sugar uptake pathways. TMG and IPTG cannot be metabolized, but they can be acetylated by the *lac* operon gene *lacA* (thiogalactoside transacetylase) (Marbach and Bettenbrock 2012). The acetylated derivatives cannot interact with the *lac* repressor anymore and are consequently transported out of the cell. The route of exit is believed to be a concentration gradient (diffusive transport) (Wilson and Kashket 1969). It is believed that catabolite activator protein is essential for expression of the *lac* operon, but not involved in catabolite repression. The main driving force of catabolite repression in the *lac* operon seems to be inducer exclusion, but is still controversial in literature (Crasnier-Mednansky 2008; Görke and Stülke 2008a; Görke and Stülke 2008b)

10.1.2 Plac system tuning on cellular level is possible using metabolisable inducers

Several authors reported that the p_{lac} system using a non-metabolisable inducer is submitted to all-or-none induction, which impedes tuning on cellular level (Afroz et al. 2014b; Narang and Pilyugin 2008; Novick and Weiner 1957; Ozbudak et al. 2004; Rao and Koirala 2014; Savageau 2011; Siegele and Hu 1997). However, recently Afroz et al. outlined that the induction characteristics of this system are more complex: Tuning on cellular level is possible using lactose as metabolisable inducer (Afroz et al. 2014b), which stands in contrast to the all-or-none response found through TMG and IPTG induction (Marbach and Bettenbrock 2012). The authors concluded that the all-or-none response is due to active transport of IPTG or TMG (Afroz et al. 2014b).

This has the practical implication that high catabolic activity paired with a low transport activity results in a graded response (lactose case), whereas a low catabolic activity paired with

a high transport activity yields an all-or-none response (IPTG and TMG case) for the p_{lac} system (Afroz et al. 2014b).

As regards the use of lactose as metabolisable inducer, *lacZ*$^-$ mutant strain were reported to show all-or-none behaviour when induced by lactose (Afroz et al. 2014b). Hence, for tuning on cellular level in the respective system via lactose as inducer, *lacY*$^+$ *lacZ*$^+$ strains are obligatory.

10.1.3 Plac system tuning on cellular level is possible using lacY mutants

Next to using metabolisable inducers (lactose), tuning on cellular level using the p_{lac} system can be achieved using *lacY*$^-$ mutant strains (lactose transport deficient strains) with gratuitous inducers (Jensen et al. 1993; Marbach and Bettenbrock 2012). Furthermore, Marbach et al. reported that LacA (thiogalactoside transacetylase) activity leads to a decrease in expression levels as the inducer concentration at single cell level is decreasing (Marbach and Bettenbrock 2012). Therefore, while knock-out of *lacY* is mandatory, also *lacA* knock-out is recommended when using IPTG as non metabolisable inducer. A similar behaviour is expected for TMG, however to our knowledge not proven yet.

10.1.4 Tuning of the lac operon – application in bioprocesses

Striedner et al. reported transcription tuning of human superoxide dismutase using T7 polymerase under control of lacUV5 in *E. coli* K12 strain HMS174(DE3) (intact lac operon) with IPTG (Striedner et al. 2003). In fed-batch experiments with an exponential feed of substrate IPTG was fed in accordance to the expected biomass at a constant ratio of 0.9 µmol IPTG per g biomass. They were able to maintain the productivity of the culture for a longer period compared to a process with conventional one-point addition of inducer and observed a 3.5-fold increase of overall product titer. However, a proof of tuning on cellular level was not provided. Afroz et al. reported that *E. coli* strains with an intact *lac* operon cannot be tuned with IPTG as inducer due to the all-or-none induction phenomenon (Afroz et al. 2014b). Hence, it is to assume that inducer titration using IPTG with intact *lac* operon (as applied in this case) does not result in tuning on cellular level.

In the same study Striedner et al. used the same host, vector and product, but applied lactose as inducer instead of IPTG (Striedner et al. 2003). In fed-batch experiments with an exponential feed of substrate, lactose was fed as inducer in accordance to a constant specific lactose uptake rate. They were able to maintain the productivity of the culture for a longer period compared to a process with conventional one-point addition of inducer and the process with IPTG inducer titration and observed 2-fold increase of overall product titer. In this study, an analytical proof on single-cell level was not provided, but considering the previous discussed findings of Afroz et al. (Afroz et al. 2014b) it is plausible that the increased productivities can be attributed to transcription tuning on single-cell level in this case. In contrast to the process with IPTG, the biomass yield coefficient was constant during the whole process. This might indicate that the cells were not overburdened, since a drop in the biomass yield coefficient is reported to stand in correlation with the metabolic load imposed on the cells (Sagmeister et al. 2012).

Several other studies used *lacY* deficient strains with gratuitous inducer to achieve expression tuning (Hartinger et al. 2010; Hillier et al. 2005; Turner et al. 2005). In order to increase the amount of soluble target protein, Hartinger et al. compared a variety of process conditions and host strains including BL21(DE3) Tuner™. Tuner™ is a *lacZY* derivative of *E. coli* BL21, which is available in various types and allows tunable transcription with induction with IPTG. The inducer was added as one-point addition at an OD600 of 1, LB-medium was used and induction phase was maintained for 3 hours. With varying inducer concentration from 10 to 1000 µM they observed a correlation between IPTG concentration and titer, but no influence on solubility of the product (Hartinger et al. 2010).

Hillier et al. developed a 300-L GMP fermentation process for liver-stage antigen 1 (LSA-1) in BL21(DE3) Tuner™ (Hillier et al. 2005). Inducer was added as one-point addition at an OD600 of 8 to 10 and induction phase was maintained for 2 hours. The tunable ability of the strain

was however not exploited as the culture was induced with 1 mM IPTG, which is expected to fully induce the promoter according to other studies (Hartinger et al. 2010; Turner et al. 2005).

Turner et al. used BL21(DE3) Tuner™ to tune the expression of a target protein (cyclomaltodextrinase of thermophilic origin) with respect to solubility (Turner et al. 2005). Cultivations were performed in 100 ml shake flasks with 30 ml working volume. Induction with IPTG was performed as one-point addition at an OD620 of 0.6-0.8 in LB medium. Induction phases were maintained for 4 hours. Inducer concentrations from 5 µM to 1mM were examined. They observed an influence of transcription rate on the ratio of soluble protein and inclusion bodies. At subsaturating inducer concentrations of 50 µM higher amounts of soluble protein and activity were detected. At 100 µM ITPG the activity and the amount of soluble protein decreased, whereas the inclusion body concentration increased. These results suggest that tuning of transcription is a useful tool to suppress inclusion body formation.

10.2 araBAD system

10.2.1 General characteristics of the araBAD system

Another commonly used promoter is the pBAD promoter of the *araBAD* operon. The *araBAD* operon enables *E. coli* to take up and metabolize L-arabinose (Schleif 2000). It is induced by L-arabinose. Until now, no gratuitous inducer is reported in literature to induce the wild type promoter. The non-metabolisable L-arabinose analogue D-fucose acts as inhibitor as it binds to AraC but does not induce transcription (Greenblatt and Schleif 1971; Wilcox 1974). By mutation of *araC* it is possible to render the system inducable by D-fucose (Beverin et al. 1971). L-arabinose and D-fucose are both transported into the cell by AraE and AraFGH (Daruwalla et al. 1981). The regulation of the araBAD operon is excellently described by Schleif (Schleif 2000). It consists of two transporter genes *araE* and *araFGH* (Daruwalla et al. 1981), the genes for arabinose catabolism *araBAD* (Englesberg 1961), a gene of yet unknown function *araJ* (Fritz et al. 2014; Reeder and Schleif 1991) and the regulation gene *araC* (Saviola et al. 1998; Schleif 2010). All genes are under arabinose inducible control of AraC, which regulates

its own expression as well. Beside the positive regulation of the genes it acts negatively on *araBAD* and *araC*. *AraC* acts on *araBAD* as repressor during absence of arabinose and as activator in presence of arabinose (Schleif 2010). *It* is also dependent on the activation by cAMP receptor protein, which renders the system prone to catabolite repression. In presence of glucose or glucose-6-phosphate, the basal expression level can therefore be held at lower levels (Miyada et al. 1984). AraE and AraFGH do not act independently and AraE is the more prevailing tansporter of these two (Daruwalla et al. 1981).

Transcription kinetics for the system are reported as follows: Transcription from the p_{araBAD} promoter is turned on 1 minute after induction with arabinose (Guzman et al. 1995) and turned off in about 3 minutes after arabinose removal (Fritz et al. 2014). The fast and homogenous shut off of p_{araBAD} transcription is not due to catabolism, but is believed to stand in connection with the arabinose efflux (Fritz et al. 2014).

10.2.2 araBAD system tuning on cellular level cab be achieved by either genetic engineering or process technology

Regarding the all-or-none phenomenon, the system shows formation of subpopulations of producing and non-producing cells at low arabinose concentrations. A graded response is observed when all cells are induced at higher arabinose concentrations (Afroz et al. 2014b). Strains deficient of enzymes for arabinose catabolism are subject to all-or-none induction (Afroz et al. 2014b; Siegele and Hu 1997) and even native strains show a bistable behaviour (Fritz et al. 2014; Makela et al. 2013).

For the araBAD system, it was shown that tuning on cellular level is possible via deleting *araE* and *araFGH* and replacing them by *araE* or *araFGH* under the control of a different promoter (Afroz et al. 2014a; Fritz et al. 2014; Khlebnikov et al. 2000). This is possible since the positive feedback loop is interrupted.

A graded response of the araBAD operon can also be achieved by process technological means. Using a mixed feed strategy with glucose as main substrate and arabinose as inducing

substrate we demonstrated the tuneability of the araBAD operon on cellular level (Sagmeister et al. 2013c).

10.2.3 Induction repression ratios in the araBAD system

Basal transcription for the system is considered to be very low (Guzman et al. 1995; Miyada et al. 1984). To further reduce the basal expression levels the culture can be grown with glucose and fucose as substrate, which can be attributed to catabolite repression and competitive inducer binding (Lee et al. 1987). Induction to repression ratios are reported to vary between 250 to 1300, dependent on the medium, which was used (Guzman et al. 1995).

10.2.4 Tuning of the araBAD operon – application in bioprocesses

We recently showed that an *E. coli* C41 strain can metabolize D-glucose and L-arabinose simultaneously and that both specific uptake rates can be controlled independently in fed-batch processes, opening the way for a mixed-feed bioprocess for this systems. Prior to induction the culture was subjected to an arabinose pulse in order to adapt to arabinose metabolism (Sagmeister et al. 2013a). In a subsequent study, the tunability of this mixed-feed process was shown using green fluorescent protein under control of p_{araBAD} as model protein in fed-batch processes (Sagmeister et al. 2013c). Flow cytometry analysis revealed that the culture was tuned on cellular level, although a small subpopulation of non-induced cells was present, but independent of the expression level applied. We observed a linear relationship between specific arabinose uptake rate ($q_{s\_L\text{-}arabinose}$) and specific productivity (q_p). The specific productivity directly responded to adjustments of $q_{s\_L\text{-}arabinose}$ and within specific ranges both variables were directly proportional. Hence, an increase in $q_{s\_L\text{-}arabinose}$ resulted in an increase in q_p. Within this study it was demonstrated that tunability of p_{araBAD} in a *E. coli* with intact arabinose operon can be achieved by a mixed-feed approach (Sagmeister et al. 2013c). Furthermore, the specific growth rate was observed to have an effect on the specific productivity.

By constructing arabinose transport deficient strains (*araE*- and *araFGH*-), harbouring a plasmid with a copy of *araE* or under the control of a separate promoter, Khlebnikov et al. were able to generate a dose dependent and uniform induction on cellular level under subsaturating arabinose concentrations (Khlebnikov et al. 2000). A second plasmid containing green fluorescent protein under the control of the pBAD promoter allowed examination of single-cell expression levels by flow cytometry analysis. Experiments were performed on millilitre scale. Comparison of different kind of promoters (ptac, ptaclacUV5 and constitutive promoters of *lactococcus lactis*) and different gene dosages (low- and medium-copy number plasmid, genome integration) for araE expression yielded an influence of promoter strength on the homogeneity of induction (Khlebnikov et al. 2001; Khlebnikov et al. 2002). Strong promoters such as the IPTG inducible p_{tac} and the constitutive promoter p_{cp18} resulted in a uniform induction within the culture at arabinose concentrations from 0% to 0.2% weight per volume. Using the weaker $p_{taclacUV5}$, only a part of the culture was induced at low arabinose concentrations. The culture-averaged fluorescence level increased with increasing inducer amount (Khlebnikov et al. 2002).

With their studies, Khlebnikov et al. proved that tunable transcription with p_{araBAD} is possible when the genes encoding for arabinose transport are under control of a separate promoter. Exploitation of this system for development of a bioprocess for recombinant protein production is not yet described in literature.

10.3 rhaBAD system

10.3.1 General characteristics of the rhaBAD system

Another promoter used for recombinant protein production are the p_{rhaBAD} and the p_T promoter of the rha regulon. The *rhaBAD* regulon enables *E. coli* to metabolize L-rhamnose. It consists of a rhamnose transporter gene *rhaT* (Muiry et al. 1993; Tate et al. 1992), the genes for rhamnose catabolism *rhaBAD* (Egan and Schleif 1993) and the regulation genes *rhaR* and *rhaS* (Tobin and Schleif 1987; Tobin and Schleif 1990a; Tobin and Schleif 1990b; Vía et al. 1996;

Wickstrum et al. 2009). The regulon is controlled by an induction cascade, which is triggered by L-rhamnose. In presence of rhamnose RhaR acts as inducer of *rhaS*, which itself induces transcription of *rhaBAD* and *rhaT* (Egan and Schleif 1993; Vía et al. 1996). Cyclic AMP receptor protein (CRP) functions as co-activator for the transcription of all three operons *rhaBAD*, *rhaT* and *rhaS*, which renders the system susceptible to catabolite repression (Holcroft and Egan 2000a; Holcroft and Egan 2000b; Wickstrum et al. 2005). RhaS itself is capable of activating *rhaSR* transcription, but due to a lower CRP contribution it results in a lower transcription than by activation with RhaR. Due to differences in the Shine-Dalgarno sequences, RhaS is expressed at a higher level than RhaR. It therefore leads to a kind of negative auto-regulation which results in a decrease of *rhaSR* transcription (Wickstrum et al. 2009).

10.3.2 Induction repression ratios in the rhaBAD system

In a comparative study the basal level of expression of p_{rhaBAD} was 10 times lower than p_{araBAD} (Haldimann et al. 1998). Due to catabolite repression, it is possible to minimize the basal level of expression (Giacalone et al. 2006; Haldimann et al. 1998). However, on addition of rhamnose to cells growing in the presence of high glucose concentration, the induction levels are comparable to cells growing in absence of glucose. This suggests that the system is still inducible when being catabolite repressed (Giacalone et al. 2006).

10.3.3 rhaBAD system – offering various tunable promoters?

The rha regulon exhibits a strict all-or-none induction, hence tuning on cellular level is not possible by one point addition the inducer (Afroz et al. 2014b; Ozbudak et al. 2004; Rao and Koirala 2014). Using an expression system based on the *rhaTRS* locus, Giacalone et al. observed a dose dependent induction on cellular level (Giacalone et al. 2006). To the authors' knowledge so far no studies were conducted aiming at the knock-out of transport proteins to achieve tunable expression using the rhaBAD system.

10.3.4 Tuning of the rhaBAD operon – application in bioprocessess

Giacalone et al. investigated the tunability of TphoA (PhoA with the toxR transmembrane domain) and green fluorescent protein expression from the *rhaT* promoter (p_{rhaT}). Different vectors (low-, medium- and high-copy plasmids) containing the reporter protein under control of p_{rhaT} and the regulatory genes *rhaR* and *rhaS* were constructed and termed pRHA. *E. coli* MG1655 (with complete rhamnose pathway) was grown in 3 ml LB medium and induced with L-rhamnose concentrations of 10, 100 and 1000 µM at an OD_{600} of 0.1. The authors observed a dependency of production level on inducer concentration and plasmid copy number. Flow cytometry analysis revealed that the cellular induction level indeed did vary with inducer concentration, but a fraction of non-induced cells remained and did increase with decreasing inducer concentrations. In our understanding these results suggest that the culture is tuned on population level and a fraction of the cells is tuned on cellular level. These ambivalent findings need to be addressed in further studies in order to define whether transcription tuning on cellular level is possible or not in this system.

Wagner et al. constructed a BL21(DE3) derivative strain, termed Lemo21(DE3). Using this strain, the possibility to adjust the T7RNAP levels by co-expression of T7 lysozyme under control of the p_{rhaBAD} promoter is reported (Wagner et al. 2008). This strain harbours two plasmids. One plasmid called pLemo contains the regulatory genes *rhaS* and *rhaR* and a variant of T7 lysozyme (LysY) under control of the p_{rhaBAD} promoter. The second plasmid harbors the gene of interest under control of the p_{T7} promoter. The actual tuning is performed with the p_{rhaBAD} promoter and rhamnose as inducer. The resulting T7 lysozyme concentrations reduce the amount of T7RNAP and consequently the expression level of the target protein under control of p_{T7}. This system was used for membrane protein production (Schlegel et al. 2012; Wagner et al. 2008) and recombinant protein production in the periplasm of *E. coli (Schlegel et al. 2013).* In shake flasks, Schlegel et al. investigated the influence of different rhamnose concentrations (0 – 2000 µM) on culture homogeneity after 8 hours of induction with 0.4 mM

ITPG (Schlegel et al. 2012). They observed sub-populations of induced and non-induced cells at 0 µM rhamnose, when lysozyme expression was not induced. At increasing rhamnose concentrations they observed a decrease of the non-induced fraction. Until at a certain rhamnose concentration, a uniform culture was attained. The authors attributed the non-induced fraction of the culture to outgrowth of segregants. Using the Lemo21(DE3) strain the authors were able to identify the optimal conditions for membrane protein expression and therefore to achieve higher titers of correctly folded product and a more stable production period (Schlegel et al. 2012).

Since two different promoter systems (p_{lacUV5} and p_{rhaBAD}) are used, both have to be considered as possible causes of non-uniformity across the culture. In this respect the system is highly complex in respect to tunability on cellular level, especially considering the findings of Afroz et al. that uniform transcription with IPTG is only possible in *lacY* deficient strains and that the rhamnose utilization pathway typically responds in a strictly all-or-none fashion (Afroz et al. 2014b).

10.4 proU Operon

10.4.1 General characteristics of the proU Operon

The osmo-regulated proU operon is one of three proline transport systems in *E. coli* providing the cell with the ability to respond to changes in osmolarity in its environment (osmotic stress). The system was reviewed by Lucht et al. (Lucht and Bremer 1994). The operon encodes three proteins: ProV (May et al. 1989) ProW and ProX (Breed et al. 2001) which regulate the transport of glycine-betaine and other osmoprotectants into the cytoplasm at high osmolarity (Gowrishankar 1985; Stirling et al. 1989). To sustain the inner cell pressure (turgor), *E. coli* can import K^+ ions via several K^+ transport systems as a response to a change of osmolarity in its environment (Booth 1990; Epstein 1986; Sutherland et al. 1986). The import of K^+ is accompanied by production of glutamate as counter ion (Measures 1975). At higher

osmolarities, the cell replaces K+ ions by compounds that do not disturb metabolic activities, so called compatible solutes or osmoprotectants (Booth 1990; Epstein 1986; Sutherland et al. 1986). Induction of proU is assumed to be a mixture of elevated potassium-glutamate levels (Leirmo et al. 1987; Lucht and Bremer 1994), changes in DNA super coiling (Higgins et al. 1988; Lucht and Bremer 1994) and a repression mechanism, that only functions at low osmolarities (Lucht and Bremer 1994). Transcription cannot be induced at limiting K+ concentration in the medium (Sutherland et al. 1986).

10.4.2 proU Operon – promising candidate for tunable recombinant protein expression

The system is not responsible for the import of its inducer. According to the conclusions of Afroz for other systems it is therefore not expected to show all-or-none behaviour (Afroz et al. 2014b; Rao and Koirala 2014). It directly responds to changes in osmolarity and expression is maintained as long as the osmotic stress exists (Herbst et al. 1994; Lucht and Bremer 1994; Walawalkar et al. 2013).

10.4.3 Tuning of the proU operon – application in bioprocesses

Herbst et al. constructed a set of expression vectors (termed pOSEX) containing proV and the target genes under control of the p_{proU} promoter (Herbst et al. 1994). With these pOSEX vectors the expression of β-galactosidase (LacZ) and a carboxyltransferase (GcdA) from *Acidamococcus fermentans* in *E. coli* MKH13 [*ΔputPA101*, *ΔproP2*, *ΔproU608*], a derivative of *E. coli* K-12, was studied. 7 ml cultures were grown for 3 to 4 hours on LB-medium with NaCl concentrations varying from 0 to 300 mM. By SDS-PAGE analysis the authors observed a correlation of target protein concentration and osmolarity of the growth medium. Higher osmolarities resulted in higher target protein concentrations. To be able to attribute these findings to transcription tuning on cellular level, culture uniformity (Afroz et al. 2014b; Rao and Koirala 2014) needs to be addressed by single-cell analytics in further studies. Whether the impact of osmotic stress on overall metabolism limits the applicability of the system or not,

needs to be investigated (Cheung et al. 2009; Roth et al. 1985; Walawalkar et al. 2013; Weber et al. 2006). Future investigations will also have to consider the impact of osmotic stress on overall metabolism and cell growth (Cheung et al. 2009; Roth et al. 1985; Walawalkar et al. 2013; Weber et al. 2006).

10.5 pprpB

The *prp* regulon was first described and studied for *Salmonella typhimurium* (Hammelman et al. 1996). A closely related operon was found in *E. coli* with a high identity by genomic sequencing (Blattner 1997) and radioactive labelling experiments (Textor et al. 1997). The tunablity of the system was investigated in *E. coli* (Lee and Keasling 2005). The regulon enables *E. coli* to metabolize propionate and is induced in the presence of propionate. The regulon was intensively investigated by the group of Escalante-Semerena (Grimek et al. 2003; Hammelman et al. 1996; Horswill and Escalante-Semerena 1997; Horswill and Escalante-Semerena 1999a; Horswill and Escalante-Semerena 1999b; Horswill and Escalante-Semerena 2001; Palacios 2004; Palacios and Escalante-Semerena 2000; Tsang et al. 1998). It consists of the two operons, *prpR* and *prpBCDE* (Horswill and Escalante-Semerena 1997). Under the control of its own promoter $p_{prpR,}$ the *prpR* operon encodes the transcriptional activator for *pprpB* of the sigma-54 family, which is essential for p_{prpB} transcription (Horswill and Escalante-Semerena 1997; Palacios and Escalante-Semerena 2000). The p_{prpR} promoter is not dependent on propionate but is activated by cAMP receptor protein and therefore believed to be solely controlled by catabolite repression (Lee et al. 2005). *PrpBCDE* encodes most of the enzymes of the 2-methylcitric acid cycle for oxidation of propionate to pyruvate (Brock et al. 2001; Brock et al. 2002; Grimek et al. 2003; Horswill and Escalante-Semerena 1999b; Horswill and Escalante-Semerena 2001; Palacios 2004). The single steps of the cycle are well described by Brock et al. (Brock et al. 2002).

P_{prpB} is not directly addressed by propionate. Propionate is converted to 2-methylcitrate (2-MC) in two consequent steps by PrpE and PrpC (Tsang et al. 1998). In presence of 2-MC PrpR initiates transcription of *prpBCDE* (Palacios 2004; Palacios and Escalante-Semerena 2000). Beside of PrpR and its co-activator the sigma-54 transcription factor and the integration host factor (IHF) are needed for *prpBCDE* transcription (Palacios and Escalante-Semerena 2000). The p_{prpB} promoter itself is directly dependent on the activation by CRP. This renders the system prone to catabolite repression (Lee et al. 2005). As propionate is only the pre-cursor of the actual inducer (2-MC), the system could be induced by endogenous metabolic pathways leading to propionate and propionyl-CoA . One such pathway is responsible for the conversion of succinate to propionate (Haller et al. 2000; Lee and Keasling 2005). By taking a look at the net reaction equation of this pathway (propanoyl-CoA + succinate ↔ propanoate + succinyl-CoA) (Keseler et al. 2012) we conclude that no pre-cursor for p_{prpB} induction can synthesized de-novo, at least by this pathway. However, possible alternative routes have to be considered. The cell wall is permeable for propionate (Salmond et al. 1984), no other active import system has been reported to exist in *E. coli* up to now. Intracellular inducer concentration can decrease in course of the process due to metabolization of propionate and dilution due to cell growth.

10.5.1 prpBCDE operon - tuning on cellular level is possible

Lee et al. observed a graded induction on cellular level (Lee and Keasling 2005) in response to the extracellular propionate concentration. With no traits of auto-catalytic functionality within its genome (Carrier and Keasling 1999; Rao and Koirala 2014) the *prpBCDE* operon is a promising tunable system.

10.5.2 prpBCDE operon - increasing strength

By using PrpR and p_{prpB} of *S. typhimurium* in *E. coli* the expression strength was increased in contrast to the *E. coli* inherent analogues (Lee and Keasling 2006a; Lee and Keasling 2008). A threefold higher green fluorescent protein production was observed. However, this increase came along with an increase in basal expression levels (Lee and Keasling 2006a).

10.5.3 Tuning of the prpBCDE operon – application in bioprocessess

Lee et al. constructed several vectors containing *prpR* (activator protein for p_{prpB}) and the target gene under control of the *prpBCDE* promoter (p_{prpB}), termed pPro (Lee and Keasling 2005). *E. coli* DH10B with pPro vectors harbouring green fluorescent protein as marker protein was grown in 5 ml LB-medium. At an OD600 of 0.5 cultures were induced by one-point addition of propionate in a concentration range from 0 to 50 mM. Culture uniformity was verified by flow-cytometry measurements 2 and 6 hours after induction. The study revealed that the culture was induced uniformly and that the GFP expression level is a function of the propionate concentration. In a consequent study Lee et al. investigated pPro vectors containing *prpR* and the *prpBCDE* promoter from *S. typhimurium* (Lee and Keasling 2006a; Lee and Keasling 2006b). Comparison with the *E. coli* based pPro vectors revealed a threefold higher GFP expression when using the *Salmonella* based pPro system. As a second step the tunability on transcriptional level of the *Salmonella* based pPro system was confirmed by expressing two plant genes encoding coclaurine *N*-methyltransferase (CMT) and norcoclaurine synthase (NCS) in shake flasks (20 ml working volume) (Lee and Keasling 2008). In 2012 the pPro system was registered for patent approval (Jay D. Keasling 2012).

Table 1: Quick recap of in literature reported systems with respect to tunability. "Proof of tunability" refers to whether or not tunability on cellular level was demonstrated for the respective system.

Promoter	Genoytpe	Add. plasmid	Inducer	Proof of tunability	Comments	Reference
p_{lac}	complete	-	lactose	no	According to lactose p_{lac} induction mechanistic (Afroz et al. 2014b), probably tunable	(Striedner et al. 2003)
	complete	-	IPTG/TMG	no	According to IPTG p_{lac} induction mechanistic (Afroz et al. 2014b), probably not tunable	(Striedner et al. 2003)
	lacY-	-	IPTG/TMG	yes		Tuner™, (Turner et al. 2005)
	lacY- lacA-	-	IPTG/TMG	yes		
p_{araBAD}	complete	-	arabinose mixed feed	yes		(Sagmeister et al. 2013c)
	araE- araFGH-	*araE* under control of different promoter	arabinose	yes		(Khlebnikov et al. 2002)
	araBD-	-	arabinose	no	According to arabinose p_{araBAD} induction mechanistic (Afroz et al. 2014b), probably not tunable	(Sommer et al. 2010)
	araBAD-	-	arabinose	no		
p_{rhaBAD}	complete	pLemo	rhamnose/IPTG	/		(Wagner et al. 2008)
p_{rhaT}	complete	-	rhamnose	/		(Giacalone et al. 2006)
p_{proU}	complete	-	NaCl	no	According to NaCl p_{roU} induction mechanistic (Lucht and Bremer 1994), probably tunable	(Herbst et al. 1994)
p_{prpB}	complete	-	propionate	yes		(Lee and Keasling 2005)

11 Process technological aspects of expression tuning

One-point additions of varying non-metabolisable inducer concentrations is the most commonly applied method to achieve expression tuning (Khlebnikov et al. 2002; Lee and Keasling 2005; Wagner et al. 2008). This is typically used for investigative studies with small-scale experiments and short production periods. It is usually assumed that the concentration of inducer per cell (specific inducer concentration) is constant. While this assumption is justifiable for small-scale shake flask experiments, it definitely does not hold true for industrial fermentation processes. Here, the specific concentration of active inducer can be submitted to change due to catabolism, dilution by growth and inactivation, for example through acetylation (Marbach and Bettenbrock 2012; Novick and Weiner 1957).

When exploiting tunable systems for industrial bioprocesses with extended production phase a continuous inducer supply is necessary to ensure a constant inducer concentration within the cell. As this criterion is hard to meet a possible simplification is to neglect the inducer consumption and transporting rates in and out of the cell and simply adjust the inducer amount to the biomass concentration. A method that compensates for these effects is the continuous feeding of inducer in order to achieve a constant inducer to biomass ratio (Striedner et al. 2003), which requires a continuous estimate of biomass concentration. A possible way to achieve that is to use an exponential feeding profile and adjust the inducer concentration to the calculated value according to the feeding profile. These feed-forward strategies assume a constant yield of biomass on substrate ($Y_{x/s}$) and neglect the fact that $Y_{x/s}$ can change especially in production phases. Therefore, a more accurate method is to estimate the biomass based on online accessible data with soft sensors (Luttmann et al. 2012; Paulsson et al. 2014; Sagmeister et al. 2013d; Wechselberger et al. 2013b). The type of inducer (metabolisable or non-metabolisable) results in several consequences with respect to the controllability of the system. When using a non-metabolisable inducer the induction rate can be independently controlled from the substrate uptake rate (or respectively the specific

growth rate). When using metabolisable inducers as sole carbon source the induction rate is tightly coupled to the sugar uptake rate and therefore cannot be controlled independently.

Another alternative is the application of a mixed-feeding strategy, where multiple carbon sources are fed to the cells in certain ratios. The mixed feed strategy permits the use of metabolisable inducer while retaining the advantage of independent control of induction rate and substrate uptake rate (which determines the specific growth rate). Recently, we demonstrated the tunability of a system via adjusting the uptake rates of two sugars: one acting as carbon source and the other as inducer and second carbon source (Sagmeister et al. 2013c). Independent control of sugar uptake rates can be achieved via generic control strategies for fed-batch processes (Sagmeister et al. 2013d). For mixed feed systems, catabolite repression poses a natural limitation to the application of these systems, which has to be investigated beforehand and considered for process design (Sagmeister et al. 2013a). A short outline of applied induction methods is given in Table 2.

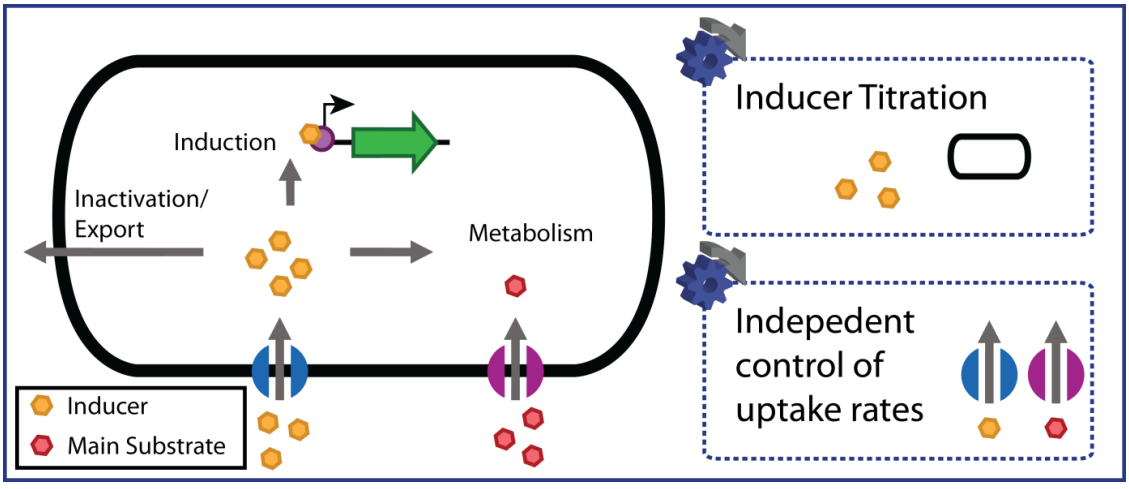

Fig. 2: In order to maintain a constant expression level under subsaturating inducer concentrations a constant inducer concentration within the cell is mandatory. Therefore all routes of transport into (active import, diffusion) and out of the cell (metabolism, inactivation, export) have to be considered. As not all of these rates are accessible a constant inducer concentration within the cell can only be approximated. When using non-metabolisable inducers this condition can be approximated by adjusting the extracellular inducer concentration to the biomass concentration. In the case of metabolisable inducers a mixed feeding strategy, where the uptake of the main substrate and inducing substrate can be controlled independently, needs to be applied in order to retain the additional degree of freedom of expression tuning.

Table 2: Process technological methods for expression tuning

Induction method	Mode of action	Comments
One point addition of inducer	The concentration of inducer is adjusted to a defined concentration in the beginning of the induction phase	Widely applied, however inducer concentration can change throughout the process due to catabolism, dilution by growth or inactivation (Marbach and Bettenbrock 2012; Novick and Weiner 1957)
Inducer titration	Non-metabolisable inducer is continuously supplied to keep the specific inducer concentration (inducer/ biomass) constant	Independent control of sugar uptake rate (growth rate) and induction rate is possible (Striedner et al. 2003)
Metabolisable inducer	Metabolisable inducer is continuously supplied and metabolized by the cells	Induction rate and uptake rate of metabolisable inducer are tightly coupled
Mixed feed	Both primary growth substrate and metabolisable inducer are simultaneously supplied and metabolized by the cells	Independent control of specific growth rate and induction rate is possible (Sagmeister et al. 2013a).

12 Discussion

12.1 Verification and evaluation of expression tuning

With respect to the definition as the adjustment of the recombinant gene transcription rate on cellular level, several things have to be considered in order to demonstrate expression tuning. First of all culture homogeneity needs to be verified in order not to mistake tuning on population level with tuning on cellular level. The use of a fluorescence reporter protein with suitable analytical methods has now established itself in order to prove expression on cellular level. Where flow cytometry (Khlebnikov et al. 2002; Lee and Keasling 2005; Sagmeister et al. 2013c; Wagner et al. 2008) is more commonly used for bioprocesses, microscopy is rather used for investigational studies on induction kinetics and behaviour (Megerle et al. 2008; Ozbudak et al. 2004; Siegele and Hu 1997). Other important aspects have to be addressed when investigating bioprocesses: Out-growth of segregants, where non-producing cells outgrow producing cells, can be caused by loss of plasmids (Krone et al. 2007; Smith and Bidochka 1998) or all-or-none induction (Novick and Weiner 1957). The transcription rate of the used promoter might change during the process according to its response time (Lee and Keasling 2005). Regarding these time-dependent effects it is necessary to gather time resolved data to be able to attribute the observed responses to the right causes. After verification of the tunable system, its performance needs to be evaluated. In order to gain physiological knowledge it is also well established to compare specific concentrations (related to biomass concentration or cell number) (Khlebnikov et al. 2002; Lee and Keasling 2005) rather than volumetric concentrations.

Furthermore, it needs to be considered that the specific cellular productivity (q_p) is varying along the production period (Sagmeister et al. 2014). This behaviour is not reflected if solely end-point specific concentrations are monitored.

In order to define comparable criteria when working with tunable systems we recommend the following strategy:

i) Use of fluorescent reporter protein and suitable analytical methods (flow cytometry or microscopy)

ii) Time-resolved acquisition of product data

iii) Specific cellular productivity as target variable

Considering the maturity of tunable expression technologies, we anticipate that in future more studies will focus on the investigation and characterization of tunable systems in industrially relevant fed-batch fermentation processes.

12.2 Prerequisites for expression tuning on cellular level

From the reviewed literature, general concepts for prerequisites for expression tuning on cellular level can be abstracted. Above all, a tunable promoter system is the fundamental requirement for expression tuning. For better control it is important to maintain a constant inducer concentration within the cell. When designing inducible systems it is therefore necessary to consider all possible routes for inducer concentration changes within the cell such as transport in and out of the cell, assimilation and change of the specific inducer concentration due to cell growth. A large dynamic range of tunability with respect to inducer concentrations is of advantage. Furthermore, plasmid stability is necessary. Otherwise, the transcription of each gene copy is controlled, but protein expression per cell varies due to different amounts of plasmid copy number and results in a inhomogeneous culture (Khlebnikov et al. 2000). Therefore, the use and proof of stable plasmids or genome integration of the expression sequence is mandatory. Despite of its stability, the plasmid copy number of the used vector itself needs to be considered. Whether the use of a low-, medium- or high-copy number plasmid is possible, depends on the strength of the used tunable promoter system. The direct controllability of protein expression by transcription tuning is only possible as long as transcription is the limiting step for recombinant protein production. It might happen that a weak promoter on a high-copy number plasmid already exceeds these limits and other steps rather than transcription become the bottleneck in recombinant protein

production. This loss of controllability can finally lead to a decrease of product quality (aggregation, amino acid incorporation, etc.) (Harris and Kilby 2014; Hartley and Kane 1991) or lead to a loss of plasmids (Chang et al. 2003). In order to receive more controllability, the tightness of the used expression system needs to be taken into consideration. Product basal expression can sometimes be a major issue as it is for e.g. membrane protein production (Giacalone et al. 2006; Wagner et al. 2007).

Other prerequisites valid for "the ideal expression system" also apply to tunable expression systems and are discussed elsewhere (Keasling 1999; Rosano and Ceccarelli 2014).

13 Tuning – paving the way for continuous processing in biopharmaceutical industry

While continuous manufacturing is widely applied across industries including the petrochemical, food and pharmaceutical sector, it is still outnumbered by batch and semi-batch processes in the biopharmaceutical industry (Konstantinov and Cooney 2014). Though downstream and product formulation unit operation already use continuous processing it is hardly employed in upstream processing. In addition to logistic barriers like challenging implementation and validation complexity continuous processing suffered from process inherent obstacles like culture instability, lack of process control and sterility issues (Farid et al. 2014; Stock et al. 2014). However the demand for flexible manufacturing facilities and reducing cost of goods is increasing due to market fluctuations and growing competition from biosimilars (Kelley 2009; Stock et al. 2014; Walsh 2014; Warikoo et al. 2012). With new technologies emerging in the course of the PAT initiative the upswing of continuous processing is also welcomed and supported by regulatory authorities (Lee et al. 2015; Myerson et al. 2015). From a regulatory or process technological point of view many obstacles did decrease or vanish. However culture stability is still an issue (Nancib and Boudrant 1992). By reducing the protein expression level cells can be longer maintained in a producing state. Expression tuning can therefore offer a great benefit and act as a enabling tool for continuous processing.

In contrast to just using a low producing mutant strain, a tunable host offers the possibility to vary the protein expression on-line and therefore provides to maneuver an out of specs process, back into the design space.

13.1 Expression tuning to speed up process development and scientific progress

Combined with process technology, tunable expression systems enable the control of the recombinant protein expression rate using process-technological means. With the use of a suitable control strategy, the transcription rate can be indirectly controlled. Subsequenlty this can be considered in process development and process optimization, for example using design-of-experiment approaches (Mandenius and Brundin 2008). Expression tuning using process technological means adds a novel degree of freedom to the design of recombinant processes. Using the reviewed technologies, it is possible to fine-tune recombinant protein expression to a level which exploits the cell factory to a maximum. This optimization can take place in controlled lab-scale bioreactor experiments, which more accurately reflects industrial processes that screening studies in shake flasks.

In the field of enzyme control analysis it is necessary to use promoters of different strengths in order to investigate different molecular fluxes within the cell (Jensen et al. 1993). This approach involves the construction of different constructs for different concentrations of observed enzymes. We anticipate that the construction of only one tunable construct to cover all cases would be a great benefit for the investigation and optimization of metabolic pathways. Possible fields of applications for expression tuning are summarized in Table 3.

Table 3: Use cases for expression tuning

Use case	Mode of action	Reference
Increase soluble protein titer	Prevent unwanted inclusion body formation through down regulation of expression	(Baig et al. 2014; Hartley and Kane 1991)
Increase active product amount	Debottleneck translocation for correct disulfide bond formation in periplasm	(Baneyx and Mujacic 2004)
Toxic protein expression	Tune toxic protein expression to a level which is tolerated by the host	(Doherty et al. 1993; Dong et al. 1995)
Reduce metabolic load	Enable longer production periods and increase product quality by lowering the burden on the host and its protein expression machinery	(Bentley et al. 2009; Bienick et al. 2014; Glick 1995; Mairhofer et al. 2013)
Substitute and supplement to promoter libraries	Facilitate enzyme control analysis by using a tunable promoter instead of several promoters of different strengths	(Alper et al. 2005; Dehli et al. 2012; Mey et al. 2007)

14 Conclusions and Outlook

Within this contribution, we provide a comprehensive overview of state of the art genetic- and process technological methods and technologies for expression tuning. Furthermore, we attempt to provide a clear and precise definition for expression tuning: As the etymology of “tuning” meets more the conception of stepping on the accelerator pedal than on building a whole new car in order to make your car go faster, we anticipate to use the term tuning as the purposeful adjustment of the recombinant gene transcription rate on cellular level.

As reviewed, a broad spectrum of *E. coli* promoter systems capable of expression tuning is reported. To purposefully apply these systems for the reviewed benefits of i) higher overall productivities, ii) debottlenecking of transport pathways and iii) avoid protein aggregation, it is necessary to consider promoter system specific constraints that were reviewed within this contribution. To date, one point addition of inducer is the most prevailing process technological method to achieve expression tuning. However, due to inherent problems such as degradation of inducer and a lack of process technological control over the tuning process, we anticipate that other process technological methods such as the use of metabolisable inducers, inducer titration and mixed feed strategies will gain in importance in the future. Furthermore, as reviewed, a variety of control methods is reported in literature that allow to i) control a constant level of inducer to biomass ratio in industrial fed-batch processes as well as to ii) control specific growth rates and specific uptake rates of inducers. We anticipate that expression tuning will unfold its full benefits only in combination with adequate control strategies. Hence, these methods will be essential for the industrial exploitation of tunable expression systems.

Considering the broad spectrum of mature methods and technologies as well as the broad scientific knowledge available, we anticipate that expression tuning will soon be adapted by industry as generically applicable tool to enable and optimize the production of a broad spectrum of products in *E. coli.* With its benefit of on-line controllability of protein expression we believe that expression tuning is able to tackle the issues of constant product quality and culture long term stability and therefore paves the way for continuous production of biopharmaceuticals. This in turn will further progress E. coli as primary expression platform for recombinant products intended for pharmaceutical or technical use.

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Recombinant growth factor production in the periplasm of E. coli utilizing the pBAD mixed feed system

17 Abstract

VEGF-A_{165} as a major key player in angiogenesis a late-breaking therapeutic target. With its rather complicated structure of 6 intra-molecular and 2 inter-molecular disulfide bonds the dimeric protein demands an oxidizing redox environment and convenient folding conditions. The periplasm of *Escherichia coli* provides an ideal folding environment similar to the endoplasmatic reticulum in Eukaryotes. Goal of this study was to investigate and develop a production process for recombinant VEGF based on a tunable recombinant protein expression system (pBAD mixed feed) in *E. coli* periplasm. Within this framework it was tried for first time to tackle the problems occurring during periplasmic expression and protein folding with the promising technology of transcription tuning.

In order to produce VEGF-A_{165} in *E. coli* in a soluble and active form an innovative experimental design approach was performed. In a first step critical process parameters (CPPs) influencing the critical quality attributes of this project (solubility, correct folding, activity) were identified. The influence on product titer and quality of the three most promising CPPs namely the specific growth rate, specific inducer uptake rate and temperature were investigated in a design of experiments. Based on mechanistic considerations two factorial designs each for either co- or posttranslational translocation to the periplasm were performed. The independent control of specific inducer uptake rate and specific growth rate was enabled by using the pBAD mixed feed system. Due to use of a tunable host the control of the inducer uptake rate enabled the control of the transcription rate on cellular level.

Beside of the observation of quality and quantity dependencies on the investigated CPPs it was demonstrated that the product transcription rate was indirectly included in an experimental design by the successful use of a tunable promoter system in *E. coli*.

18 Introduction

A major issue in recombinant protein production in *E. coli* is the solubility of the target protein. For reasons of codon usage, sequence of the translation initiation region and disulfide bridge formation only a small fraction target proteins can be expressed in soluble form (Correa and Oppezzo 2015). The soluble form of these proteins can often be expressed in higher yields in eukaryotic cells (Delic et al. 2014). To still profit from the benefits of *E.coli* as production host these challenges needs to be addressed.

Some recombinant proteins require the formation of disulfide bridges to reach their native and active conformation. A disulfide bridge is a covalent bond between the thiol groups of two cystein residues which is formed in an oxidation reaction (Kadokura and Beckwith 2010). For this reaction to take place an oxidizing environment is favorable (Salinas et al. 2011). Disulfide bond formation can take place spontaneously in vitro or catalyzed by specific enzymes in vivo (Bardwell et al. 1991). Dependent on whether the linked cysteins are part of the same or of different polypeptides the disulfide bridges are called intra- or inter-molecular. Disulfide bridges can fulfill catalytic, signaling and structural functions in proteins. In oxidoreductases disulfide bridges act catalytic in form of electron donors and acceptors, when reduced. They can react to the redox state of the environment and therefore act as signals (Berkmen 2012). For recombinant protein production, the structural role of disulfide bonds for the target proteins is commonly prevailing. They can be necessary for the protein to reach the correctly folded conformation or increase the proteins thermo stability (Beeby et al. 2005; Clarke and Fersht 1993). Therefore, not formed or incorrectly formed disulfide bridges can results in mis-folding and aggregation (Kane and Hartley 1991).

Transcription and translation in *E.coli* takes place in the cytoplasm, which is a reducing environment. Within the cytoplasm the glutathione (GSH) and the thioredoxin systems (Trx) regulate the redox condition, prevent oxidation of proteins and keep the thiol residues in a reduced state (Makrides 1996; Salinas et al. 2011). Both pathways are dependent on NADPH to

reduce their substrates (Prinz et al. 1997). The redox environment, the amino acid sequence of the active site and the stability of the redox states influence whether an oxidoreductase acts as reductase or oxidase. Enzymes that act as reductases in the cytoplasm could act as oxidases in a more oxidizing environment (Berkmen 2012; Debarbieux and Beckwith 1998).

For these reasons, recombinant proteins with complicated tertiary structures dependent on disulfide bridges cannot be produced in the cytoplasm and alternatives are required that allow correct disulfide bridge formation.

There are three strategies to enable the production of disulfide containing products in *E. coli*: i) genetically modified strains with an oxidizing cytoplasm, ii) translocation to periplasm and iii) export out of the cell. For more detailed information refer to works of Berkmen et al., Delic et al. and Correa et al. (Berkmen 2012; Correa and Oppezzo 2015; Delic et al. 2014).

A lot of factors do influence the expression of soluble and correctly folded proteins: the engineering of the cells redox potential, co-expression of varieties of chaperones, deletion of genes coding for proteases, fusion tags, promoter strength, codon usage and the structure of the recombinant protein itself (primary structure, non-consecutive disulfide bridges, etc.). As the structure of the recombinant protein often cannot be altered for reasons of product activity when developing an originator product or product similarity when producing a biosimilar, the degree of freedoms of process design are restricted to the aforementioned external factors. Nevertheless, the variety of degree of freedoms is large and combinations are infinite. Neglecting the fact that there might be a better solution among these combinations for each specific product, we anticipated using *E. coli* periplasm as naturally given disulfide formation environment for a medical highly relevant protein.

In the following section a brief overview on methodologies for production of disulfide containing proteins in *E. coli* will be given. Existing transport pathways and the periplasm itself as ideal folding environment will be discussed in detail. The introduction will be closed with a short presentation of the recombinant product, followed by experimental part of the study.

18.1 Genetically modified strains with an oxidizing cytoplasm

Introduction of mutations into the genes encoding glutathione reductase (gor) and thioredoxin reductase (trxB) turns *E. coli* cytoplasm into a less reducing environment and enables the successful expression of disulfide containing proteins (Salinas et al. 2011). The *gor⁻, trxB⁻* strains are deficient in growing aerobically as the essential enzyme aerobic ribonucleotide reductase cannot be reduced (Prinz et al. 1997). However a mutation in the *ahpC* restores the ability to grow. The mutation turns AhpC from a hydrogen peroxide reductase to a disulfide reductase that is most likely to substitute the function of Gor by using NADPH instead of NADH as electron source for the glutathione pathway. However the thioredoxin pathway remains aberrated and it is even stated that the thioredoxins act as active catalysators for disulfide formation (Ritz et al. 2001; Stewart et al. 1998). These *gor⁻, trxB⁻, ahpC** strains are commercially available as Origami™, Origami B, Rosetta-gami™, Rosetta-gami 2 (Novagen). If wrong cystein residues are paired the protein remains irreversibly mis-folded in these strains. The development of a strain (Shuffle™ strain, New England Biolabs)that expresses DsbC a disulfide isomerase in the cytoplasm, which enables the isomerization of incorrectly oxidized disulfide bridges, circumvented this drawback (Lobstein et al. 2012).

Though for some processes higher yields have been observed with these strains than with periplasmic expression strains soluble expression of the target protein cannot be guaranteed (Bessette et al. 1999; Salinas et al. 2011).

18.2 Translocation to periplasm

The periplasmic space is a cell compartment with an oxidizing redox environment and enzymes that enables disulfide formation in prokaryotes similar to the endoplasmatic reticulum in eukaryotes (Kemp et al. 2008).

It is bounded by the cytoplasmic membrane and the outer membrane which separates it from the extracellular space. The cytoplasmic membrane is impermeable for ions, proteins and other macromolecules, which enables the existence of two compartments with different redox conditions, whereas the outer membrane is permeable to small molecules (< 600 Da) (Decad and Nikaido 1976; Natale et al. 2008; Sochacki et al. 2011).

i) Compared to the cytoplasm the periplasm harbours several advantages. In contrast to the crowded cytoplasm in which macromolecule concentrations can reach 300 to 400 mg/ml only 4 % of total cellular proteins are found in the periplasm (Berlec and Strukelj 2013; Ellis and Minton 2003). As the content of the periplasm is accessible by biological, chemical and physical methods the majority of impurities can easily be separated from the protein of interest which facilitates the downstream process (Ewis and Lu 2005; Robbens et al. 1995).

ii) Fewer proteases are found in contrast to the cytoplasm reducing the risk of target protein degradation (Makrides 1996).

iii) An authentic N-terminus of the target protein is achieved as the signal sequence is cleaved of upon translocation by specific peptidases (Baneyx 1999). Otherwise the initiator metheonine might negatively influence the function and the stability of the target protein (Liao et al. 2004).

iv) iv) The periplasm holds several chaperones and folding catalysts that facilitate protein folding by protection of reactive sites and acceleration of rate limiting steps (Baneyx and Mujacic 2004; Goemans et al. 2013). A good review is given by Goemans et al (Goemans et al. 2013). Two chaperone pathways seem to exist

where SurA transports its substrates through the periplasm and is assisted by FkpA. Whereas Skp and DegP take on the proteins that SurA fails to process (Goemans et al. 2013). SurA and FkpA act as both chaperones and as peptidyl cis-trans isomerases (PPIase), that catalyze the isomeration of proline including peptide bonds (Bitto and McKay 2002; Lazar and Kolter 1996; Saul et al. 2004). Skp is a chaperone that likely belongs to the holdase family and has a wide substrate specifity (Goemans et al. 2013; Walton and Sousa 2004). DegP is a heat shock protein with either chaperone or protease activity, which is activated at temperatures higher than 28 °C (Spiess et al. 1999)

v) v) Due to its importance the Dsb system, which supports disulfide formation, is covered in its own section.

18.2.1 Dsb system in *E.coli* periplasm

Within the periplasm the Dsb system, a group of five proteins (DsbABCDG) promotes the formation of correct disulfide bonds. DsbA and DsbB are oxido reductases that introduce disulfide bridges into their substrates. DsbC and DsbG are disulfide isomerases that are able to rearrange incorrect disulfide bonds. An extensive review on the Dsb system is given by Kadokura et al (Kadokura and Beckwith 2010).

Upon translocation the unfolded proteins are processed by DsbA. With a standardard redox potential of -120 mV DsbA is among the most reactive oxidants that introduce disulfide bridges (Huber-Wunderlich and Glockshuber 1998; Wunderlich and Glockshuber 1993). A reason for its high reactivity is its unstable disulfide bridge, which destabilizes the folded form of DsbA and is a driving force for electron transfer to its substrates (Zapun et al. 1993). Due to its flexibility (Vinci et al. 2002) and conformation (Paxman et al. 2009) DsbA can interact with a large number of substrates.

After DsbA transferred its electrons to a substrate, it needs to be reoxidized. By natural means this is done by DsbB, which is linked to the cytoplasmic membrane. DsbB itself is reoxidized by

ubiquinone under aerobic conditions and menaquinone under anaerobic conditions (Bader et al. 1999). In DsbB negative strains can be reoxidized by oxidants in the fermentation medium (Bardwell et al. 1993).

Despite its high reactivity and broad substrate specifity DsbA often introduces wrong disulfide bonds as it usually, but not exclusively, links cysteine residues in a consecutive way as they are translocated into the periplasm (Kadokura and Beckwith 2010). In addition to that disulfide formation is influenced by the folding mechanism of the protein and cystein residues need to be close to each other (Salinas et al. 2011).

Therefore DsbC and DsbG catalyze the correction of these mis-linked cysteines. DsbC, a dimeric isomerase with additional chaperone activity, is able to identify mis-folded proteins with its hydrophobic binding site as the hydrophic core of mis-folded proteins is exposed (Berkmen 2012; Sun and Wang 2000). The isomerization can take place in two ways: i) DsbC only reduces the mis-linked disulfide bridge and the correct one is introduced by DsbA or ii) DsbC reduces the uncorrect bond and consequently introduces the right one (Kadokura and Beckwith 2010).

The activity of DsbC is restored by DsbD which donates electrons to DsbC. DsbD is linked to the cytoplasmic membrane and is itself reduced by cytomplasmic thioredoxin. It therefore is indirectly dependent on NADPH (Rietsch et al. 1997; Stewart et al. 1999).

DsbG is a reductase that reduces cysteines back to their thiolate state, acts as oxidation protection for cysteine residues and together with DsbC controls the sulfenic acid content of the periplasm (Depuydt et al. 2009).

19 Periplasmic expression

Protein transcription and translation take place in the cytoplasm of *E. coli*. Periplasmic or membrane proteins need to be transported across the cytoplasmic membrane following a signal sequence, which is recognized by corresponding translocation channels and cleaved of upon translocation (Choi and Lee 2004; Paetzel et al. 2002).

In the following section the three translocation mechanisms to the periplasm will be described and related challenges and their possible solutions will be discussed.

19.1 Translocation pathways

19.1.1 Sec translocase

The Sec translocase is a protein complex consisting of the SecYEG translocon, the ATPase SecA, and the membrane associated proteins SecDF. Extensive reviews are given by Denks et al and Kudva et al (Denks et al. 2014; Kudva et al. 2013), but do not cover most recent findings (Bauer et al. 2014).

The model of translocation by SecYEG is still an open research field. Recently, Park et al investigated the mechanism SecYEG by cryo-electron microscopy and presented an animation of their model (Park et al. 2014). SecYEG is a hetero-trimer consisting of three subunits SecY, SecE and SecG (van den Berg et al. 2004). The pore for translocation is believed to be solely formed by SecY (Cannon et al. 2005; van den Berg et al. 2004). However pores formed by oligomerized translocons has been reported as well (Denks et al. 2014). SecY is composed of two halves which in its open form a channel. In its closed form a short hydrophobic helix (the so-called plug) lies in the cavity on the periplasmic side. SecE partly encompasses both halves, provides stability and prevents degradation of SecY by the membrane protease FtsH (Kihara et al. 1995). For translocation the channel needs to be widened and the plug needs to be removed (Park et al. 2014; Spiess 2014). Within the channel a ring of isoleucines acts as seal around the passing protein, which prevents the unwanted passage of small solutes during the

translocation process (van den Berg et al. 2004). SecY can either interact directly via its ribosome binding site with the ribosome (Denks et al. 2014; Frauenfeld et al. 2011) or with SecA to drive the translocation either in a co-translational or post-translational way (Bauer et al. 2014). The translocation process is started by opening of the SecY pore, which might be triggered by the entrance of the polypeptide chain (Park et al. 2014) Dependent on the signal sequence proteins can either be transported through SecY into the periplasm or leave the SecY channel laterally into the membrane (Kudva et al. 2013).

The ATPase SecA acts as targeting factor and motor for protein translocation. It consists of two nucleotide binding domains (NBD1 and NBD2), a peptide-cross-linking domain (PBD), which acts as pre-protein binding site, a helical wing domain and a helical scaffold domain, which itself harbors a two-helix finger motif (Bauer et al. 2014; Sardis and Economou 2010). Not all the functions of these domains have been identified. SecA can form a complex with SecYEG, which involves the association of several domains (Zimmer et al. 2008). Considering its function as targeting factor it has not been clarified whether signal sequence binding happens at the translocon or at the ribosome. In the first case the polypeptide is targeted to SecA by other chaperones, whereas in the second case SecA itself binds to the ribosome, recognizes mildly hydrophobic nascent chains and targets them to the translocon (Castanié-Cornet et al. 2014; Denks et al. 2014). SecA is believed to be mainly involved in post-translational translocation, but is also able to act in a co-translational manner (Zhou et al. 2014).

SecDF is a protein complex located in the cytomplasmic membrane next to SecYEG, consisting of several sub-domains including a flexible “head” domain that is located on the periplasmic side and can swing above the SecY channel. Tsukazaki et al suggest a model in which the head subunit catches the trespassing protein. A conformational change of SecDF avoids a backward motion of the polypeptide. As no ATP is available in the periplasm the whole process is driven by the proton motif force (PMF) (Tsukazaki et al. 2011). SecDF therefore acts as pulling force

and might prevent a backward diffusion during the “sliding” phase. However it seems that not every SecYEG complex is in contact with a SecDF unit (Kudva et al. 2013).

Polypeptides can be targeted to the Sec translocon by two mechanisms: co-translational or post translational. The decision by which mechanism a protein is transported is made at the ribosome during translation. At the exit of the ribosomal tunnel the ribosomal protein L23 acts as a protein docking site, which can interact with specific targeting factors and molecular chaperones (Castanié-Cornet et al. 2014; Kudva et al. 2013). Interacting partners include the chaperone Trigger Factor (TF), the signal recognition particle (SRP), SecA and SecY (Kudva et al. 2013). Beside the “key factors” other chaperones like DnaK and GroEl do assist in protein translocation. An extensive review is given by Castanié-Cornet et al (Castanié-Cornet et al. 2014). Which targeting factors and chaperones do participate is dependent on the leader peptide of the nascent polypeptide that determines which route is chosen (Kudva et al. 2013).

19.1.1.1 Co-translational translocation

Co-translational translocation is mainly used for highly hydrophobic proteins such as inner membrane proteins and some secretory proteins in order to prevent mis-folding and unintended aggregation. Signal sequences (or signal anchors in the case of membrane proteins) for co-translational export are more hydrophobic than signal sequences for post-translational export (Denks et al. 2014; Luirink et al. 2012).

Co-translational translocation is initiated by signal recognition protein (SRP). SRP consists of the protein Ffh and the 4.5S RNA (Driessen et al. 2001). Ffh itself is made up of two domains, whereby one is responsible for binding the signal sequence and the other one a GTPase is responsible for binding to the SRP receptor FtsY. The 4.5S RNA interacts with Ffh and supports signal sequence binding and stabilizes the complex of SRP with its receptor (Denks et al. 2014; Zheng and Gierasch 1997). The SRP receptor FtsY binds to negatively charged phospholipids and to the same residues of SecY, which are contacted by the ribosome (Denks et al. 2014).

SRP binds to the translating ribosome before the nascent chain emerges from the ribosomal tunnel (“standby mode”). Dependent on the emerging signal sequence SRP can be switched from “standby” to “targeting mode” (Holtkamp et al. 2012). In the case of a hydrophobic signal sequence the binding of SRP to the ribosome is stabilized and SRP is switched to “targeting mode”, whereas in the case of a less hydrophobic signal sequence the binding affinity of SRP is decreased, which leads to a fast dissociation of SRP (Holtkamp et al. 2012; Kudva et al. 2013). In “targeting mode” SRP binds the signal sequence and consequently targets FtsY, which dissociates from SecY providing space for the ribosome. The ribosome associates with SecY and SecE. The SRP – FtsY hetero-dimer binds GTP with its GTPase site initiating the transfer of the nascent chain to the Sec translocon. Upon conformational changes caused by the Sec translocon GTP is hydrolyzed and the SRP-FtsY hetero-dimer dissociates.

Despite the common binding site of SecA and the ribosome to SecY, SecA also takes part in co-translational translocation. SecA can associate to ribosomes and target the nascent chains to the translocon in a co-translational way. However participation of SRP and FtsY turn the process more efficient (Zhou et al. 2014).

19.1.1.2 Post-translational translocation

Post-translational translocation is mainly used for periplasmic proteins and outer membrane proteins. Polypeptide chains are kept in a translocation-competent shape by chaperones and are guided to the translocon when translation is finished (Denks et al. 2014). Among these chaperones TF is reported to play a major role.

TF consists of 3 domains; one responsible for binding to the ribosomal L23 domain, a PPIase and a domain harboring binding sites for polypeptides (Castanié-Cornet et al. 2014). Beside its role for translocation it supports folding of cytosolic proteins and it is believed that 60-70% of cytosolic proteins could reach their native form only by interaction with TF (Castanié-Cornet et al. 2014). It was observed that TF deletion strains show a higher amount of co-translational translocation than wild-type strains therefore suggesting that TF is a key factor for post-

translational translocation (Oh et al. 2011; Ullers et al. 2007). TF probably binds to the ribosome after about 100 amino acids of the nascent chain have emerged (Oh et al. 2011) and a model in which TF keeps the nascent chains in a translocation competent state itself or transfers it to other chaperones such as DnaKJE or SecB in order to transfer it to SecA seems likely (Castanié-Cornet et al. 2014).

The homo-tetrameric chaperone SecB can keep proteins in a competent state for translocation. It does not specifically bind signal sequences but recognizes hydrophobic regions within the emerging polypeptide (Knoblauch et al. 1999; Sala et al. 2014). Due to the higher affinity of its SecA binding site to membrane-bound SecA SecB is able to target the protein to the translocon and transfer the polypeptide to SecA (Sala et al. 2014). Besides of targeting the polypeptide to the translocon, SecB can also be recruited by SecA-polypeptide complexes at the ribosome, also leading to post-translational translocation (Castanié-Cornet et al. 2014) Despite its abilities SecB is however not mandatory for targeting proteins to the translocon (Denks et al. 2014).

Bauer et al suggest that the ATP dependent SecA drives the translocation of polypeptides through SecY by a “push and slide” mechanism. When ATP is bound its helix-finger domain can interact with specific amino acids of the translocating polypeptide and drag it into the channel (“pushing motion”). If the finger domain encounters amino acids with which it cannot interact (e.g. glycine) the polypeptide can move by passive diffusion in both directions (“sliding motion”). In its ADP-bound form the interaction between finger domain and the polypeptide is weaker, which as well results in a “sliding motion”. During this process SecA does not dissociate and rebinds after each ATP hydrolysation, but it remains bound to SecY for about 260 ATP hydrolysis cycles until it de- and re-associates. Due to the fact that SecA is mostly in its ADP state, the “sliding” phases might prevail the “push” motions (Bauer et al. 2014). However several models for driving the translocation have been proposed (Denks et al. 2014).

19.1.2 Twin arginine translocation (Tat) system

In contrast to the Sec translocon involved pathways the Tat pathway in *E. coli* translocates fully folded proteins to the periplasm (Patel et al. 2014). Its translocation process is driven by the PMF (Bageshwar and Musser 2007). Compared to Sec signal sequences Tat pathway signal peptides are less hydrophobic and contain basic residues, which rarely occur in similar regions of Sec signal sequences (Patel et al. 2014). In addition to that they contain a conserved twin arginine pair (DeLisa et al. 2002), giving the system its name. The translocon is composed of 3 transmembrane proteins. TatB and TatC form the tight complex TatBC and act as receptor for Tat signal sequences (Behrendt and Brüser 2014). TatA is able to associate to TatBC and harbors a ampiphatic helix (APH) domain, that lies along the surface on the cytosolic side of the membrane (Patel et al. 2014). Tat mediated translocation is often supported by specific chaperones called redox enzyme maturation proteins (REMP) and the common chaperones DnaKJ and GroEL (Robinson et al. 2011). The translocation process is believed to be initiated by the recognition of the signal sequence by TatBC. Though TatBC assembles in complexes of several copies, signal sequence binding only takes place at a single TatBC molecule (Patel et al. 2014). After the recruitment of the signal sequence TatA molecules associate with TatBC. For the subsequent translocation two models have been proposed: i) the “membrane weakening model”, ii) the “trapdoor model”. Within the “membrane weakening model” it is assumed that TatA forms unordered aggregates within the membrane in direct vicinity to TatBC, resulting in a destabilization of the lipid-bilayer and making the membrane permeable for the secretory protein. The translocation then might be mediated by a pulling event by TatC (Brüser and Sanders 2003). The “trapdoor model” relies on the observation that TatA can form aggregates of various sizes, that act as translocation pores. These pores would be covered by the APH domain, which could be flipped inside acting like a “trapdoor” and providing the pore with a hydrophilic interior (Gohlke et al. 2005; Gouffi et al. 2004). Though clear evidence is still

missing accumulated evidence is favoring the “membrane weakening” model (Behrendt and Brüser 2014).

19.2 Periplasmic expression - challenges and solutions

19.2.1 Challenges

Beside of the nature of *E. coli* as host additional factors affecting product quality and quantity need to be addressed when expressing the target protein in *E.coli* periplasm. i) Incomplete or lacking cleavage of signal sequences may lead to mis-folding or loss in product activity (Choi and Lee 2004). ii) Weak translocation efficiency can cause inclusion body formation or product degradation by proteases in the cytoplasm and therefore reduce the yield of soluble and correctly folded product (Baneyx and Mujacic 2004). iii) Periplasmic proteases such as DegP, Prc, DegS, DegO, Protease III and OmpT might lead to product degradation and therefore diminish product titer (Jones et al. 2002; Walsh et al. 2003). iv) Formation of inclusion bodies in the periplasm can be caused by high expression levels and could make another downstream step mandatory (Pizarro et al. 2010). v) Incorrect formation of disulfide bonds may lead to an inactive product or even product degradation (Choi and Lee 2004). vi) Membrane jamming can be caused by highly hydrophobic proteins and can be toxic and lead to cell lysis (Baneyx and Mujacic 2004). These above mentioned challenges are listed in Table 4.

Table 4: Several challenges do arise additionally when targeting a recombinant protein to *E. coli* periplasm.

Incomplete cleavage of signal sequences
Weak translocation efficiency
Product degradation
Formation of inclusion bodies
Incorrect formation disulfide bonds
Membrane jamming

19.2.2 Solutions

In order to tackle these challenges several genetic and process technological solutions are available. Genetic solutions cover engineering of the host or the product itself. i) By changing the signal sequence the target protein can be targeted to a different translocation pathway in order to prevent membrane jamming and increase translocation efficiency (Bowers et al. 2003; Marco 2009). ii) To prevent product degradation and therefore increase product titer the deletion of genes encoding periplasmic proteases is an alternative. However negative effects on cell growth have to be taken into account when using such mutant strains (Baneyx and Mujacic 2004; Ignatova et al. 2003; Park et al. 1999). iii) Co-expression of cytoplasmic, periplasmic and eukaryotic chaperones or other folding catalysts can improve the yield of recombinant protein by support in protein folding, reduction of product degradation and formation of inclusion bodies (Baneyx and Mujacic 2004; Delic et al. 2014). However this approach is not generic and the effects strongly depend on the co-expressed chaperones, the nature of the secretory protein and the used signal sequence (Bergès et al. 1996). iv) Engineering of the translocation pathway can lead to higher translocation rates (Nouwen et al. 1996). v) Possible bottlenecks like translocation or folding can be circumvented genetically by

changing to a weaker promoter or process technological by using a tunable promoter system (Marco 2009; Sagmeister et al. 2013c) (PART I). vi) As temperature has a major influence on protein solubility, low temperatures can favor higher product yields and decrease inclusion body formation (Mujacic et al. 1999; Rodríguez-Carmona et al. 2012; Schein and Noteborn 1988). vii) As the outer cell-membrane is permeable to small molecules (<600 Da) additives to the culture medium are reported to result in higher yields of correctly folded product (Bowden and Georgiou 1988; Marco 2009).

Table 5: Various possibilities to circumvent impediments in periplasmic protein expression are available.

	Genetic engineering	Process technology
Change of translocation pathway	X	
Protease negative strains	X	
Co-expression of chaperones and folding catalysts	X	
Engineering of the translocation pathway	X	
Modulation of expression level	X	X
Temperature		X
Additives to culture medium		X

20 Vascular endothelial growth factor (VEGF) - A

Angiogenesis is involved in embryogenesis (Peters et al. 1993), wound healing (Nissen et al. 1998) and several pathogenic mechanisms such as tumor growth and metastase formation (Dvorak 2015; Koch et al. 2014), ischemia (Crafts et al. 2015; Yang et al. 1998), rheumatoid arthritis (Brenchley 2000), atheroma formation (Ho-Tin-Noé and Michel 2011) and psoriasis (Qi et al. 2014) and has therefore become an interesting therapeutic target (Ferrara and Kerbel 2005; Sivakumar et al. 2004). A major role in angiogenesis play members of the vascular endothelial growth factor (VEGF) family containing several growth factors (VEGF-A, -B, -C, -D, -E, -F, PlGF and their receptors) (Tammela et al. 2005; Yamazaki and Morita 2006). Among these VEGF-A, also known as vascular permeability factor (VPF) is most commonly related to angiogenesis and is a common target of anti-VEGF treatments(Bhisitkul 2006). VEGF-A itself or its antagonists can act as therapeutic agent (Nissen et al. 1998; Weidemann et al. 2013). The dimeric VEGF-A is encoded by one gene and occurs in 9 different isoforms resulting from alternative splicing leading to homo- and hetero-dimers (Keyt et al. 1996; Park et al. 1993). Among these splicing isoforms VEGF-A_{165} is the most prevailing (Yamazaki and Morita 2006). The homo-dimeric VEGF-A_{165} consists of two 165 amino-acid-monomers, which both form a receptor binding domain and a heparin binding domain (Keck et al. 1997). The part of the monomer, forming the receptor binding domain contains a conserved cystein knot motif, made up of 3 intra-molecular disulfide bonds (McDonald and Hendrickson 1993; Murray-Rust et al. 1993), and two cystein residues linking the monomers by two inter-molecular disulfide bridges. The part forming the heparin binding domain possesses another 8 cystein residues (Keck et al. 1997). In addition to that each monomer contains a glycosylation site at Asn-74 (Claffey et al. 1995). Whereas the disulfide bonds are necessary for its biological activity (Claffey et al. 1995), glycosylation is not mandatory (Peretz et al. 1992; Yang et al. 1998), paving the way for recombinant protein production in *E. coli*.

20.1 Recombinant production of VEGF-A

Various hosts have been used for VEGF-A production including *Chinese hamster ovary cells* (CHO) (Lee et al. 2008b), insect cell suspensions cultures (Lee et al. 2006), transgenic rice cell suspension cultures (Chung et al. 2014), *Pichia pastoris* (Mohanraj et al. 1995) and *E.coli.* Among *E. coli* following strains have been used: *E. coli* TOP10 (Kang et al. 2013), *E. coli* DH5(α), *E. coli* AD494 (DE3) pLysS (Kim et al. 2007), *E.coli* BL21(DE3) (Siemeister et al. 1996), *E.coli* BL21(DE3)LysS (Backer and Backer 2001) and *E.coli* 64D1 (W3110 ΔfhuA (ΔtonA) ptr3 lacIq lacL8 ΔompT(nmpc-fepE) ΔdegP ilvG2096(IlvG+; Valr) ΔmalE ΔrhaR) (Pizarro et al. 2010). Most studies are based on uncontrolled shake flasks experiments and only in a few studies fermentations were performed in bioreactors: CHO was cultivated in lab scale reactors (7,5 L) (Lee et al. 2008b) and *E.coli* in lab scale (Kim et al. 2007) and pilot-scale (Pizarro et al. 2010) (7 and 1000 L). Soluble product titers varied between 20 and 80 mg/l, whereas highest product titers of 9.3 g/l were achieved in inclusion body processes (Pizarro et al. 2010).

One-point addition of inducer was applied in every study and so far no more sophisticated induction strategy has been used.

21 Material and Methods

21.1 Strains

Escherichia coli C41 (OverExpress™ C41 (DE3)): F– ompT hsdSB (rB- mB-) gal dcm (DE3) – obtained from Lucigene, Middleton, WI, USA (Lucigene, 2011) (Lucigen, Middleton,Wisconsin, USA) provided by BIRD-C GmbH (BIRD-C, Vienna, Austria) was used. *E. coli* C41 strains have an intact L-arabinose metabolism. Two plasmids originating from pBAD24 (provided by BIRD-C, Vienna, Austria) both carrying a gene encoding for VEGF-A_{165} under the control of the L-arabinose inducible araBAD promoter were constructed. The plasmids differed in the signal sequence fused to the N-terminal end of VEGF-A_{165}. Two strains were constructed each carrying a plasmid harboring a VEGF-A_{165} GIII fusion or a VEGF-A_{165} DsbA fusion .

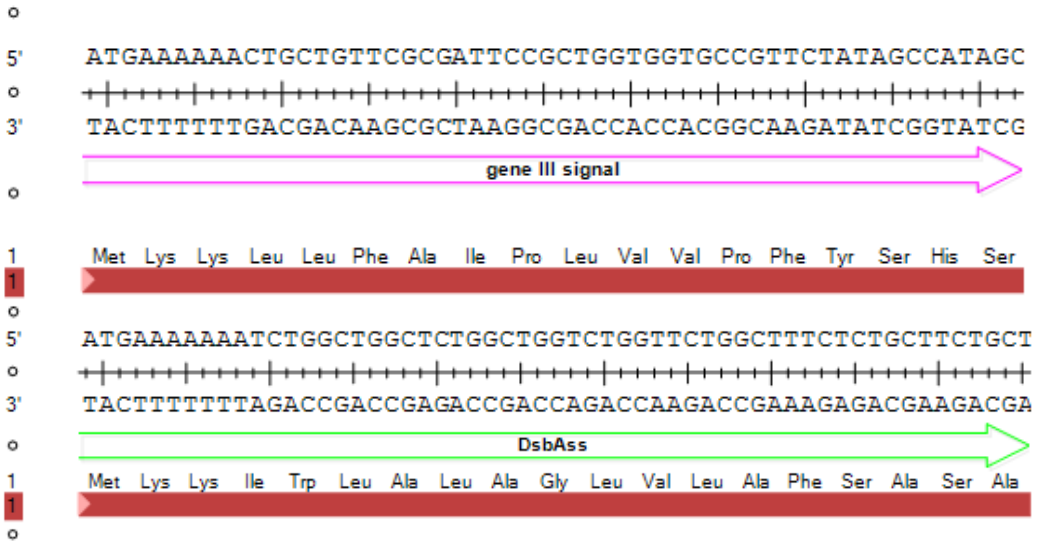

Fig. 3: DNA and amino sequence of the investigated signal sequences.

21.2 Medium

A defined minimal medium according to DeLisa et al was used (Table 6) (DeLisa et al. 1999). $MgSO_4$ * 7 H_2O, Fe(III) citrate, $Zn(CH_3COO)_2$ * 2 H_2O, Thiamine HCl, Gentamicin sulfate and Kanamycin sulfate were prepared as stock solutions and separately auto-claved or sterile-filtered, if heat unstable. $CoCl_2$ * 6 H_2O, $MnCl_2$ * 4 H_2O, $CuCl_2$ * 2 H_2O, H_3BO_3 and Na_2MoO_4 * 2 H_2O were prepared as one trace element solution and auto-claved. For the induced fed-batch phase the ratio of the mixed glucose arabinose feed was adjusted for each fermentation in accordance to the intended ratio of the specific growth rate (μ) and specific arabinose uptake rate (q_{s_ara}) (Table 7) (Eq. 3, Eq. 4, Eq. 5 and Eq. 6).

$$\mu = Y_{x/s} \cdot q_{s\_total} \qquad \textbf{Eq. 3}$$

$$q_{s\_total} = q_{s\_gluc} + q_{s\_ara} \qquad \textbf{Eq. 4}$$

$$c_{ara} = \frac{q_{s\_ara} \cdot c_{total}}{q_{s\_total}} \qquad \textbf{Eq. 5}$$

$$c_{gluc} = \frac{q_{s\_gluc} \cdot c_{total}}{q_{s\_total}} \qquad \textbf{Eq. 6}$$

Table 6: Components of Delisa media.

	Pre-culture (per l)	Batch (per l)	Feed (per l)
Glucose	8.8 g	22 g	400 g (non-induced
Mix			400 g (induced fed-
KH_2PO_4	13.3 g	13.3 g	-
$(NH_4)_2HPO_4$	4 g	4 g	-
Citric acid	1.7 g	1.7 g	-
$MgSO_4 * 7 H_2O$	1.2 g	1,2 g	20 g
Fe(III) citrate	0.1 g	0.1 g	40 mg
EDTA	8.4 mg	8.4 mg	13 mg
$Zn(CH_3COO)_2 * 2$	13 mg	13 mg	16 mg
$CoCl_2 * 6 H_2O$	2.5 mg	2.5 mg	4 mg
$MnCl_2 * 4 H_2O$	15 mg	15 mg	23,5 mg
$CuCl_2 * 2 H_2O$	1.2 mg	1.2 mg	2,2 mg
H_3BO_3	3 mg	3 mg	5 mg
$Na_2MoO_4 * 2 H_2O$	2.5 mg	2.5 mg	4 mg
Thiamine HCl	4.5 mg	4.5 mg	-
Gentamicin sulfate	20 mg	20 mg	-
Kanamycin sulfate	50 mg	50 mg	-

21.3 Bioreactor-setup

100 ml pre-cultures were grown in 1000 ml Erlenmeyer flasks with cotton plugs in a shaking incubator Infors HR Multitron (Infors, Bottmingen, Switzerland).

Fed-batch experiments were carried out in a DASGIP multi-bioreactor system consisting of four glass bioreactors with a working volume of 2 l each (Eppendorf; Hamburg, Germany) equipped with baffles and three disk impellers. The DASGIP control software v4.5 revision 230 was used to adjust the process parameters: pH (Hamilton, Reno, USA) and pO_2 (Mettler Toledo; Greifensee, Switzerland; module DASGIP PH4PO4), temperature and stirrer speed (module DASGIP TC4SC4) and aeration (module DASGIP MX4/4). Feed and base (12.5 NH_4OH) were added using the pump module DASGIP MP8. Reactors were sterilized at 121 °C for 20 min. CO_2, O_2, and mass flow in the off-gas were quantified by a gas analyzer (module DASGIP GA4) using the non-dispersive infrared and zircon dioxide detection principle, respectively.

21.4 Fermentation parameters

Pre-cultures were performed at 35 °C and 200 rpm for a maximum of 11 hours.

During the fed-batch experiments the pH was maintained at 7.2. The reactors were sparged with air at an aeration rate of 90 sl/h. The dissolved oxygen (DO_2) level was kept above 60% saturation by addition of pure oxygen to the in-gas stream (100% were set before inoculation at 35 °C, pH 7.2 and an aeration rate of 90 sl/h). The stirrer speed was adjusted to 1600 rpm. Throughout the batch and the non-induced fed-batch phase the temperature was kept at 35 °C. In the induced fed-batch phase the temperature was adjusted to the design of experiments (DOE) (Table 7). In the lysis phase the temperature was increased to 42 °C. Set-point of the non-induced and induced fed-batch phases (2 and 3) were controlled via feed forward control exponential feeding (Eq. 7 and Eq. 8), following the assumption that the biomass on substrate yield coefficient is constant over the time of the experiment and 0.5 g/g under non-induced and 0.4 g/g under induced conditions. The values for the biomass yields were taken from Ehgartner et al (Ehgartner 2013).

$$F = F_0 \cdot e^{\mu t} \qquad \textbf{Eq. 7}$$

$$F_0 = \frac{x_0 \cdot \mu \cdot V_0}{c_{s_{total}} \cdot Y_{\frac{x}{s}}} \qquad \textbf{Eq. 8}$$

21.5 Determination of sampling intervals

Comparison of processed data (metabolic rates and yield coefficients) demands comparable signal-to-noise ratios (SNRs) of rates obtained from off- and online data (Wechselberger et al. 2013a). To achieve similar SNRs from off-line data at varying specific growth rates, sampling intervals were approximated according to Eq. 9 (Wechselberger et al. 2013a) with a desired SNR of 12 and an expected error on the biomass measurements of 1 %.

$$Sampling\ Interval = \frac{SNR \cdot Error_{Biomass}}{\mu \cdot 67} \qquad \textbf{Eq. 9}$$

21.6 Offline analytical methods

21.6.1 Biomass

At the end of each phase the biomass concentration was determined by optical density measurements at 600 nm using Eq. 10 taken from Ehgartner et al.

$$BM = 0.46 \cdot OD600 \qquad \text{Eq. 10}$$

For cell dry weight (CDW) measurements two 2 ml samples were taken at each sampling point. The samples were centrifuged for 10 min at 5000 rcf. The supernatant was discarded and the pellet was washed with de-ionized (DE) water. After a second centrifugation step the pellets were dried at 105 °C for 72 hours. The dried pellets were cooled to room temperature in an exsiccator and weighed. Samples were taken in duplicates. The error of cell dry-weight determination reveals a median of 1.16 % (Fig. 4)

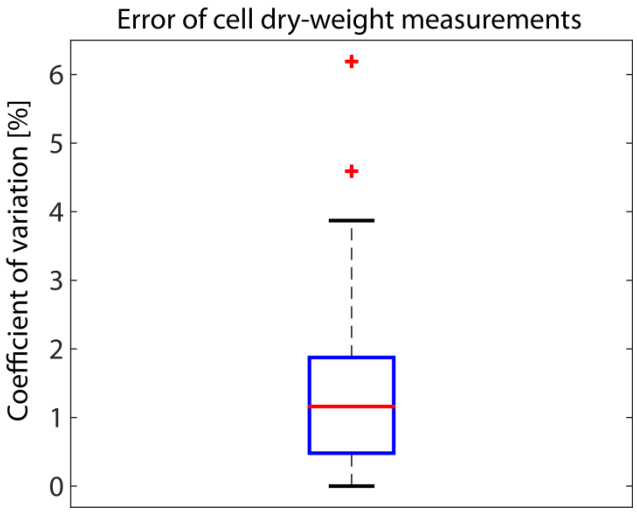

Fig. 4: The boxplot shows the distribution of coefficients of variation of each sampling duplicate. The error of cell dry-weight determination reveals a median of 1.16 %-

21.6.2 Homogenization and sample preparation

2 ml samples were centrifuged at 5000 rcf for 10 min, washed with DE water and centrifuged again. The supernatant resembling the extracellular content was frozen and stored at -20 °C. The pellet was further processed and re-suspended in 20 ml of 50 mM Tris, 1 mM EDTA and 5 mM DTT, pH 8 buffer and homogenized for 8 passages at 1600 ± 200 bar (Avestin EmulsiFlex, Ottawa, Canada). The homogenized sample was aliquoted to more handy volumes (ranging from 1 to 4 ml) and consequently centrifuged at 5000 rcf for 10 min. The supernatant resembling the soluble cell content and the pellet resembling the insoluble fraction were separated and stored at -20 °C for further analysis. The workflow is illustrated in Fig. 5.

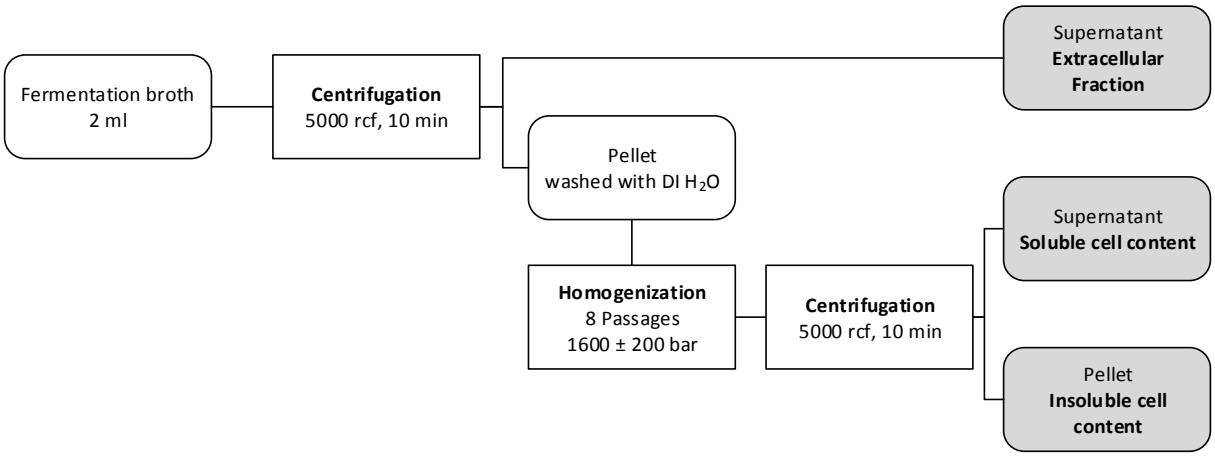

Fig. 5: Workflow of sample preparation. Each sample was divided in 3 fractions containing the extracellular, the soluble intracellular and insoluble intracellular fraction of the cell.

21.6.3 Electrophoresis

The extracellular content and the soluble cell fraction were directly mixed with Laemmli buffer. The insoluble content was re-suspended in Tris-buffered saline (TBS, pH 7.4) beforehand. SDS-PAGE performed with minor deviations as described by Laemmli (LAEMMLI 1970). The samples were incubated at 95°C for 10 min. Iod acetamid was added subsequently to the hand warm samples to a final concentration of 1M. After an incubation time of 15 min at room

temperature the samples were loaded on the gels. If reduced samples were needed β-mercapto-ethanol was added to a final concentration of 5 % prior to the heating step.

Any kD™ Mini-Protean® TGX™ precast polyacrylamide gels (Bio-Rad Laboratories, Hercules, USA) were used together with a Mini-Protean® Tetra cell (Bio-Rad Laboratories, Hercules, USA). Electrophoresis was performed at a constant voltage of 160 V for 45 minutes. The PageRuler Unstained Protein Ladder was used as marker (Thermo Fisher Scientific Inc., Schwerte, Germany).

21.6.4 Westernblot

Westernblots were performed according to Towbin et al with minor deviations (Towbin et al. 1979). The used antibodies were: Vascular Endothelial Growth Factor A Antibody (Thermo Scientific, Pierce Antibodies, PA1-16948) and Goat anti-Rabbit IgG (H+L) Secondary Antibody HRP conjugate (Thermo Scientific, Pierce Antibodies, #31460). Amersham Protran® nitrocellulose membranes (BA83, pore size 0.2 µm) were used together with a Mini Trans-Blot® Cell (Bio-Rad Laboratories, Hercules, USA). Blots were performed at a constant current of 350 mA for 100 minutes. Images were taken and densidometric measurements were performed with the gel imaging system Gel Doc™ XR System (Bio-Rad Laboratories, Hercules, USA). For quantitative analysis standards in 3 different concentrations were applied to the gel.

21.6.5 Data processing

On-line and off-line recorded data was processed in InCyght® (Exputec, Vienna). Physiological meaningful information was extracted by calculating specific uptake and formation rates and yields from the available raw data.

22 Results and discussion

22.1 Process design and development

The production of VEGF-A_{165} in soluble and active form in the periplasm of *E. coli* was the main subjective of this project. Pre-determined by the industrial project was the use of a GIII and DsbA signal sequence construct in order to investigate post and co-translational translocation of VEGF-A_{165}. Following Quality by Design (QbD) principles critical quality attributes (CQAs) were defined (ICH). In a second step a risk assessment was performed in order to link process parameters to the given CQAs. Three critical process parameters (CPPs) were chosen for a design of experiments in order to create process understanding.

22.1.1 Identification of critical quality attributes (CQAs)

Within this project the CQAs were already pre-defined namely product solubility and correctly folded quaternary structure.

22.1.2 Identification of critical process parameters (CPPs)

In a risk assessment three critical steps for soluble recombinant protein production in *E. coli* periplasm were identified. i) Protein expression in the cytoplasm (transcription and translation), ii) translocation to periplasm, iii) correct folding in the periplasm (Fig. 6).

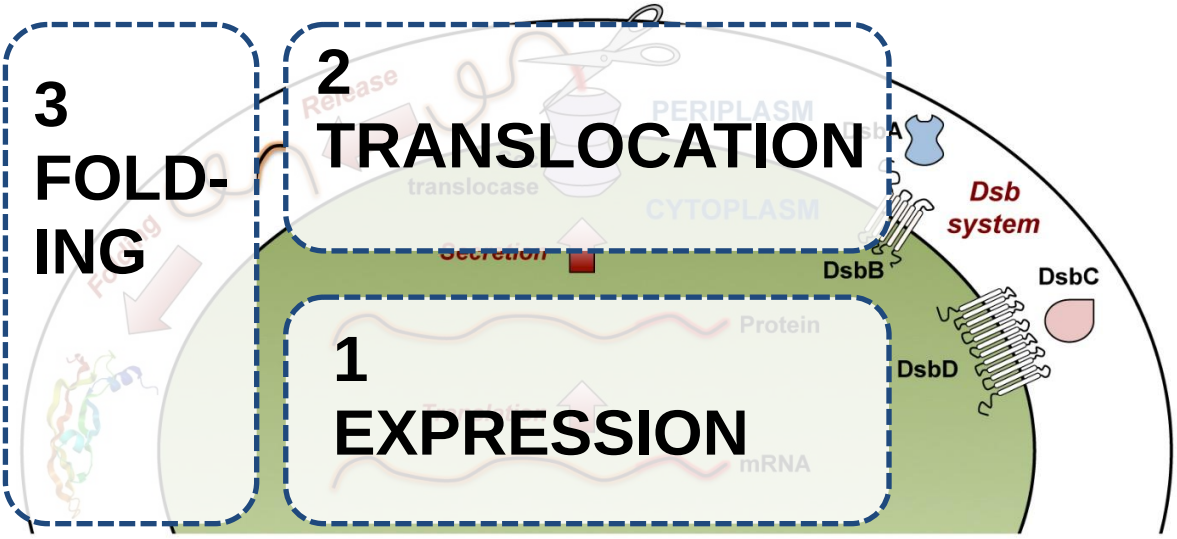

Fig. 6: 3 critical steps for recombinant protein produciton in *E. coli* periplasm were identified. The image was taken and modified from Klint et al. (Klint et al. 2013).

Three CPP which were believed to influence these key steps the most were selected as factors for the factorial design. i) By impacting factors like the ribosomal content (Singh et al. 2012) and metabolic load (Gnoth et al. 2008) the specific growth rate μ has an major influence on the overall expression level and product quality (Sagmeister et al. 2013c). ii) In a tunable promoter system the inducer uptake rate directly controls the transcription rate of the recombinant protein (Sagmeister et al. 2013c)(Part I). iii) Temperature impacts recombinant protein solubility, aggregation, global protein expression, protease activity and plasmid stability and therefore is highly suitable for the intended purpose (Rosano and Ceccarelli 2014; Spiess et al. 1999; Vera et al. 2007). In addition to their influence on the critical quality attributes the three chosen CPPs can easily be controlled by process technological means. The independent control of growth rate and inducer uptake rate was enabled by applying a mixed-feed platform

technology with glucose and arabinose developed by Sagmeister et al. (Sagmeister et al. 2013c).

22.1.3 Design of experiments

In order to avoid experimental biases and keep the number of experiments as low as possible two factorial designs were performed for both signal sequences (Mandenius and Brundin 2008). A 2^3 factorial design (3 factors at 2 levels) for GIII and 2^2 factorial design in the case of DsbA signal sequence were conducted, each with a set of 3 center points (at a level resembling the median of both levels of each factor) (Fig. 8).

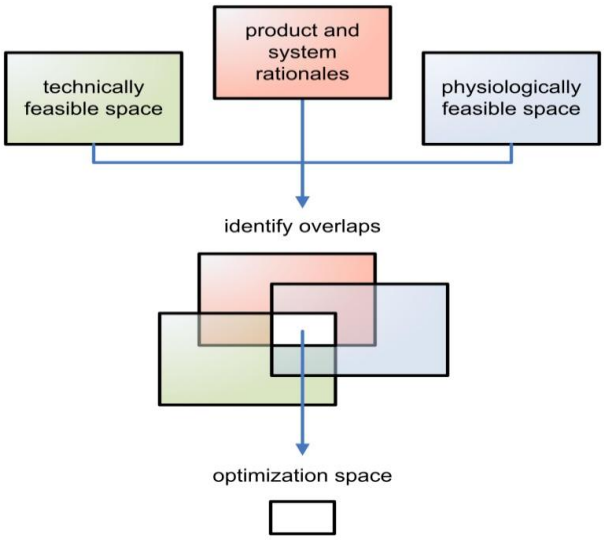

Fig. 7: Deriving the optimization space. The levels of the selected factors were chosen based on constraints predefined by the used equipment, host strain and product (CQAs). With the permission of the author the image was taken from Sagmeister (Sagmeister 2015)

The levels of the selected factors were chosen in dependence on the technically feasible space (e.g. maximal heat transfer rate, maximal oxygen transfer rate), system rationales of the pBAD mixed feed system (investigated limits of the pBAD mixed feed system in reference to all-or-none induction) and product rationales (e.g. optimal folding conditions for proteins) (Kiefhaber et al. 1991; Sagmeister et al. 2013a; Sagmeister et al. 2013b; Sagmeister et al. 2013c; Vallejo and Rinas 2013).

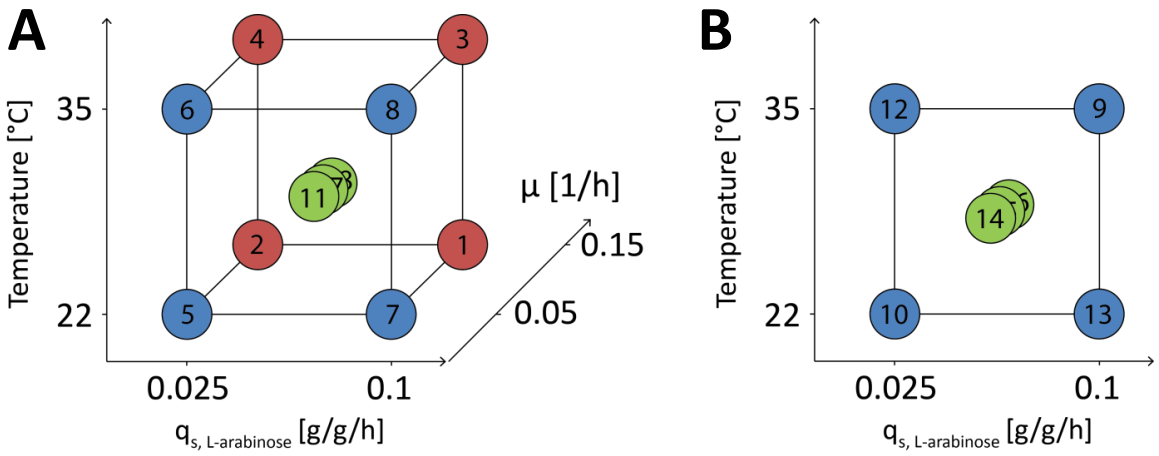

Fig. 8: The experimental design for constructs with GIII signal sequence consisted of 11 bioprocesses. 3 factors were varied between 3 levels (A). The design for constructs with DsbA signal sequence consisted of 7 bioprocesses. In contrast to the 3 dimensional DOE for GIII signal sequence μ was fixed to 0.5 1/h and only 2 factors were varied with (B). Bioprocesses with a μ of 0.15 1/h are shown in red, with a μ of 0.05 1/h in blue and center points in green. As a consequence of the dimensions of both experimental designs their center points differ in μ (0.1 1/h for the three dimensional design and 0.05 for the two dimensional design). The numbers within the circles represent the sequence in which the experiments were conducted.

Table 7: Fermentation list and parameters of performed DOE designs.

Fermentation	μ [1/h]	q_{s_ara} [g/g/h]	T [°C]	Sequence
1	0,15	0,1	22	GIII
2	0,15	0,025	22	GIII
3	0,15	0,1	35	GIII
4	0,15	0,025	35	GIII
5	0,05	0,025	22	GIII
6	0,05	0,025	35	GIII
7	0,05	0,1	22	GIII
8	0,05	0,1	35	GIII
9	0,05	0,1	35	DsbA
10	0,05	0,025	22	DsbA
11	0,1	0,0625	28,5	GIII
12	0,05	0,025	35	DsbA
13	0,05	0,1	22	DsbA
14	0,05	0,0625	28,5	DsbA
15	0,05	0,0625	28,5	DsbA
16	0,05	0,0625	28,5	DsbA
17	0,1	0,0625	28,5	GIII
18	0,1	0,0625	28,5	GIII

22.1.4 Process design and control strategies

The fed-batch experiments consisted of 4 phases: (1) batch on glucose, (2) non-induced fed-batch on glucose, (3) induced fed-batch on a mix of glucose and arabinose. In the batch phase the culture was grown till all glucose was consumed (to a total biomass of 9 g). The non-induced fed-batch was performed for about 3.5 hours according to one generation time from 9 to 18 g total biomass where μ was fixed to 0.2 1/h. During the induced fed-batch μ and q_{s_ara} were adjusted according to the design of experiments and the duration was again fit to one generation time from 18 to 36 g total biomass. Therefore durations varied with the applied μ (Table 7). Whereas batch and non-induced fed-batch solely served for accumulation of biomass, the actual experiment started when applying the mixed feed in the induced fed-batch phase.

Throughout the two multivariate studies, three parameters were examined: μ, q_{s_ara} and T. The parameters of each experiment are shown in Table 7. The process design for the bioprocesses is shown in Fig. 9 and two experimental designs are shown in Fig. 8. Center-points were performed as triplicates.

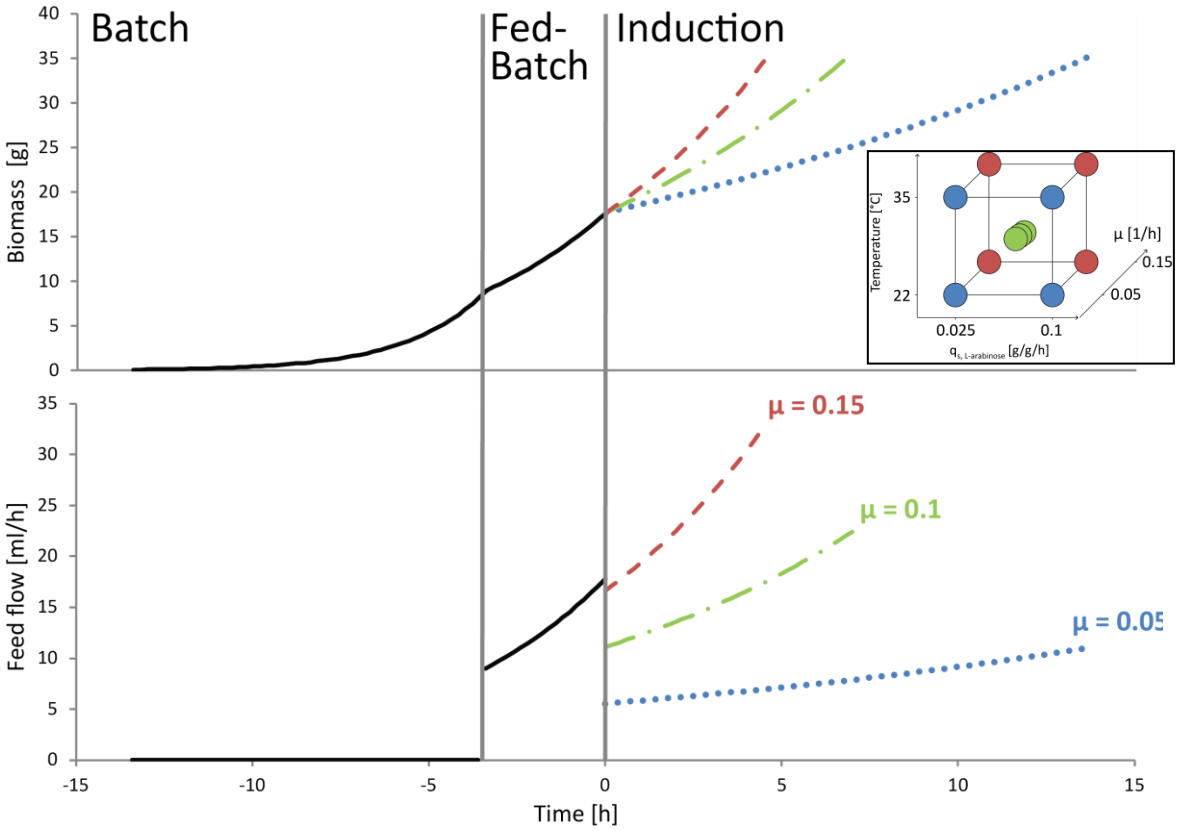

Fig. 9: The graphic shows the intended biomass trend and the therefore needed feed flow of mixed-substrate feed. Each bioprocess consists of 3 phases: batch, biomass accumulation (fed-batch) and recombinant product production (induction). The duration of the batch phase is variable due to variations in inoculum amount. The duration of induction phase varies between 3 values according to the DoE.

22.2 Qualitative analysis

Qualitative analysis is solely based on western blots and only available for 8 bioprocesses of the factorial design for the GIII sequence. It has to be stated that the western blots, on which the analysis is based, were only intended for providing a yes or no question, whether product has been produced or not. Therefore the applied sample amounts were not normalized to the biomass concentration. In addition to that, the contrast was adapted for each plot individually. For these reasons comparisons between lanes have to be made with caution and comparison between several blots is impossible. As described previously soluble and insoluble fraction were separated after the homogenization step. Whereas the soluble fraction showed no detectable product in westernblot analysis (Fig. 10 C and D), the insoluble fraction yielded bands at 18 and 36 kDa, which were attributed to VEGF monomer and dimer (Fig. 10 A and B). No product bands were observed under non-induced conditions (Fig. 10 B), indicating a tight promoter. This is not the case for fermentations 1 to 4, where dimer bands are visible, but can be attributed to the fact that the sample was taken 10 minutes after induction start. The two major protein bands of the insoluble cell fraction were assumed to be two kinds of inclusion bodies: periplasmic inclusion bodies consisting of aggregated dimers and cytoplasmic inclusion bodies consisting of aggregated monomers (Arié et al. 2006; Bowden et al. 1991; Joly et al. 1998; Lee et al. 2008a). Though these western blots were not determined for quantification some trends can be still be derived.

Higher temperatures lead to higher volumetric product titer (periplasmic as well as cytoplasmic inclusion bodies). However, it might be possible that this increase in volumetric product titer is not attributed to the temperature but to a higher biomass concentration, as the same sample volume was applied for each fermentation. This should be verified by an example. Fermentation 3 is the counterpart at higher temperatures to fermentation 1 in the fractorial design (Fig. 10). If the higher volumetric product titer in fermentation 3 was attributed to more biomass, the biomass concentration in fermentation 3 (17.8 g/l) would

need to be higher than in fermentation 1 (24.3 g/l). As this is not the case – the concentration is in this case even lower – the observed trend can be attributed to temperature. This observation applies as well to the other high temperature fermentations (4, 6 and 8) as their biomass concentration are either in a similar range or even lower than their low-temperature counter-parts (Fig. 10 A and B).

Higher inducer uptake rates ($q_{s,L\text{-arabinose}}$) as well lead to increased product amounts. However the trend is less marked than the previous described temperature influence.

At high growth rate and high temperature (fermentation 3 and 4) amounts of soluble monomer were observed (Fig. 10). However, the lack of similar monomer bands at low growth rate (Fig. 10 D) might be attributable to the processing of the image, i.e. the contrast might be too low. For this reason this observation has to be handled with care.

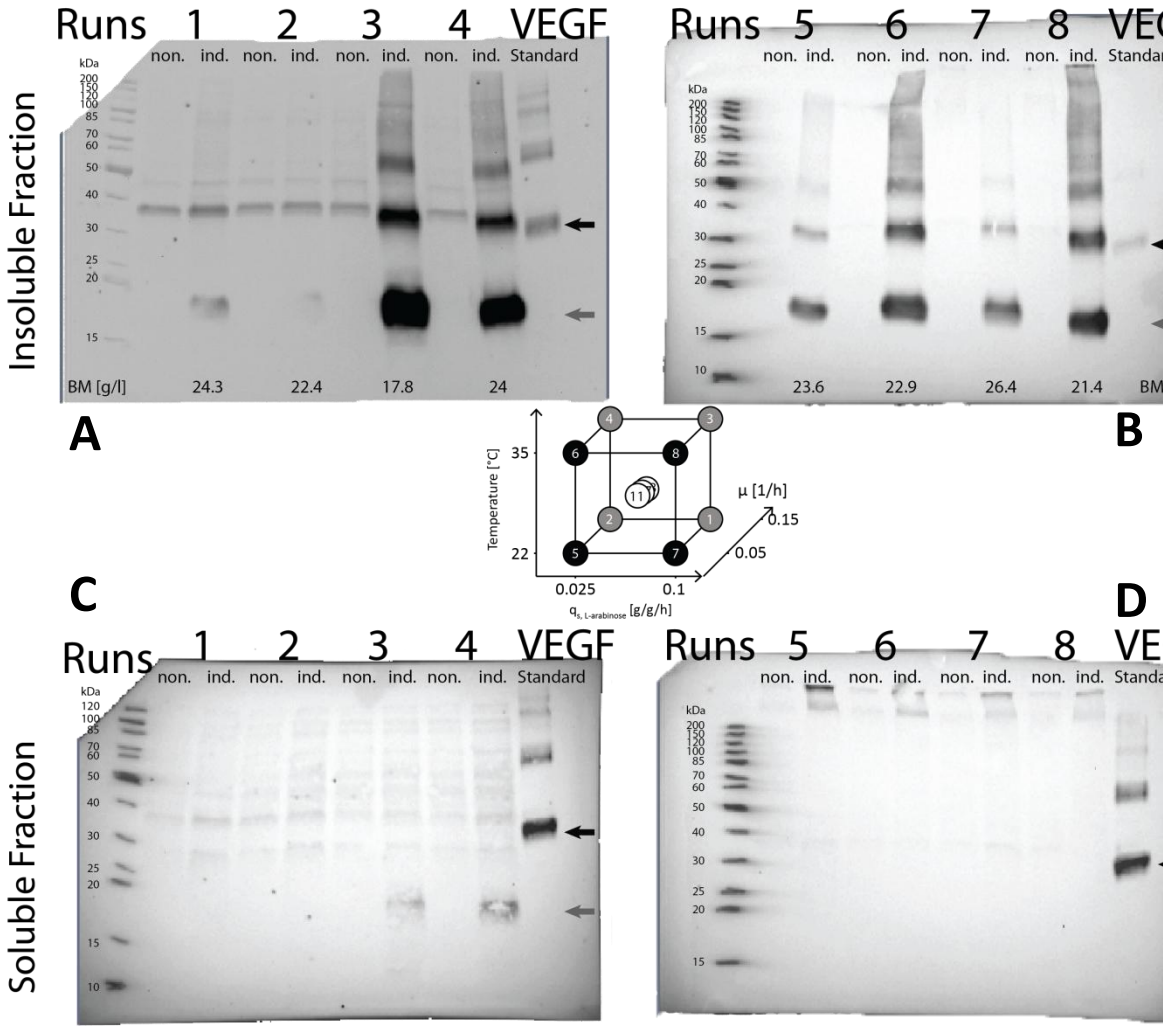

Fig. 10: Westernblots of the insoluble (A and B) and soluble fraction (C and D) of fermentation runs 1 to 8. Per run 2 lanes are plotted showing a sample before induction with L-arabinose (non.) and a sample at the end of the induction phase (ind.). In each blot the standard was loaded in the last lane on the right hand side and the dimer band at 36 kDa is marked with a black arrow. The same volumetric amount of standard and samples were loaded in each blot. The differences in intensity of the standard dimer bands result from contrast adjustments during image processing. The biomass concentration for the end-of-induction samples are shown in the corresponding lanes. (A) Two intense bands are visible at 18 and 36 kDa, which are attributed to VEGF monomer and dimer. The visible dimer bands in non-induced cultures can be attributed to the fact that the samples were taken 10 min after induction. (B) Two intense bands are visible at 18 and 36 kDa, which are attributed to VEGF monomer and dimer. No bands are visible under non-inducing conditions indicating a tight promoter. (C) Monomer bands are visible in the soluble fraction at high temperature and high growth rate. (D) No bands are visible in the soluble fraction at low growth rate, which might be attributable to image processing.

Extracellular cell content was also investigated by western blotting. In all of the 8 investigated fermentations, extracellular product was observed (Fig. 11). However, the bands where slightly

shifted to a higher molecular weight with respect to the standard, indicating an incomplete cleavage of the signal sequence.

Higher specific growth rates lead to higher volumetric titers of extracellular product. For the same reasons described previously this observation is not related to a higher biomass concentration, indicating that the specific titer (per biomass) is increased as well. These findings are in line with other studies where lysis independent leakage to the culture medium has been reported (Marco 2009). Besides substrate limitation in fed-batch conditions the specific growth rate is reported to influence outer membrane composition leading to periplasmic leakage (Bäcklund et al. 2008; Shokri and Larsson 2004; Shokri et al. 2002; Shokri et al. 2003). Other factors as the aeration rate are also reported to impact periplasmic leakage (Ukkonen et al. 2013).

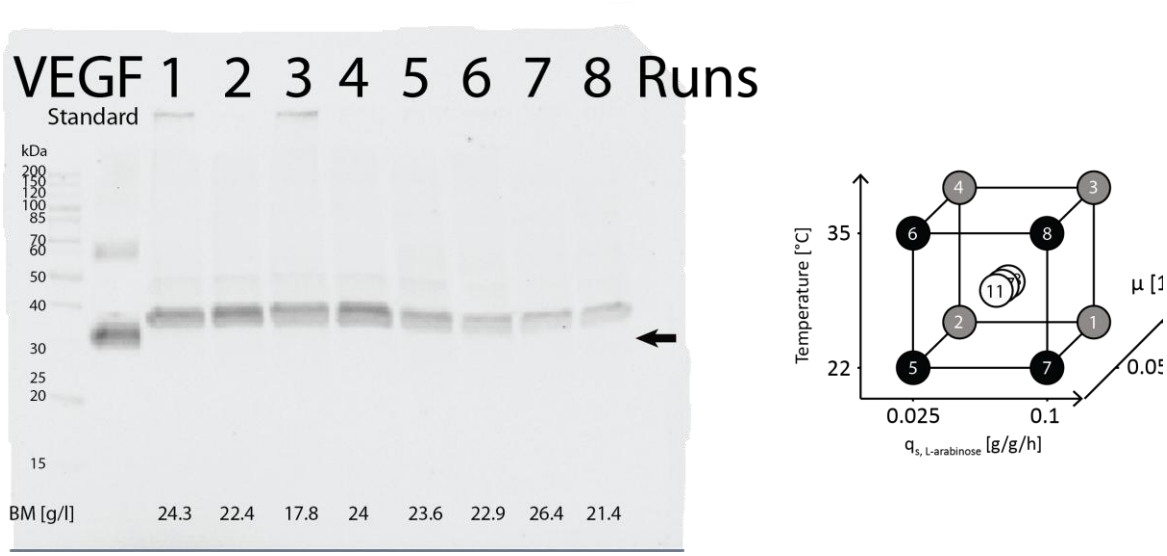

Fig. 11: Westernblot of the extracellular content of fermentation runs 1 to 8. The standard was loaded in the second lane on the left hand side and the height of the standard dimer band at 36 kDa is marked with a black arrow. In each lane a sample at the end of the induction phase was loaded. Extracellular product was observed in each of the 8 runs. The shift of the bands to higher molecular weights with respect to the standard might be attributable to a missing cleavage of signal sequence. At higher growth rate more intense dimer bands were observed (fermentations 1 to 4) indicating a higher volumetric extracellular product concentration.

22.3 Semi-quantitative analysis

For the quantification of VEGF no quantitative HPLC method was available and the development of an analytical method was not in the scope of this thesis. In order to get semi-quantitative data on product titer, western blots of all end-of-fermentation samples were performed and measured densidometrically. Westernblots and corresponding calibration curves are shown in Fig. 13 and Fig. 14 (26 Supplements). The calculated specific titers are summarized in Table 9. In order to determine western blot reproducibility, one sample was applied on every western blot and served as quality benchmark. The corresponding specific titers, which were expected to yield similar results, differed significantly (Fig. 12). With a coefficient of variation of 116 % the method was not reproducible and the calculated specific titers could therefore not be used for data evaluation of the performed DOEs. In addition to the 4 shown plots another 4 plots were performed, yielding no reproducible results (data not shown).

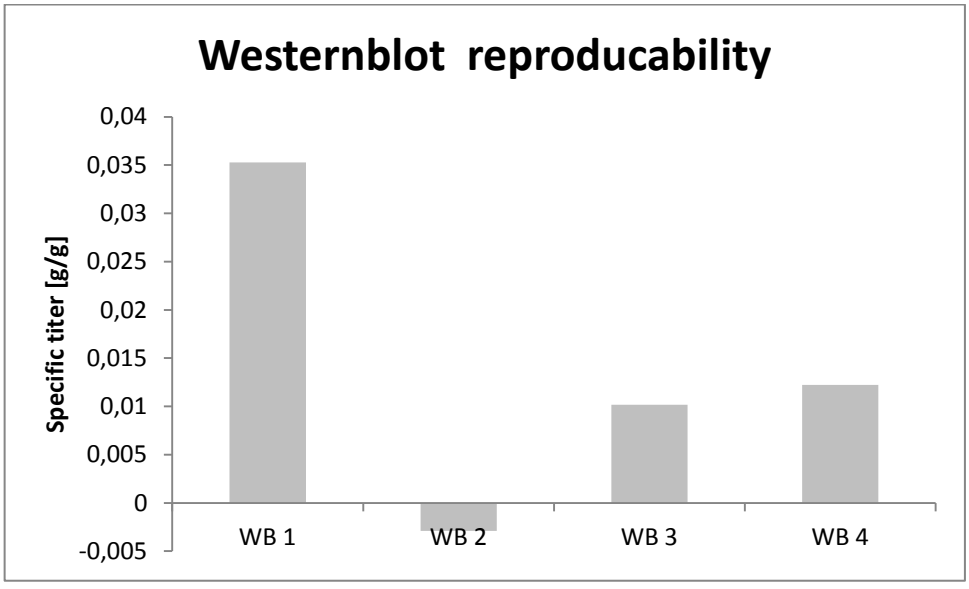

Fig. 12: The same sample was applied on each westernblot in order to determine the reproducibility of the method. The calculated mean is 0.014 with a corresponding CV of 116 %.

23 Conclusions

Within this study, the applicability of a tunable promoter system for process development of a medical highly relevant product was investigated. The pBAD mixed feed strategy (Sagmeister et al. 2013c) was used to produce the disulfide containing VEGF-A_{165} in *E. coli* periplasm. Three critical process parameters (μ, $q_{s,L\text{-arabinose}}$ and temperature) possibly affecting product quality (solubility and activity) and quantity were investigated for two signal sequences in factorial designs (2^3 and 2^2) . In addition to that two signal sequences (GIII and DsbA), each mediating protein translocation either post- or co-translational, should have been compared.

Due to the lack of a suitable quantification method multivariate data analysis for the fractional design could not be performed. A qualitative analysis could only be performed for the corner points of the factorial design for the GIII sequence. Here, interesting trends were observed.

- Product location

  Based on western blot analytics it was concluded that the product is located extracellular and intracellular in the periplasm and cytoplasm.

- Product state

  Based on western blot analysis, some soluble monomer was detected. However, the product was mostly found as monomers aggregated as inclusion bodies within the cytoplasm. In the periplasm, only insoluble dimer was found, probably assembled as inclusion bodies. Soluble dimer was found extracellular (Table 9).

Table 8: Product state within the different compartments.

		Cytoplasm	Periplasm	Extracellular
soluble	**monomer**	**X**	**-**	**-**
	dimer	**-**	**-**	**X**
insoluble	**monomer**	**X**	**-**	**-**
	dimer	**-**	**X**	**-**

- Influence of process parameters
    - Higher product titers were observed at higher temperatures and higher inducer uptake rates
    - Soluble monomer in the cytoplasm was observed at high temperatures and high growth rates.
    - More extracellular product was observed at higher growth rates

23.1 Limitations of the current work and recommendations for further improvement

Although the work was carried out to the best of knowledge, this does not relieve it from experimental limitations that offer space for improvement.

- Product analytics

Due to the fact that a solid product quantification method was missing the performed DOEs could not be evaluated as originally intended. As an alternative it was tried to quantify the product form western blots. A beforehand established quantification method for product monomer and dimer should have been the standard approach, but analytical method development was not in the scope of this thesis.

In addition to that a separated processing of cytoplasm and periplasm would have been a major benefit as well. Several methods for cytoplasm and periplasm separation do exist (Boock et al. 2015; Derman et al. 1993a; Derman et al. 1993b). However this approach was not desired by the industrial partner.

- Feed forward control strategy

As described in 22.1.4 a feed forward control strategy was applied. The applied control strategy demands several assumptions: i) the substrate concentration in the fermentation broth has to be constant ($dc_s/dt = 0$). ii) The substrate concentration inside the reactor is negligibly small compared to the substrate concentration in the feed. iii) The biomass yield ($Y_{x/s}$) remains constant. Despite its easy applicability the applied control strategy comes with two critical key steps: i) the estimation of the initial biomass and ii) the determination of the

correct biomass yield. According to Ehgartner et al for the experiments in this thesis a biomass yield of 0.5 g/g during non-induced fed-batch and 0.4 g/g during induced fed-batch was used. Recombinant protein production requires energy and therefore negatively affects cell growth and consequently $Y_{x/s}$ (Heyland et al. 2011). Using the same strain Ehgartner et al observed a biomass of 0.4 g/g under induced conditions, but produced a different product, bone morphogenetic protein 2 (BMP-2). For this reason the observed value may not apply for the used host in this thesis and an alternative control strategy might have been an improvement. The use of a soft-sensor based on off-gas signals and mass balances for biomass estimation does not need the assumption of a fixed biomass yield. A control strategy based on this kind of soft sensor therefore offers the advantage to adapt the feeding rate to different metabolic states

24 Novelty

For the first time it was tried to apply transcription tuning in the development of an upstream production process of a medical relevant protein. Following a QbD approach the specific inducer uptake rate controlling the transcription rate was identified as a critical process parameter and incorporated as factor in a design of experiments. This was made possible by using the *E. coli* pBAD mixed feed platform.

Furthermore it was investigated whether expression tuning can facilitate production of a rather complex and disulfide bridges containing protein in soluble and active form in the periplasm of *E coli*. This should be achieved by a combination of 2 factorial designs each itself investigating a different translocation pathway to the periplasm and both comparing the two pathways among themselves. Unfortunately the outcome of this study could not fully be evaluated due to the lack of a suitable analytical quantification method.

25 Abbreviations

μ	Specific growth rate [1/h]
BM	Biomass [g/l]
c_{ara}	L-arabinose concentration [g/l]
c_{gluc}	D-glucose concentration [g/l]
c_{total}	Concentration of total carbon source [g/l]
DOE	Design of experiments
OD600	Optical density at 600 nm
$q_{\_sara}$	Specific uptake rate of L-arabinose [g/g/h]
$q_{\_sgluc}$	Specific uptake rate of D-glucose [g/g/h]
q_{s_total}	Specific uptake rate of total carbon source [g/g/h]
SNR	Signal-to-noise ratio
T	Temperature [°C]
V_0	Volume at the end of batch phase [l]
VEGF	Vascular endothelial growth factor
x_0	Biomass at the end of batch phase [g/l]
$Y_{x/s}$	Yield [g biomass/ g substrate]

26 Supplements

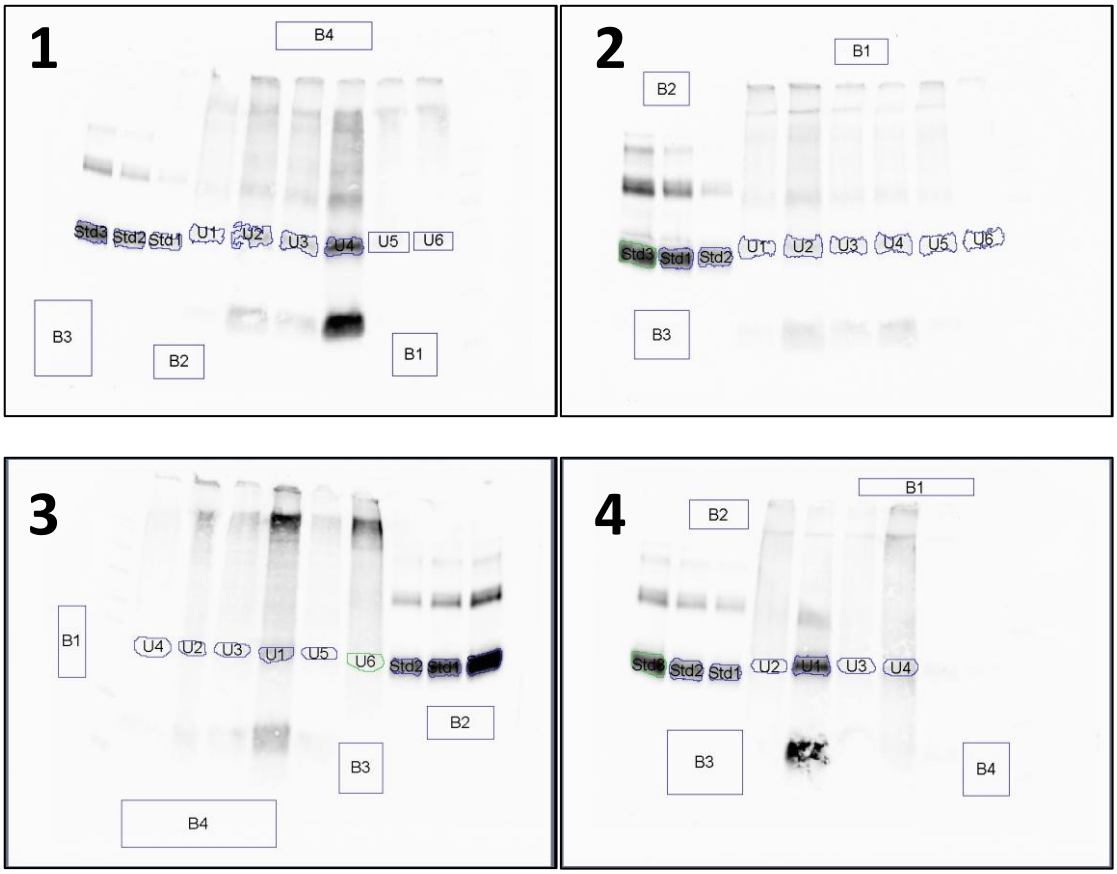

Fig. 13: Western blots for quantitave analysis. The corresponding calibration curves are shown in Fig. 14 and the identifiers are explained in Table 4.

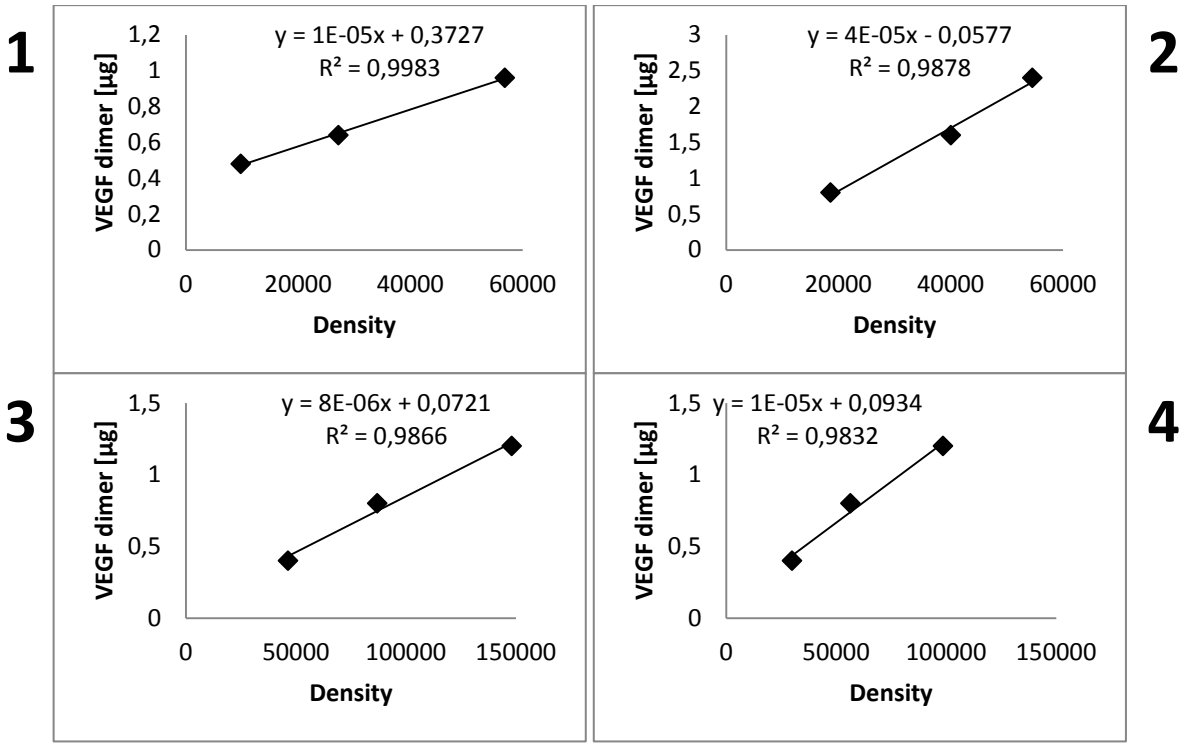

Fig. 14: Calibration curves of quantitative western blot analysis.

Table 9: Results of quantitative western blot analysis and identifiers to assign the measured samples to the bands in the western blots.

Fermentation	μ [1/h]	q_{ara} [g/g/h]	T [°C]	Specific Titer [g/g]	WB	Identifier
1	0,15	0,1	22	0,0023	1	U1
2	0,15	0,025	22	0,0035	1	U2
3	0,15	0,1	35	0,0484	1	U3
4	0,15	0,025	35	0,0744	1	U4
5	0,05	0,025	22	0,0141	1	U5
11	0,1	0,0625	28,5	0,0353	1	U6
6	0,05	0,025	35	0,0101	2	U1
7	0,05	0,1	22	0,0162	2	U2
8	0,05	0,1	35	0,0101	2	U3
17	0,1	0,0625	28,5	0,0075	2	U4
18	0,1	0,0625	28,5	0,0019	2	U5
11	0,1	0,0625	28,5	-0,0029	2	U6
9	0,05	0,1	35	0,0039	3	U4
10	0,05	0,025	22	0,0008	3	U2
12	0,05	0,025	35	0,0044	3	U3
13	0,05	0,1	22	0,0010	3	U1
14	0,05	0,065	28,5	0,0048	3	U5
11	0,1	0,0625	28,5	0,0102	3	U6
15	0,05	0,065	28,5	-	4	-
16	0,05	0,065	28,5	-	4	-
8	0,05	0,1	35	0,0377	4	U4
17	0,1	0,0625	28,5	0,0100	4	U3
18	0,1	0,0625	28,5	0,0757	4	U1
11	0,1	0,0625	28,5	0,0122	4	U2

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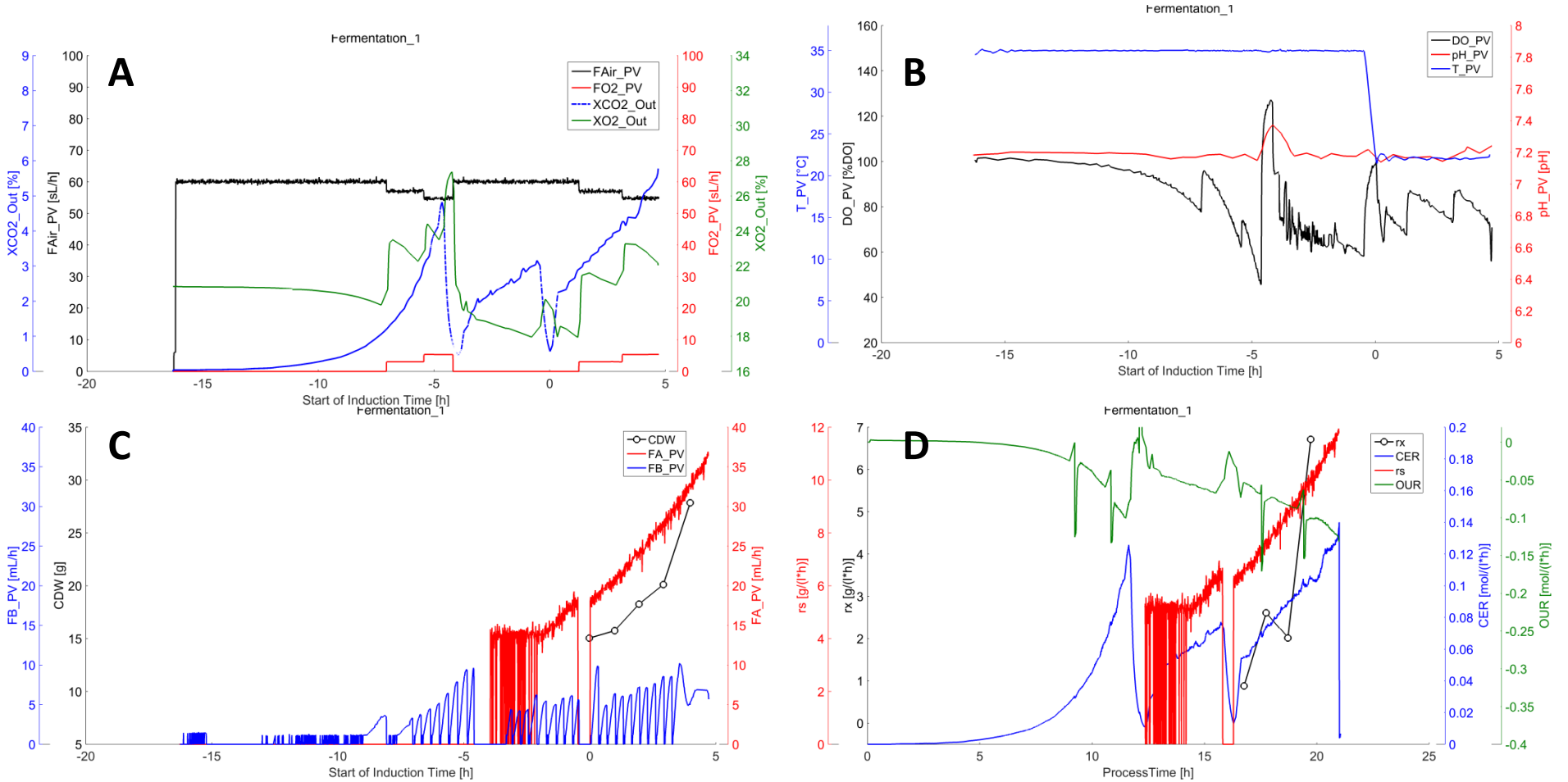

Fig. 15: Fermentation 1. (A) Air (black, Fair_PV) and oxygen (green, FO2_PV) inflow to the reactor and CO_2 (blue, XCO2_Out) and O_2 (green, XO2_Out) content in the off gas are shown in the graph at the top left corner. (B) Process parameters like temperature (blue, T_PV), pH (red, pH_PV) and dissolved oxygen (black, DO_PV) are plotted in the graph at the top right. (C) Liquid feeds like substrate feed (red, FA_PV) and base feed (blue, FB_PV) are plotted along with the cell dry weight (black, CDW) in the graph at the bottom left corner. (D) Calculated volumetric rates are shown in the graph at the bottom right corner. Volumetric growth rate (black, rx), volumetric substrate uptake rate (red, rs), volumetric carbon evolution rate (blue, CER) and volumetric oxygen uptake rate (green, OUR). All variables are plotted against the induction time. Hour represents the time of induction.

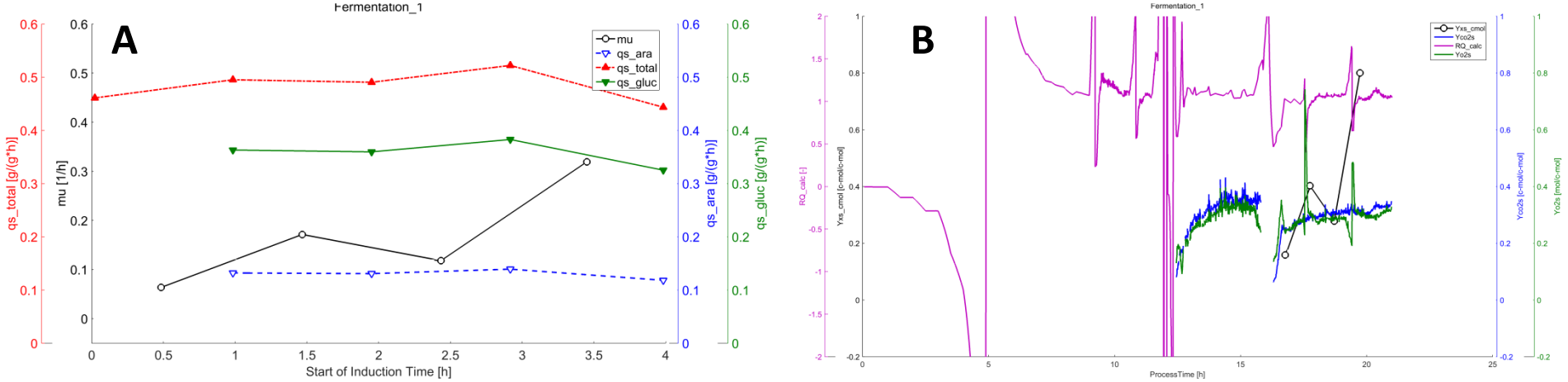

Fig. 16: Fermentation 1. (A) Specific conversion rates are shown in the graph on the left-hand side: specific growth rate (black, mu), specific total substrate rate (red, qs_total), specific arabinose uptake rate (blue, qs_ara) and specific glucose uptake rate (green, qs_gluc). (B) Yields on total substrate are displayed in the graph on the right-hand side: biomass yield (black, Yxs_cmol), CO_2 yield (blue, Yco2s), O_2 yield (green, Yo2s) and the respiratory coefficient (magenta, RQ_calc). All variables are plotted against the induction time. Hour represents the time of induction.

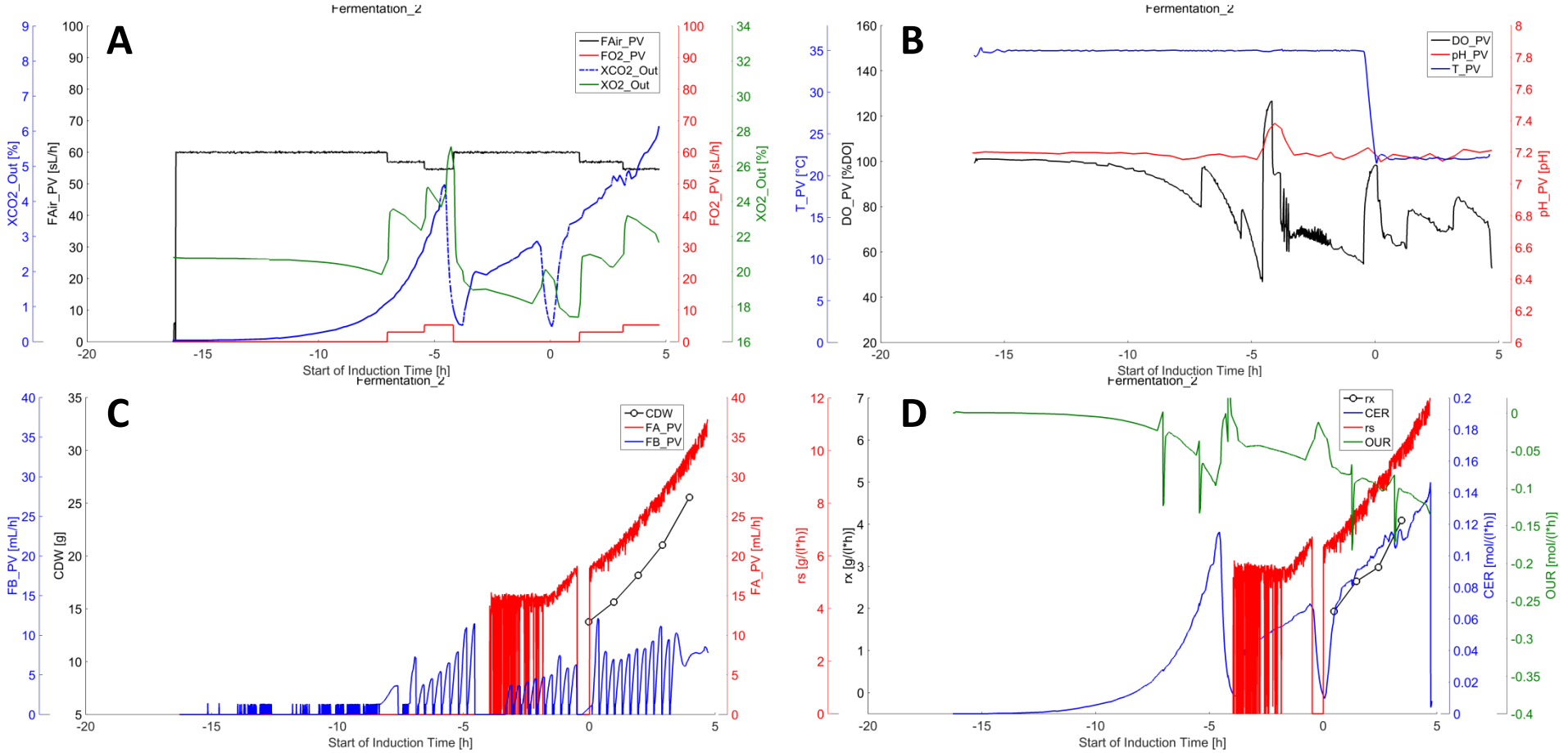

Fig. 17: Fermentation 2. (A) Air (black, Fair_PV) and oxygen (green, FO2_PV) inflow to the reactor and CO_2 (blue, XCO2_Out) and O_2 (green, XO2_Out) content in the off gas are shown in the graph at the top left corner. (B) Process parameters like temperature (blue, T_PV), pH (red, pH_PV) and dissolved oxygen (black, DO_PV) are plotted in the graph at the top right. (C) Liquid feeds like substrate feed (red, FA_PV) and base feed (blue, FB_PV) are plotted along with the cell dry weight (black, CDW) in the graph at the bottom left corner. (D) Calculated volumetric rates are shown in the graph at the bottom right corner. Volumetric growth rate (black, rx), volumetric substrate uptake rate (red, rs), volumetric carbon evolution rate (blue, CER) and volumetric oxygen uptake rate (green, OUR). All variables are plotted against the induction time. Hour represents the time of induction.

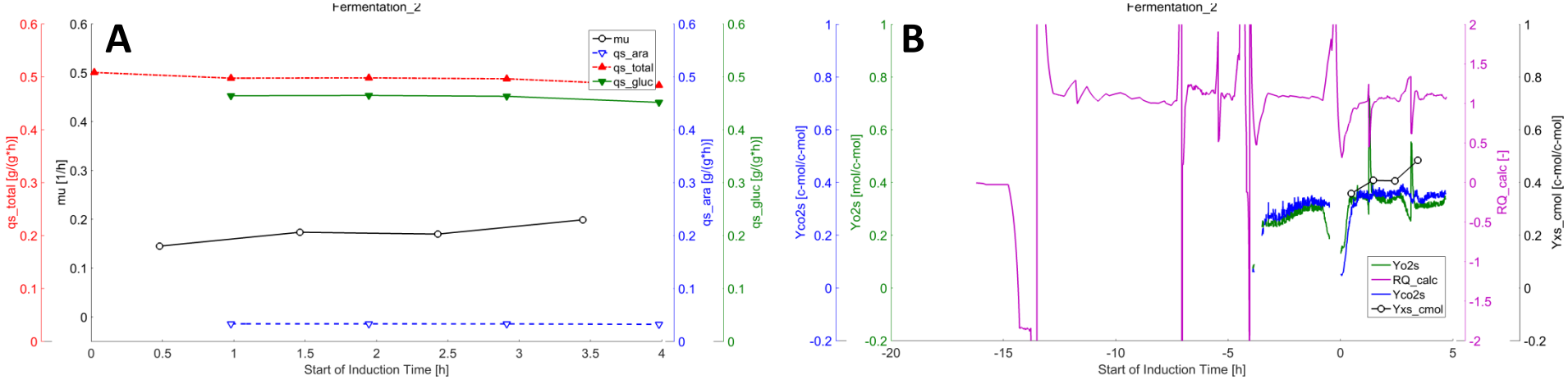

Fig. 18: Fermentation 2. (A) Specific conversion rates are shown in the graph on the left-hand side: specific growth rate (black, mu), specific total substrate rate (red, qs_total), specific arabinose uptake rate (blue, qs_ara) and specific glucose uptake rate (green, qs_gluc). (B) Yields on total substrate are displayed in the graph on the right-hand side: biomass yield (black, Yxs_cmol), CO_2 yield (blue, Yco2s), O_2 yield (green, Yo2s) and the respiratory coefficient (magenta, RQ_calc). All variables are plotted against the induction time. Hour represents the time of induction.

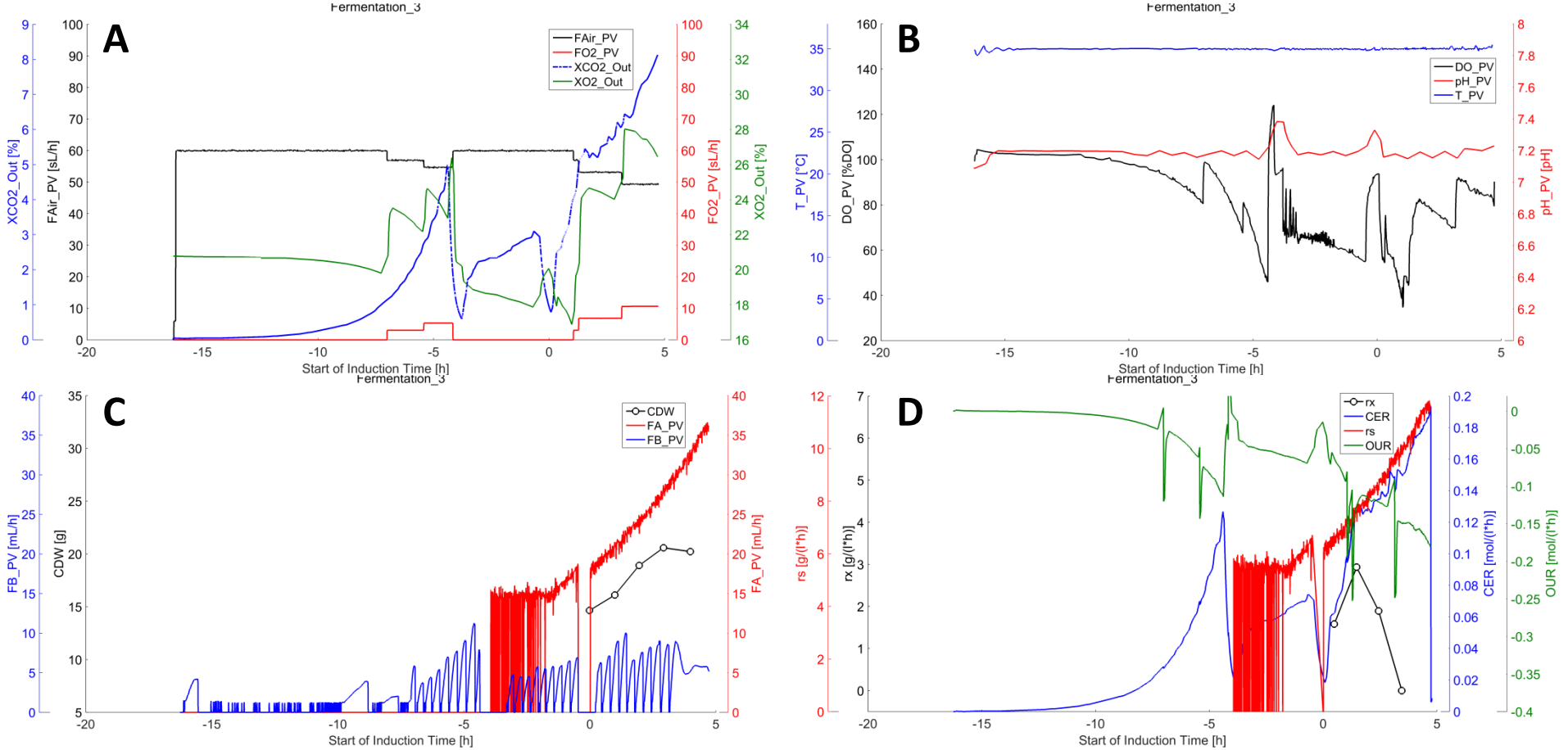

Fig. 19: Fermentation 3. (A) Air (black, Fair_PV) and oxygen (green, FO2_PV) inflow to the reactor and CO_2 (blue, XCO2_Out) and O_2 (green, XO2_Out) content in the off gas are shown in the graph at the top left corner. (B) Process parameters like temperature (blue, T_PV), pH (red, pH_PV) and dissolved oxygen (black, DO_PV) are plotted in the graph at the top right. (C) Liquid feeds like substrate feed (red, FA_PV) and base feed (blue, FB_PV) are plotted along with the cell dry weight (black, CDW) in the graph at the bottom left corner. (D) Calculated volumetric rates are shown in the graph at the bottom right corner. Volumetric growth rate (black, rx), volumetric substrate uptake rate (red, rs), volumetric carbon evolution rate (blue, CER) and volumetric oxygen uptake rate (green, OUR). All variables are plotted against the induction time. Hour represents the time of induction.

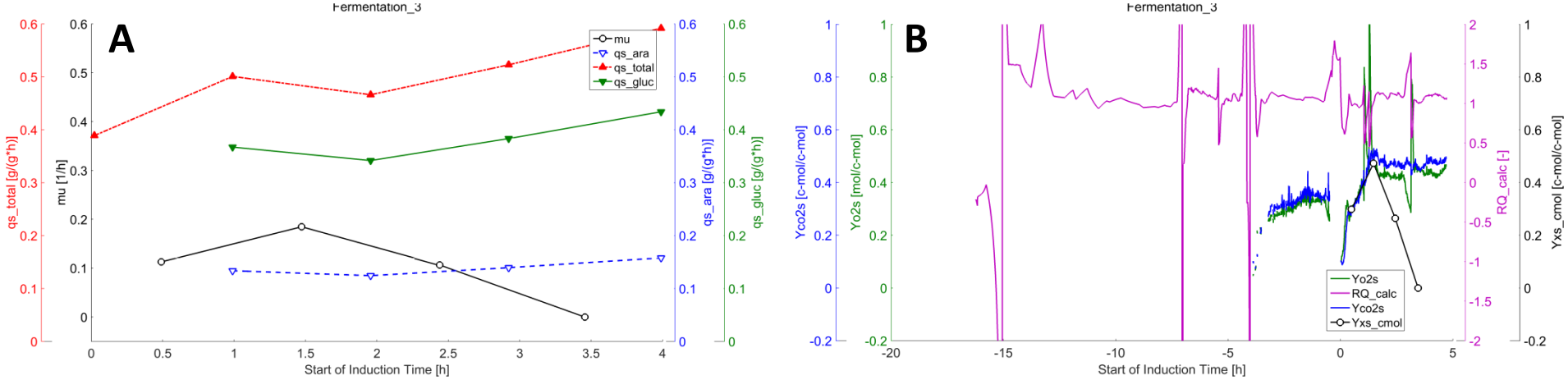

Fig. 20: Fermentation 3. (A) Specific conversion rates are shown in the graph on the left-hand side: specific growth rate (black, mu), specific total substrate rate (red, qs_total), specific arabinose uptake rate (blue, qs_ara) and specific glucose uptake rate (green, qs_gluc). (B) Yields on total substrate are displayed in the graph on the right-hand side: biomass yield (black, Yxs_cmol), CO_2 yield (blue, Yco2s), O_2 yield (green, Yo2s) and the respiratory coefficient (magenta, RQ_calc). All variables are plotted against the induction time. Hour represents the time of induction.

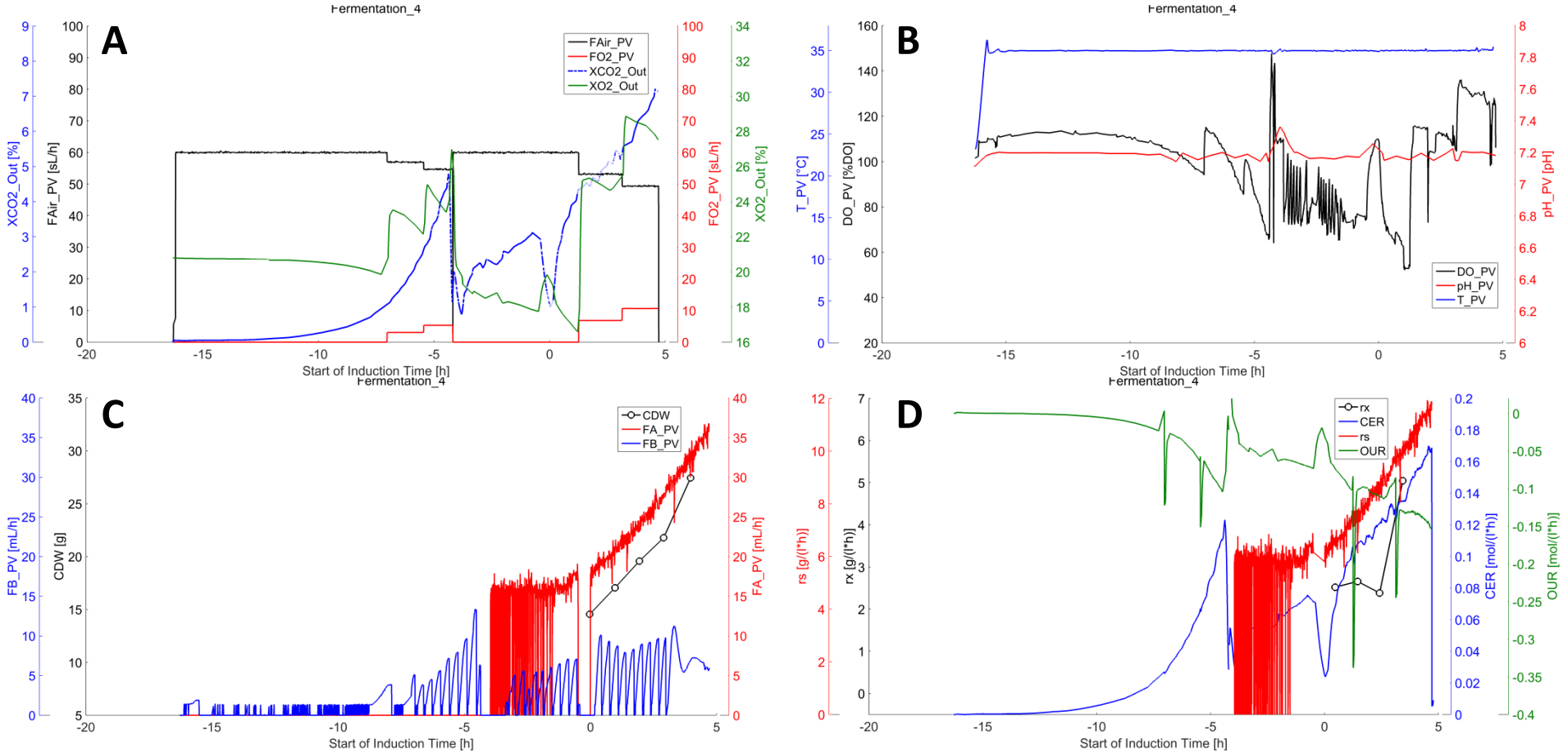

Fig. 21: Fermentation 4. (A) Air (black, Fair_PV) and oxygen (green, FO2_PV) inflow to the reactor and CO_2 (blue, XCO2_Out) and O_2 (green, XO2_Out) content in the off gas are shown in the graph at the top left corner. (B) Process parameters like temperature (blue, T_PV), pH (red, pH_PV) and dissolved oxygen (black, DO_PV) are plotted in the graph at the top right. (C) Liquid feeds like substrate feed (red, FA_PV) and base feed (blue, FB_PV) are plotted along with the cell dry weight (black, CDW) in the graph at the bottom left corner. (D) Calculated volumetric rates are shown in the graph at the bottom right corner. Volumetric growth rate (black, rx), volumetric substrate uptake rate (red, rs), volumetric carbon evolution rate (blue, CER) and volumetric oxygen uptake rate (green, OUR). All variables are plotted against the induction time. Hour represents the time of induction.

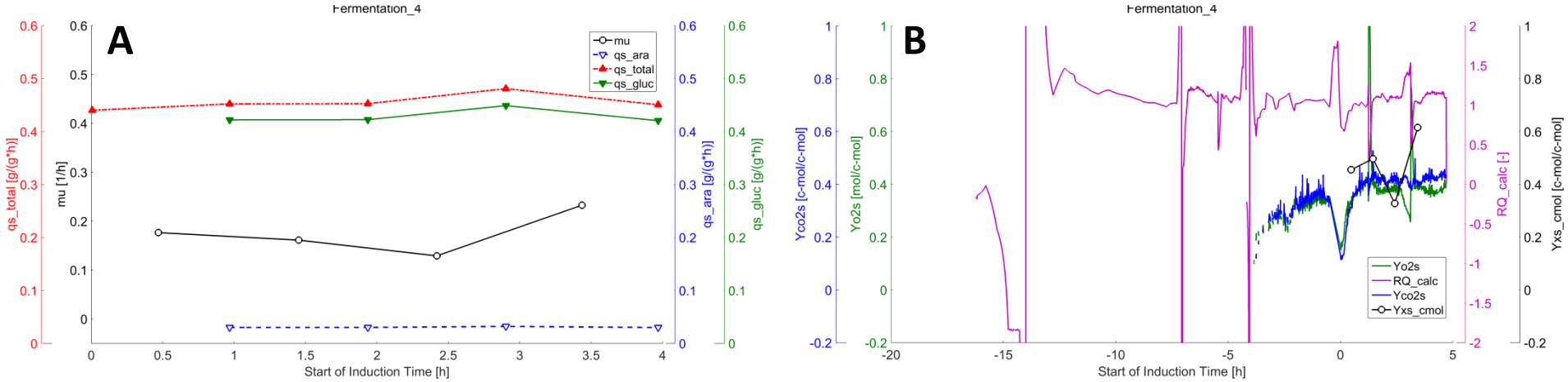

Fig. 22: Fermentation 4. (A) Specific conversion rates are shown in the graph on the left-hand side: specific growth rate (black, mu), specific total substrate rate (red, qs_total), specific arabinose uptake rate (blue, qs_ara) and specific glucose uptake rate (green, qs_gluc). (B) Yields on total substrate are displayed in the graph on the right-hand side: biomass yield (black, Yxs_cmol), CO_2 yield (blue, Yco2s), O_2 yield (green, Yo2s) and the respiratory coefficient (magenta, RQ_calc). All variables are plotted against the induction time. Hour represents the time of induction.

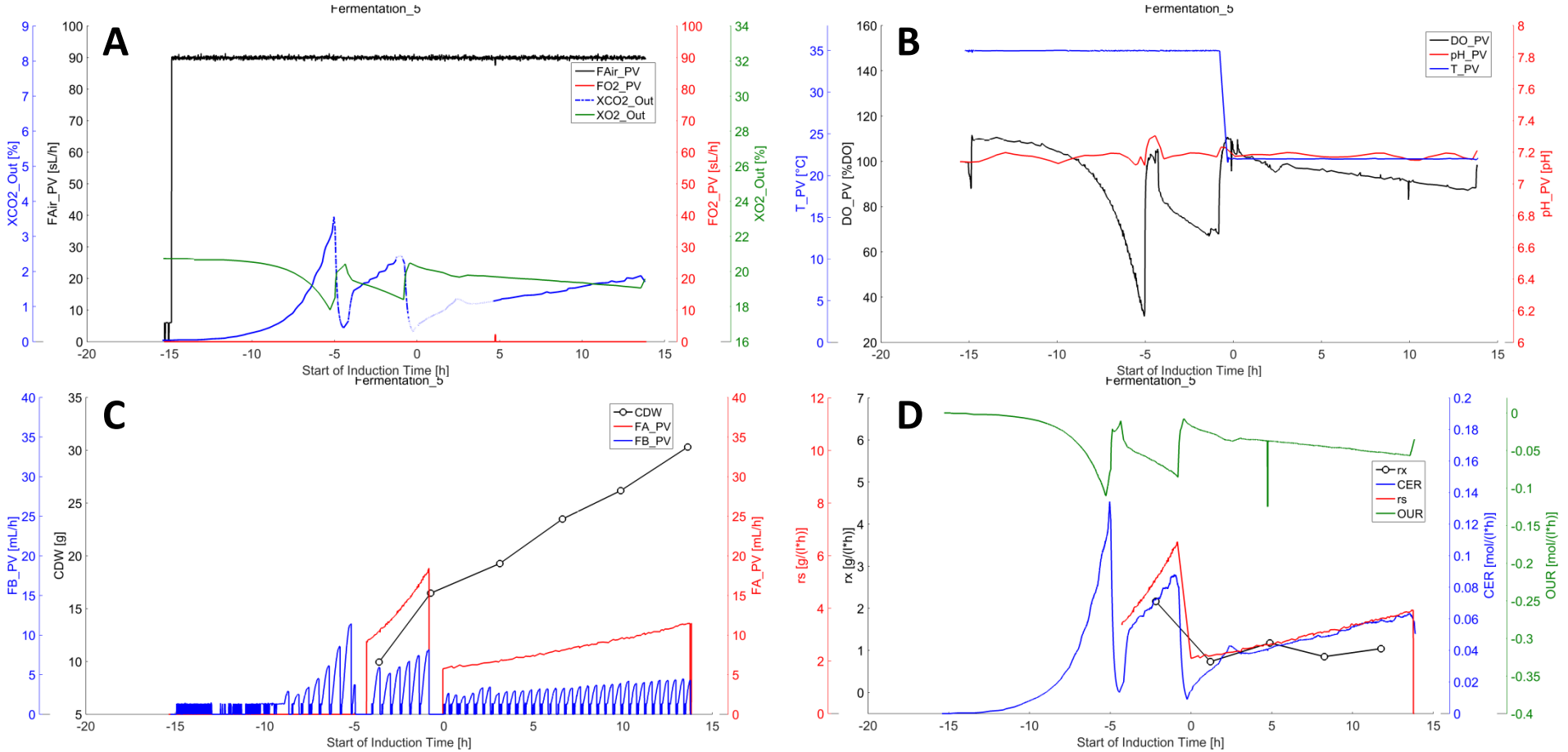

Fig. 23: Fermentation 5. (A) Air (black, Fair_PV) and oxygen (green, FO2_PV) inflow to the reactor and CO_2 (blue, XCO2_Out) and O_2 (green, XO2_Out) content in the off gas are shown in the graph at the top left corner. (B) Process parameters like temperature (blue, T_PV), pH (red, pH_PV) and dissolved oxygen (black, DO_PV) are plotted in the graph at the top right. (C) Liquid feeds like substrate feed (red, FA_PV) and base feed (blue, FB_PV) are plotted along with the cell dry weight (black, CDW) in the graph at the bottom left corner. (D) Calculated volumetric rates are shown in the graph at the bottom right corner. Volumetric growth rate (black, rx), volumetric substrate uptake rate (red, rs), volumetric carbon evolution rate (blue, CER) and volumetric oxygen uptake rate (green, OUR). All variables are plotted against the induction time. Hour represents the time of induction.

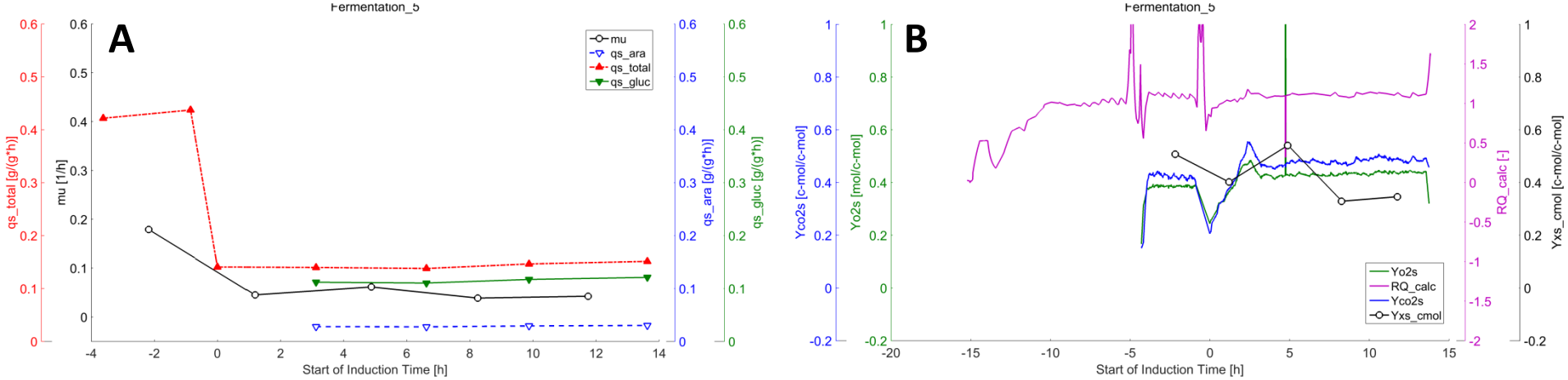

Fig. 24: Fermentation 5. (A) Specific conversion rates are shown in the graph on the left-hand side: specific growth rate (black, mu), specific total substrate rate (red, qs_total), specific arabinose uptake rate (blue, qs_ara) and specific glucose uptake rate (green, qs_gluc). (B) Yields on total substrate are displayed in the graph on the right-hand side: biomass yield (black, Yxs_cmol), CO_2 yield (blue, Yco2s), O_2 yield (green, Yo2s) and the respiratory coefficient (magenta, RQ_calc). All variables are plotted against the induction time. Hour represents the time of induction.

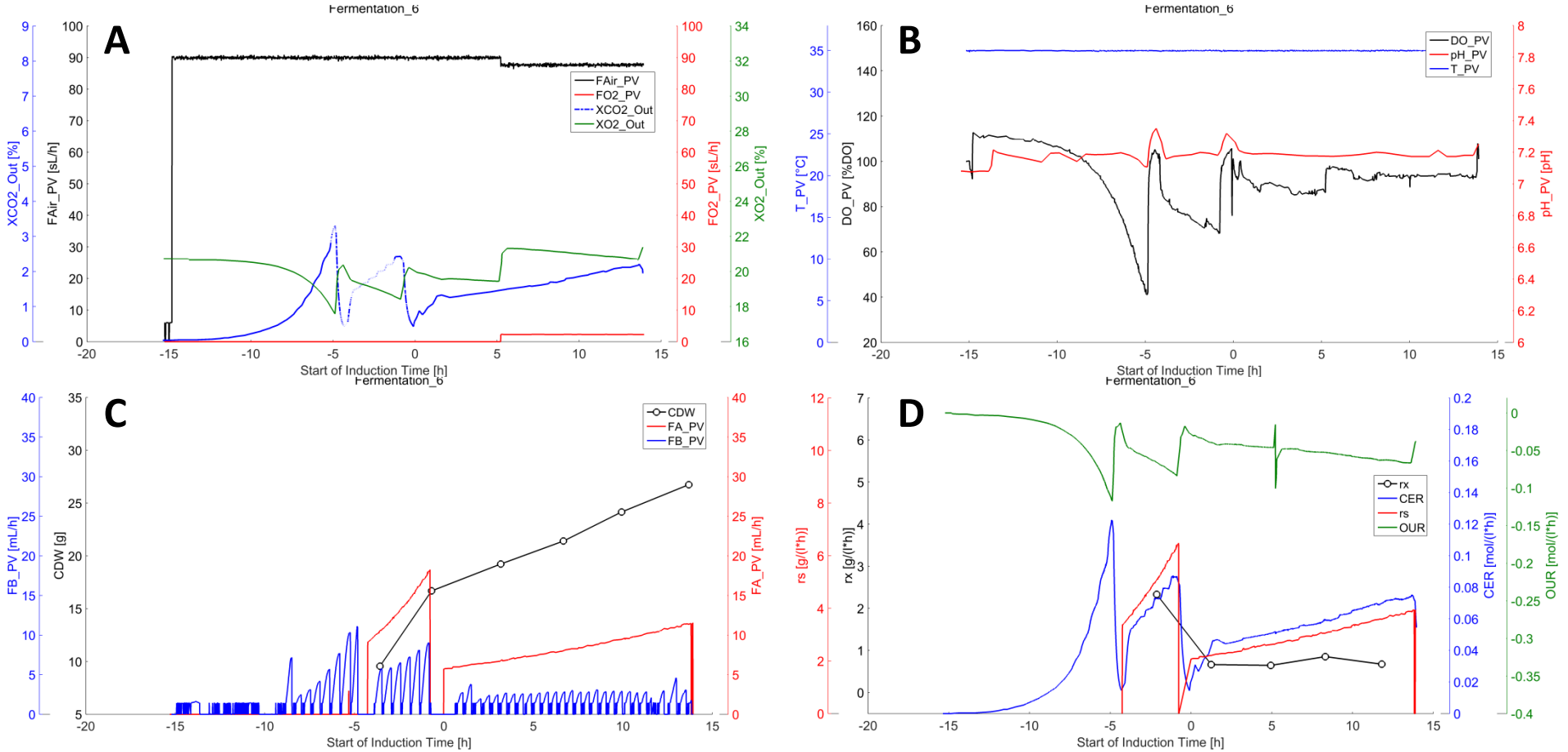

Fig. 25: Fermentation 6. (A) Air (black, Fair_PV) and oxygen (green, FO2_PV) inflow to the reactor and CO_2 (blue, XCO2_Out) and O_2 (green, XO2_Out) content in the off gas are shown in the graph at the top left corner. (B) Process parameters like temperature (blue, T_PV), pH (red, pH_PV) and dissolved oxygen (black, DO_PV) are plotted in the graph at the top right. (C) Liquid feeds like substrate feed (red, FA_PV) and base feed (blue, FB_PV) are plotted along with the cell dry weight (black, CDW) in the graph at the bottom left corner. (D) Calculated volumetric rates are shown in the graph at the bottom right corner. Volumetric growth rate (black, rx), volumetric substrate uptake rate (red, rs), volumetric carbon evolution rate (blue, CER) and volumetric oxygen uptake rate (green, OUR). All variables are plotted against the induction time. Hour represents the time of induction.

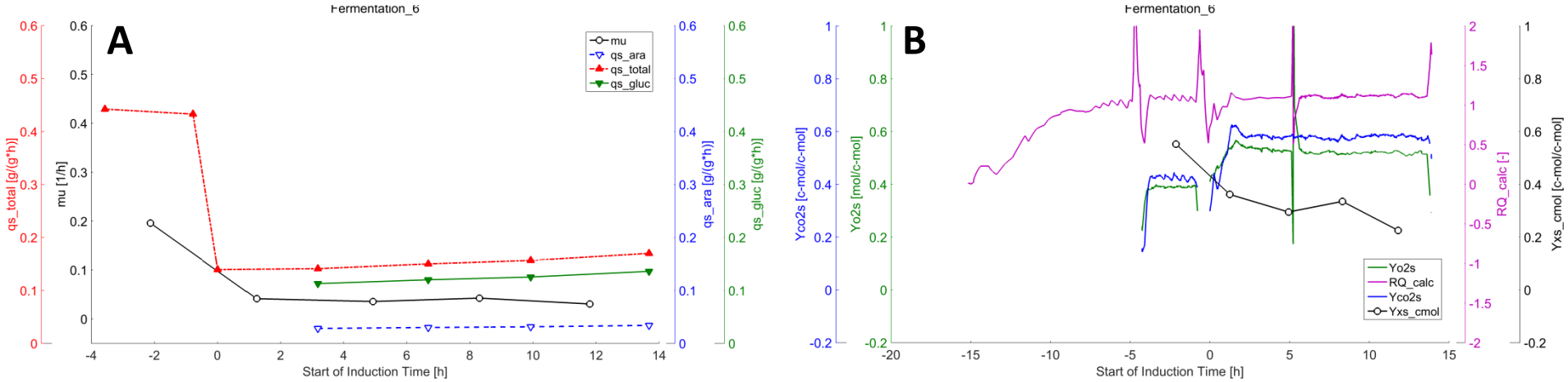

Fig. 26: Fermentation 6. (A) Specific conversion rates are shown in the graph on the left-hand side: specific growth rate (black, mu), specific total substrate rate (red, qs_total), specific arabinose uptake rate (blue, qs_ara) and specific glucose uptake rate (green, qs_gluc). (B) Yields on total substrate are displayed in the graph on the right-hand side: biomass yield (black, Yxs_cmol), CO_2 yield (blue, Yco2s), O_2 yield (green, Yo2s) and the respiratory coefficient (magenta, RQ_calc). All variables are plotted against the induction time. Hour represents the time of induction.

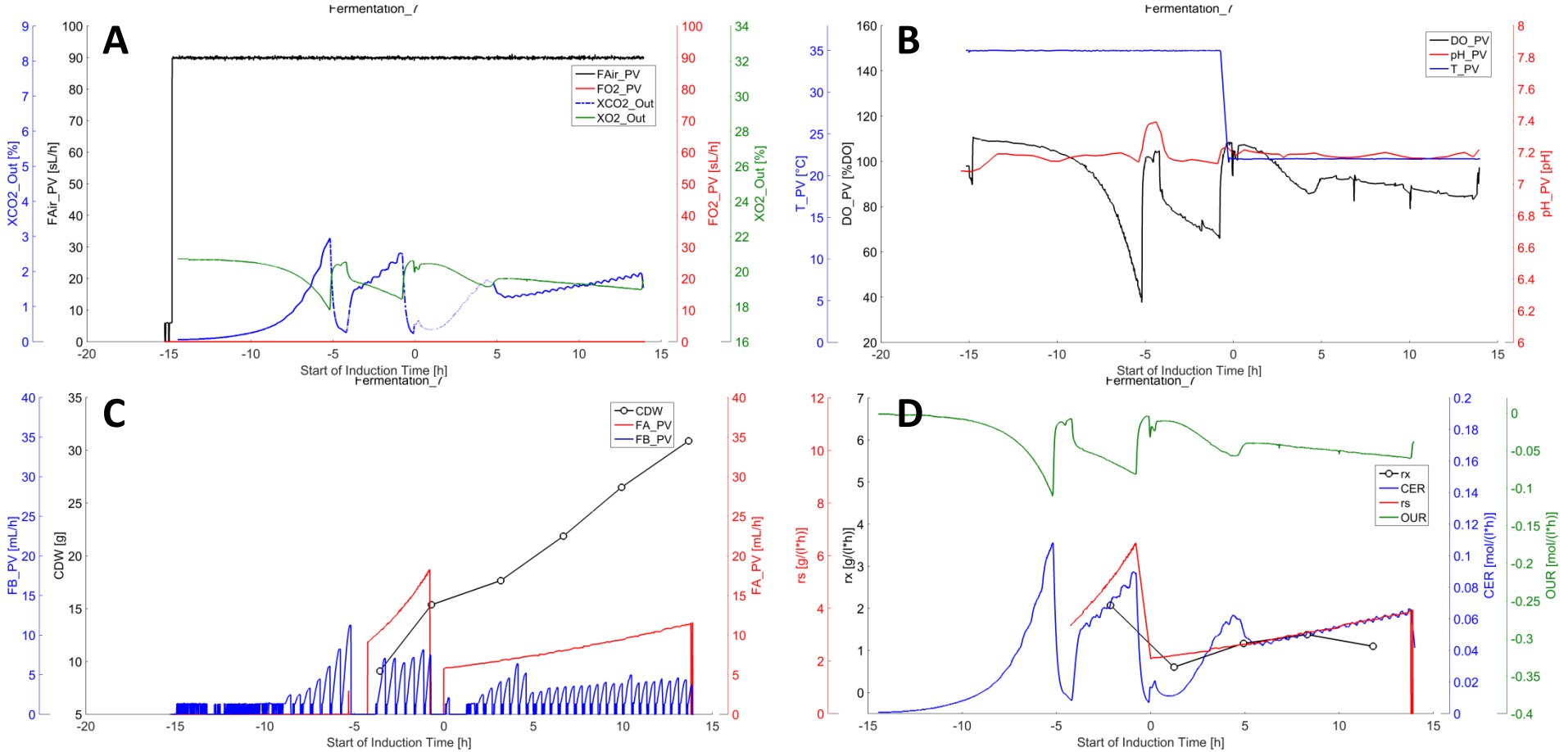

Fig. 27: Fermentation 7. (A) Air (black, Fair_PV) and oxygen (green, FO2_PV) inflow to the reactor and CO_2 (blue, XCO2_Out) and O_2 (green, XO2_Out) content in the off gas are shown in the graph at the top left corner. (B) Process parameters like temperature (blue, T_PV), pH (red, pH_PV) and dissolved oxygen (black, DO_PV) are plotted in the graph at the top right. (C) Liquid feeds like substrate feed (red, FA_PV) and base feed (blue, FB_PV) are plotted along with the cell dry weight (black, CDW) in the graph at the bottom left corner. (D) Calculated volumetric rates are shown in the graph at the bottom right corner. Volumetric growth rate (black, rx), volumetric substrate uptake rate (red, rs), volumetric carbon evolution rate (blue, CER) and volumetric oxygen uptake rate (green, OUR). All variables are plotted against the induction time. Hour represents the time of induction.

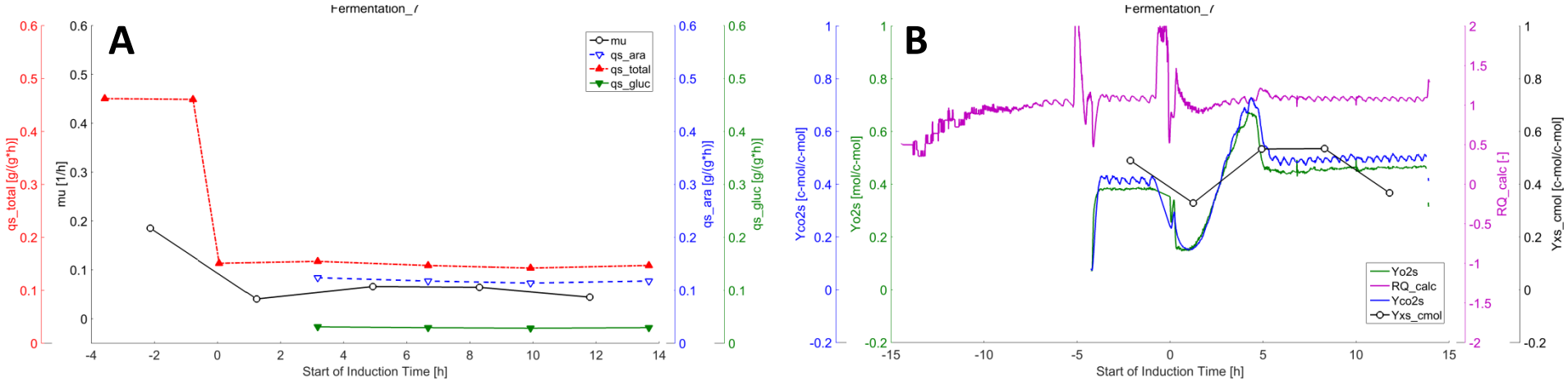

Fig. 28: Fermentation 7. (A) Specific conversion rates are shown in the graph on the left-hand side: specific growth rate (black, mu), specific total substrate rate (red, qs_total), specific arabinose uptake rate (blue, qs_ara) and specific glucose uptake rate (green, qs_gluc). (B) Yields on total substrate are displayed in the graph on the right-hand side: biomass yield (black, Yxs_cmol), CO_2 yield (blue, Yco2s), O_2 yield (green, Yo2s) and the respiratory coefficient (magenta, RQ_calc). All variables are plotted against the induction time. Hour represents the time of induction.

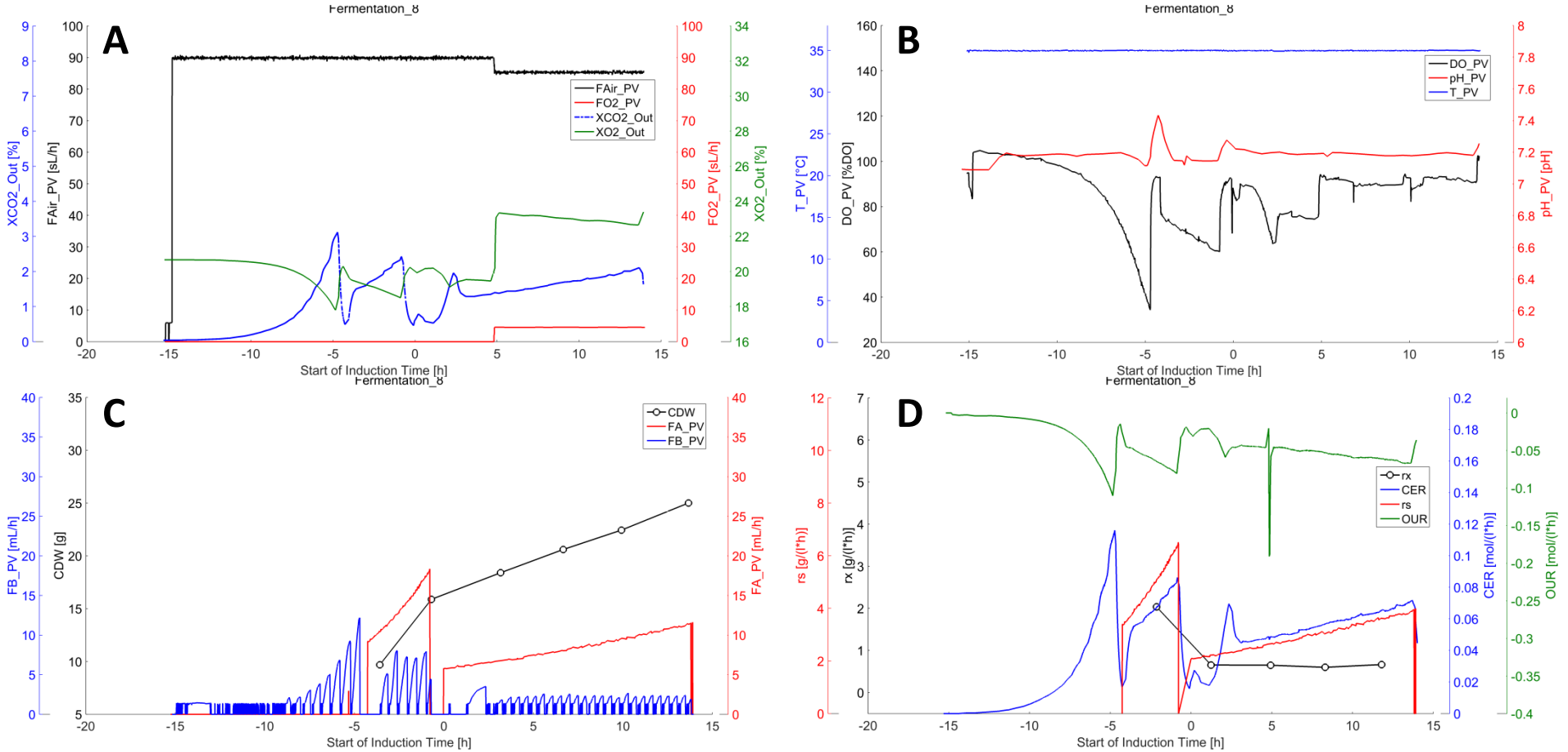

Fig. 29: Fermentation 8. (A) Air (black, Fair_PV) and oxygen (green, FO2_PV) inflow to the reactor and CO_2 (blue, XCO2_Out) and O_2 (green, XO2_Out) content in the off gas are shown in the graph at the top left corner. (B) Process parameters like temperature (blue, T_PV), pH (red, pH_PV) and dissolved oxygen (black, DO_PV) are plotted in the graph at the top right. (C) Liquid feeds like substrate feed (red, FA_PV) and base feed (blue, FB_PV) are plotted along with the cell dry weight (black, CDW) in the graph at the bottom left corner. (D) Calculated volumetric rates are shown in the graph at the bottom right corner. Volumetric growth rate (black, rx), volumetric substrate uptake rate (red, rs), volumetric carbon evolution rate (blue, CER) and volumetric oxygen uptake rate (green, OUR). All variables are plotted against the induction time. Hour represents the time of induction.

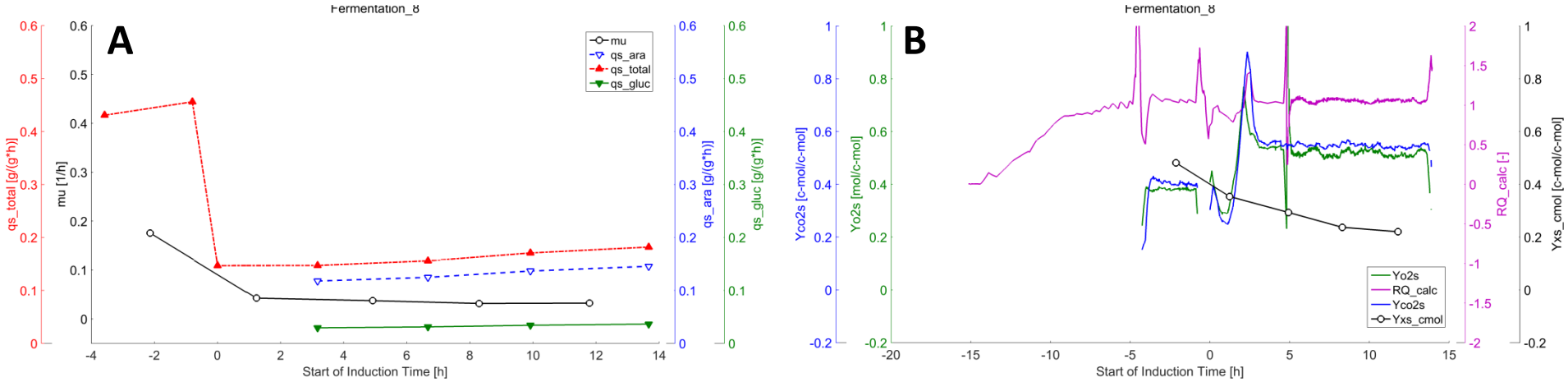

Fig. 30: Fermentation 8. (A) Specific conversion rates are shown in the graph on the left-hand side: specific growth rate (black, mu), specific total substrate rate (red, qs_total), specific arabinose uptake rate (blue, qs_ara) and specific glucose uptake rate (green, qs_gluc). (B) Yields on total substrate are displayed in the graph on the right-hand side: biomass yield (black, Yxs_cmol), CO_2 yield (blue, Yco2s), O_2 yield (green, Yo2s) and the respiratory coefficient (magenta, RQ_calc). All variables are plotted against the induction time. Hour represents the time of induction.

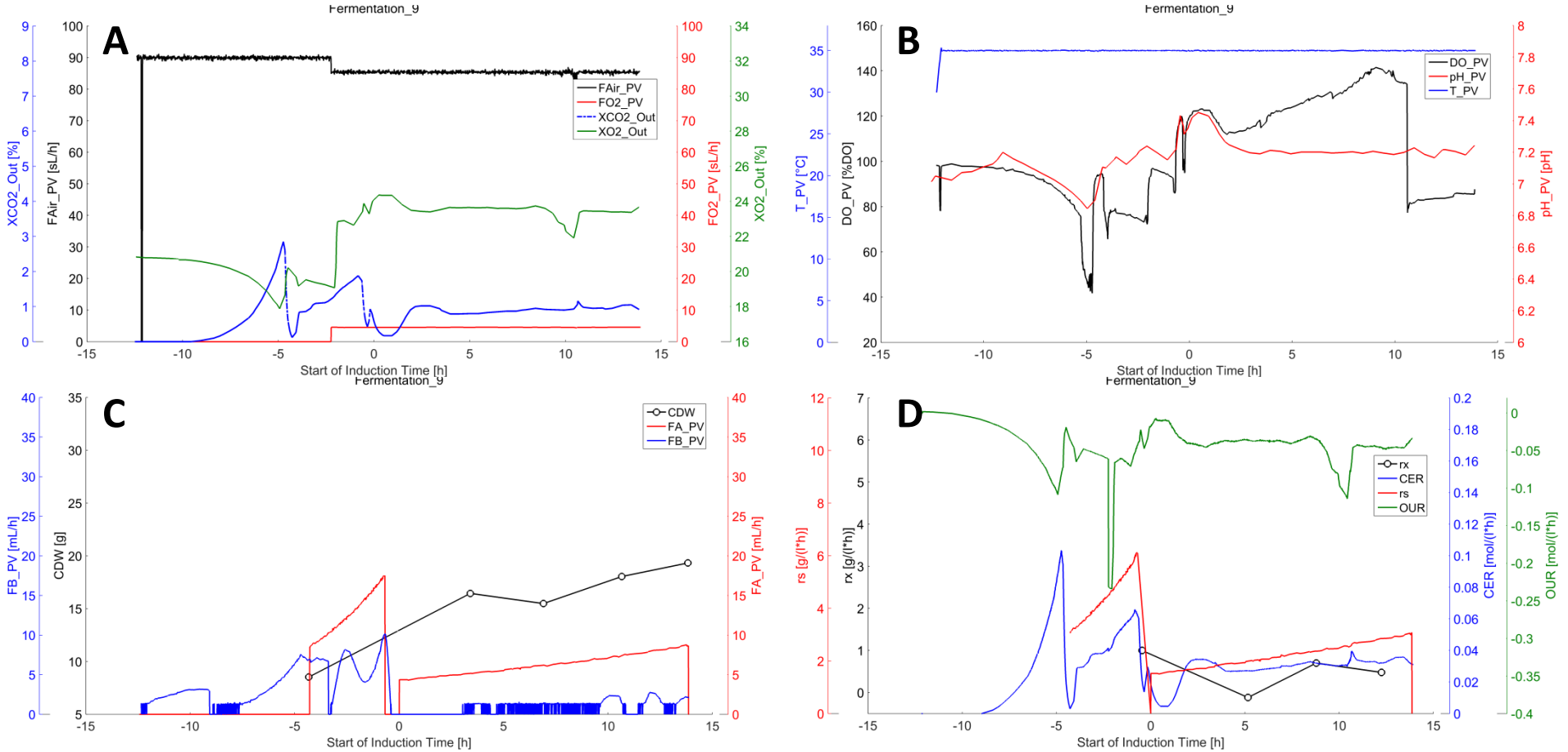

Fig. 31: Fermentation 9. (A) Air (black, Fair_PV) and oxygen (green, FO2_PV) inflow to the reactor and CO_2 (blue, XCO2_Out) and O_2 (green, XO2_Out) content in the off gas are shown in the graph at the top left corner. (B) Process parameters like temperature (blue, T_PV), pH (red, pH_PV) and dissolved oxygen (black, DO_PV) are plotted in the graph at the top right. (C) Liquid feeds like substrate feed (red, FA_PV) and base feed (blue, FB_PV) are plotted along with the cell dry weight (black, CDW) in the graph at the bottom left corner. (D) Calculated volumetric rates are shown in the graph at the bottom right corner. Volumetric growth rate (black, rx), volumetric substrate uptake rate (red, rs), volumetric carbon evolution rate (blue, CER) and volumetric oxygen uptake rate (green, OUR). All variables are plotted against the induction time. Hour represents the time of induction.

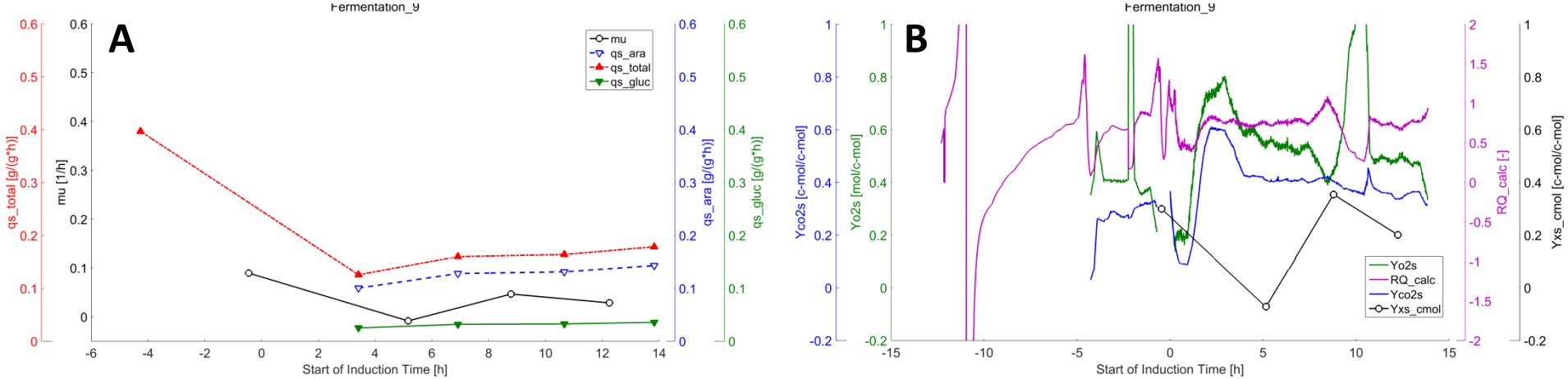

Fig. 32: Fermentation 9. (A) Specific conversion rates are shown in the graph on the left-hand side: specific growth rate (black, mu), specific total substrate rate (red, qs_total), specific arabinose uptake rate (blue, qs_ara) and specific glucose uptake rate (green, qs_gluc). (B) Yields on total substrate are displayed in the graph on the right-hand side: biomass yield (black, Yxs_cmol), CO_2 yield (blue, Yco2s), O_2 yield (green, Yo2s) and the respiratory coefficient (magenta, RQ_calc). All variables are plotted against the induction time. Hour represents the time of induction.

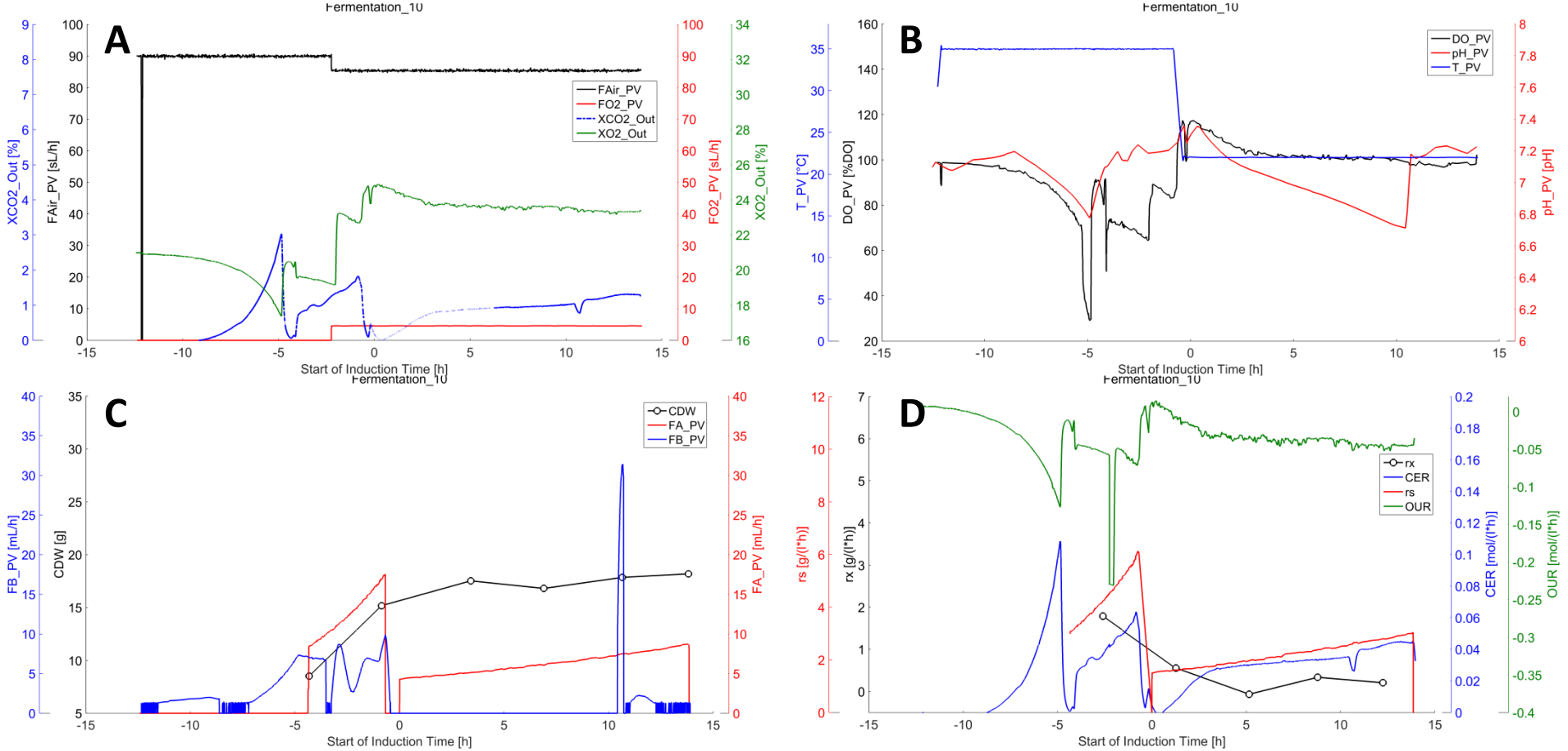

Fig. 33: Fermentation 10. (A) Air (black, Fair_PV) and oxygen (green, FO2_PV) inflow to the reactor and CO_2 (blue, XCO2_Out) and O_2 (green, XO2_Out) content in the off gas are shown in the graph at the top left corner. (B) Process parameters like temperature (blue, T_PV), pH (red, pH_PV) and dissolved oxygen (black, DO_PV) are plotted in the graph at the top right. (C) Liquid feeds like substrate feed (red, FA_PV) and base feed (blue, FB_PV) are plotted along with the cell dry weight (black, CDW) in the graph at the bottom left corner. (D) Calculated volumetric rates are shown in the graph at the bottom right corner. Volumetric growth rate (black, rx), volumetric substrate uptake rate (red, rs), volumetric carbon evolution rate (blue, CER) and volumetric oxygen uptake rate (green, OUR). All variables are plotted against the induction time. Hour represents the time of induction.

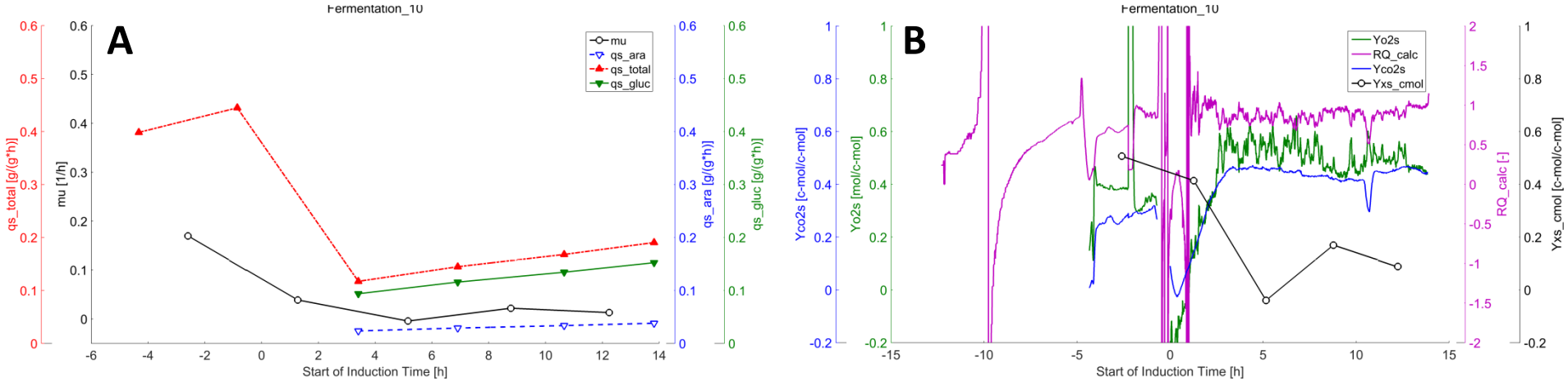

Fig. 34: Fermentation 10. (A) Specific conversion rates are shown in the graph on the left-hand side: specific growth rate (black, mu), specific total substrate rate (red, qs_total), specific arabinose uptake rate (blue, qs_ara) and specific glucose uptake rate (green, qs_gluc). (B) Yields on total substrate are displayed in the graph on the right-hand side: biomass yield (black, Yxs_cmol), CO_2 yield (blue, Yco2s), O_2 yield (green, Yo2s) and the respiratory coefficient (magenta, RQ_calc). All variables are plotted against the induction time. Hour represents the time of induction.

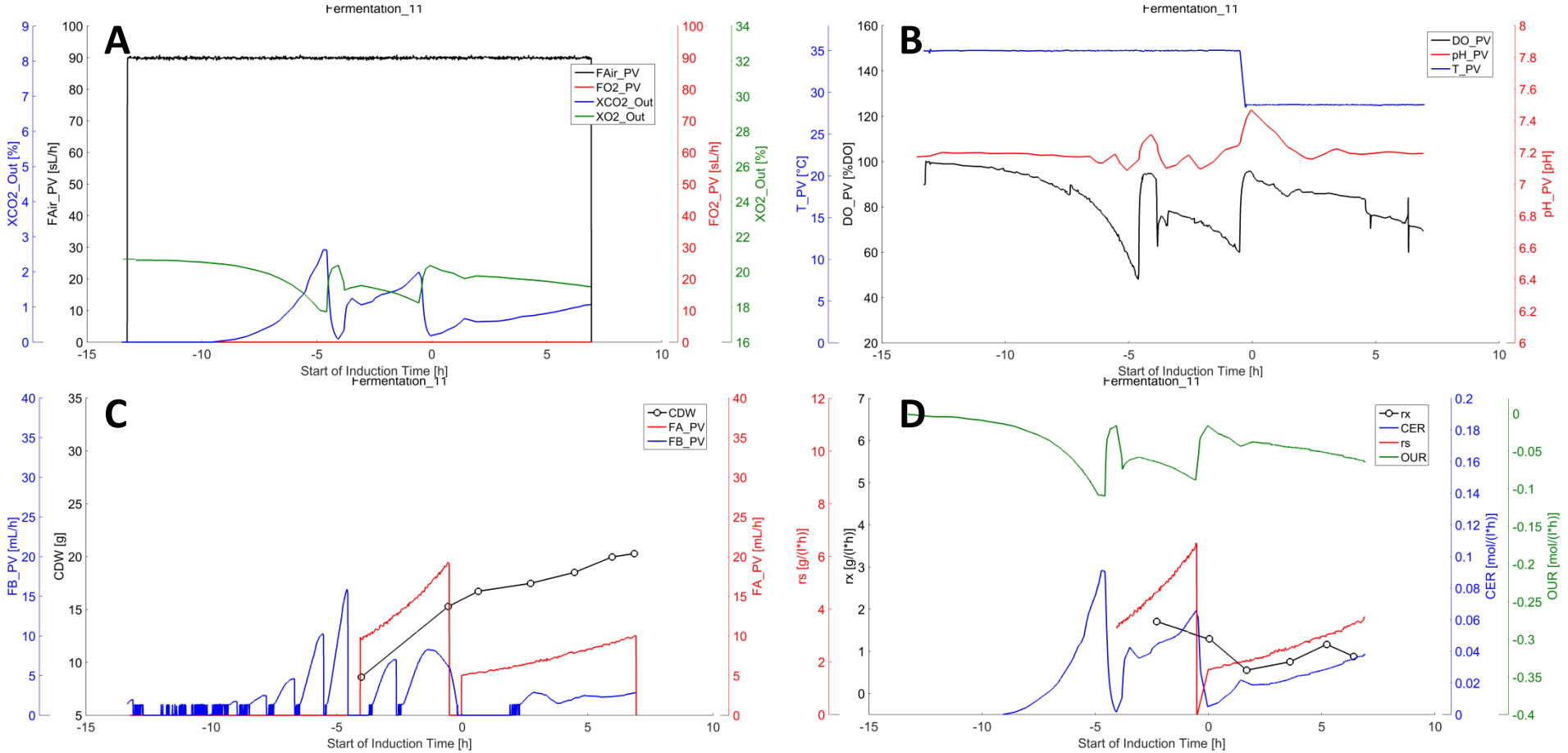

Fig. 35: Fermentation 11. (A) Air (black, Fair_PV) and oxygen (green, FO2_PV) inflow to the reactor and CO_2 (blue, XCO2_Out) and O_2 (green, XO2_Out) content in the off gas are shown in the graph at the top left corner. (B) Process parameters like temperature (blue, T_PV), pH (red, pH_PV) and dissolved oxygen (black, DO_PV) are plotted in the graph at the top right. (C) Liquid feeds like substrate feed (red, FA_PV) and base feed (blue, FB_PV) are plotted along with the cell dry weight (black, CDW) in the graph at the bottom left corner. (D) Calculated volumetric rates are shown in the graph at the bottom right corner. Volumetric growth rate (black, rx), volumetric substrate uptake rate (red, rs), volumetric carbon evolution rate (blue, CER) and volumetric oxygen uptake rate (green, OUR). All variables are plotted against the induction time. Hour represents the time of induction.

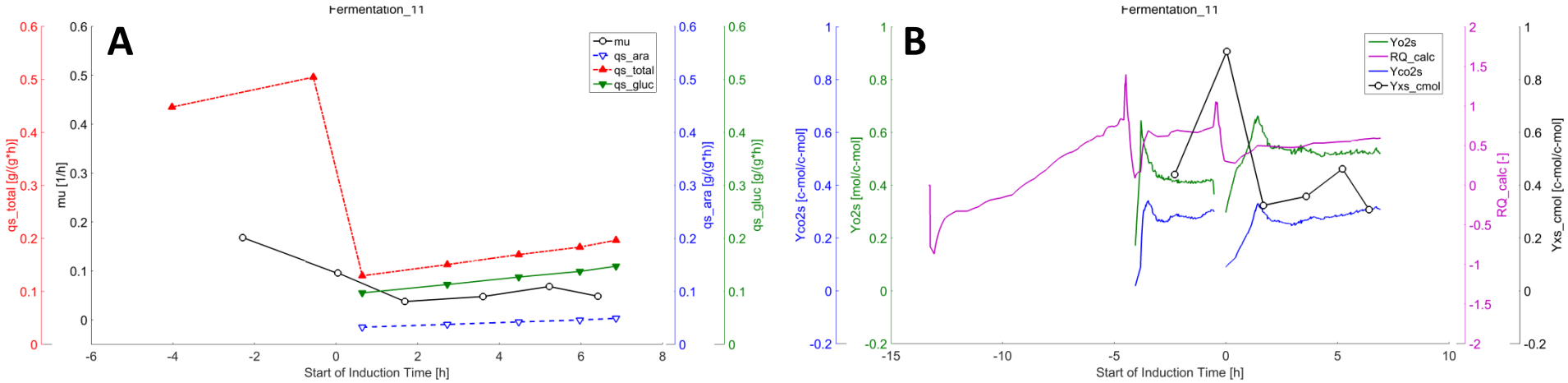

Fig. 36: Fermentation 11. (A) Specific conversion rates are shown in the graph on the left-hand side: specific growth rate (black, mu), specific total substrate rate (red, qs_total), specific arabinose uptake rate (blue, qs_ara) and specific glucose uptake rate (green, qs_gluc). (B) Yields on total substrate are displayed in the graph on the right-hand side: biomass yield (black, Yxs_cmol), CO_2 yield (blue, Yco2s), O_2 yield (green, Yo2s) and the respiratory coefficient (magenta, RQ_calc). All variables are plotted against the induction time. Hour represents the time of induction.

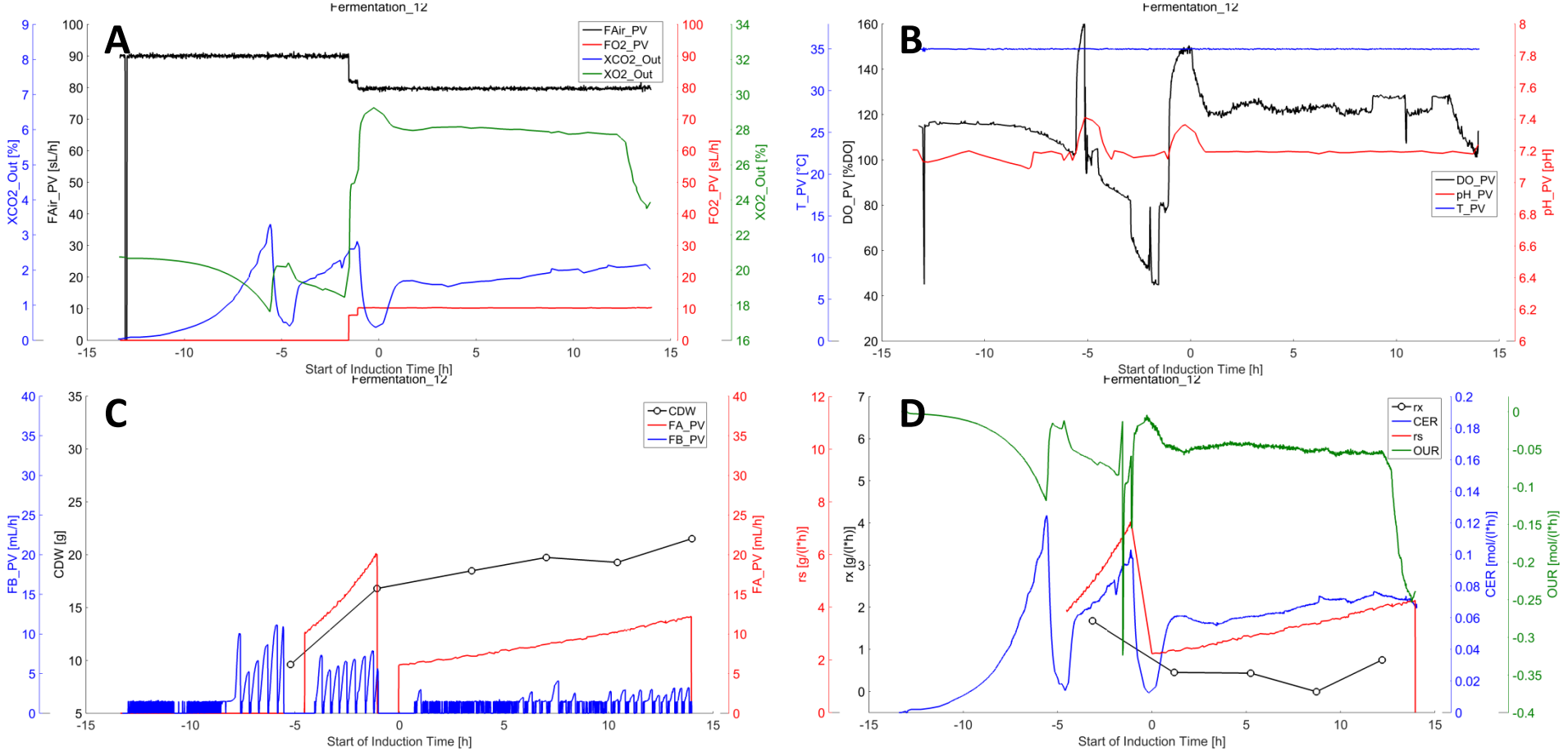

Fig. 37: Fermentation 12. (A) Air (black, Fair_PV) and oxygen (green, FO2_PV) inflow to the reactor and CO_2 (blue, XCO2_Out) and O_2 (green, XO2_Out) content in the off gas are shown in the graph at the top left corner. (B) Process parameters like temperature (blue, T_PV), pH (red, pH_PV) and dissolved oxygen (black, DO_PV) are plotted in the graph at the top right. (C) Liquid feeds like substrate feed (red, FA_PV) and base feed (blue, FB_PV) are plotted along with the cell dry weight (black, CDW) in the graph at the bottom left corner. (D) Calculated volumetric rates are shown in the graph at the bottom right corner. Volumetric growth rate (black, rx), volumetric substrate uptake rate (red, rs), volumetric carbon evolution rate (blue, CER) and volumetric oxygen uptake rate (green, OUR). All variables are plotted against the induction time. Hour represents the time of induction.

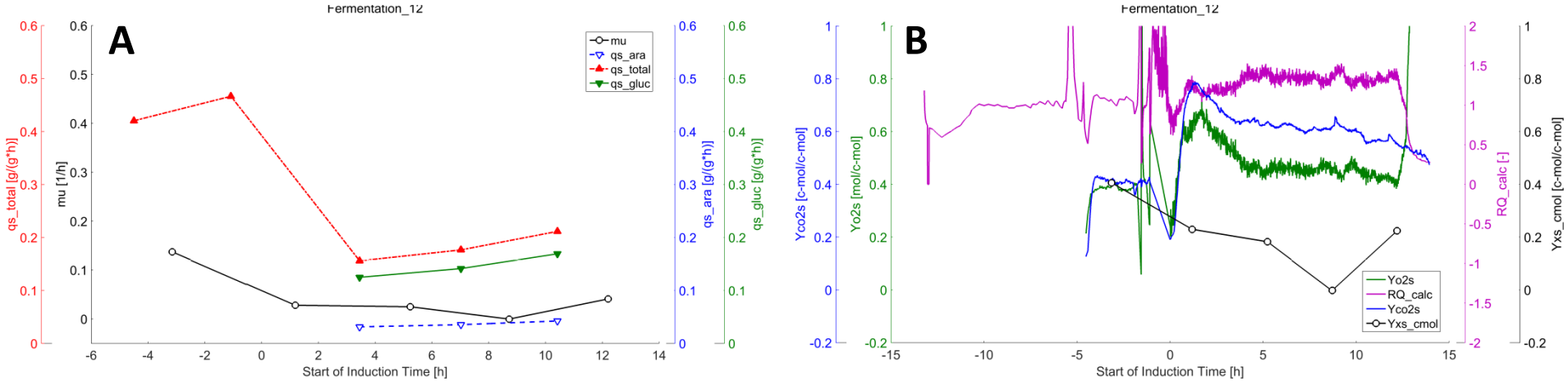

Fig. 38: Fermentation 12. (A) Specific conversion rates are shown in the graph on the left-hand side: specific growth rate (black, mu), specific total substrate rate (red, qs_total), specific arabinose uptake rate (blue, qs_ara) and specific glucose uptake rate (green, qs_gluc). (B) Yields on total substrate are displayed in the graph on the right-hand side: biomass yield (black, Yxs_cmol), CO_2 yield (blue, Yco2s), O_2 yield (green, Yo2s) and the respiratory coefficient (magenta, RQ_calc). All variables are plotted against the induction time. Hour represents the time of induction.

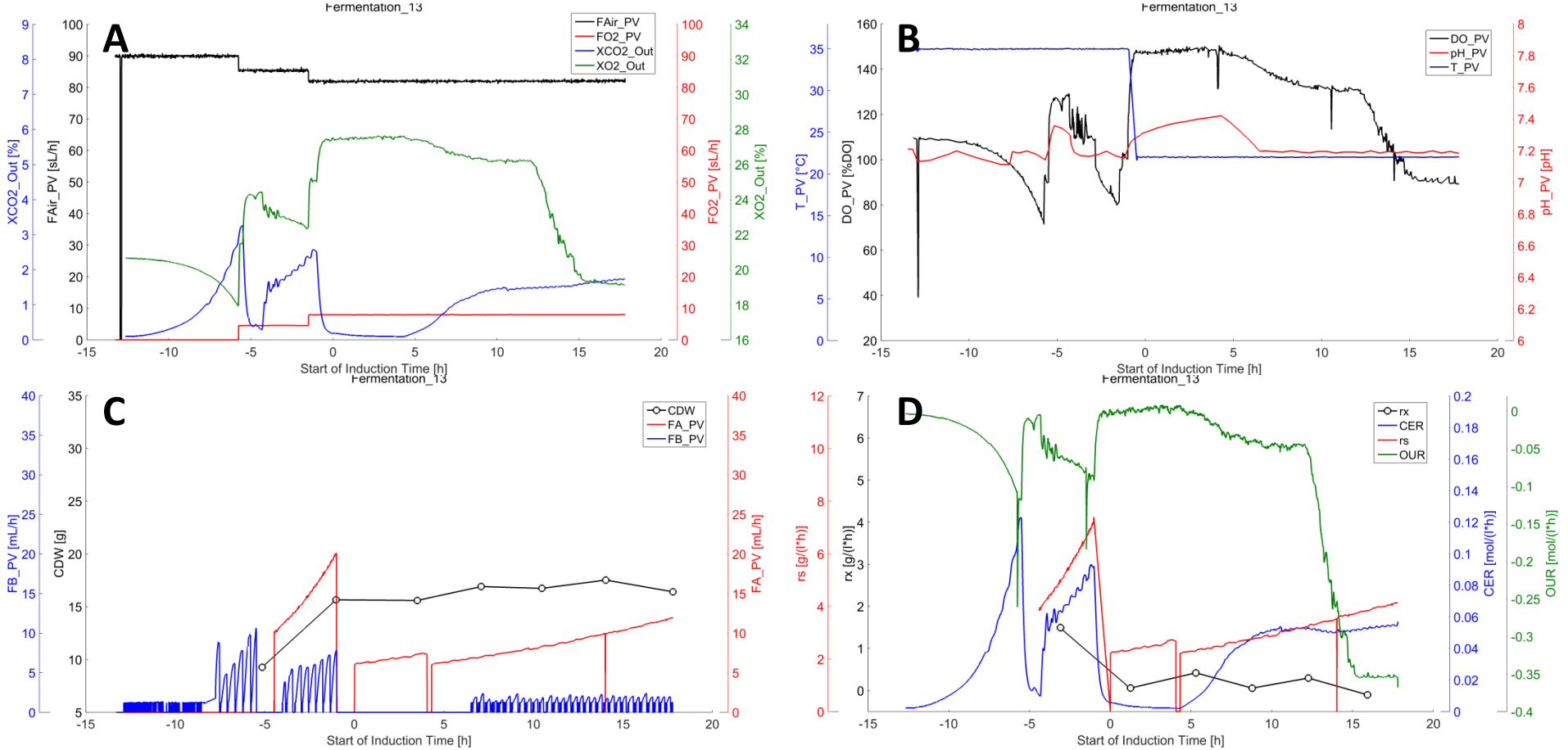

Fig. 39: Fermentation 13. (A) Air (black, Fair_PV) and oxygen (green, FO2_PV) inflow to the reactor and CO_2 (blue, XCO2_Out) and O_2 (green, XO2_Out) content in the off gas are shown in the graph at the top left corner. (B) Process parameters like temperature (blue, T_PV), pH (red, pH_PV) and dissolved oxygen (black, DO_PV) are plotted in the graph at the top right. (C) Liquid feeds like substrate feed (red, FA_PV) and base feed (blue, FB_PV) are plotted along with the cell dry weight (black, CDW) in the graph at the bottom left corner. (D) Calculated volumetric rates are shown in the graph at the bottom right corner. Volumetric growth rate (black, rx), volumetric substrate uptake rate (red, rs), volumetric carbon evolution rate (blue, CER) and volumetric oxygen uptake rate (green, OUR). All variables are plotted against the induction time. Hour represents the time of induction.

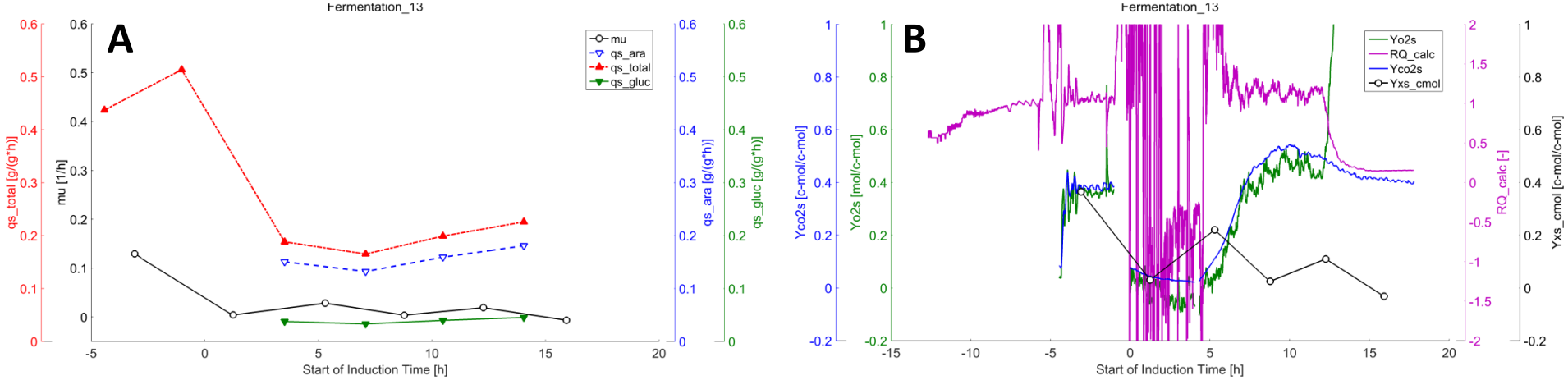

Fig. 40: Fermentation 13. (A) Specific conversion rates are shown in the graph on the left-hand side: specific growth rate (black, mu), specific total substrate rate (red, qs_total), specific arabinose uptake rate (blue, qs_ara) and specific glucose uptake rate (green, qs_gluc). (B) Yields on total substrate are displayed in the graph on the right-hand side: biomass yield (black, Yxs_cmol), CO_2 yield (blue, Yco2s), O_2 yield (green, Yo2s) and the respiratory coefficient (magenta, RQ_calc). All variables are plotted against the induction time. Hour represents the time of induction.

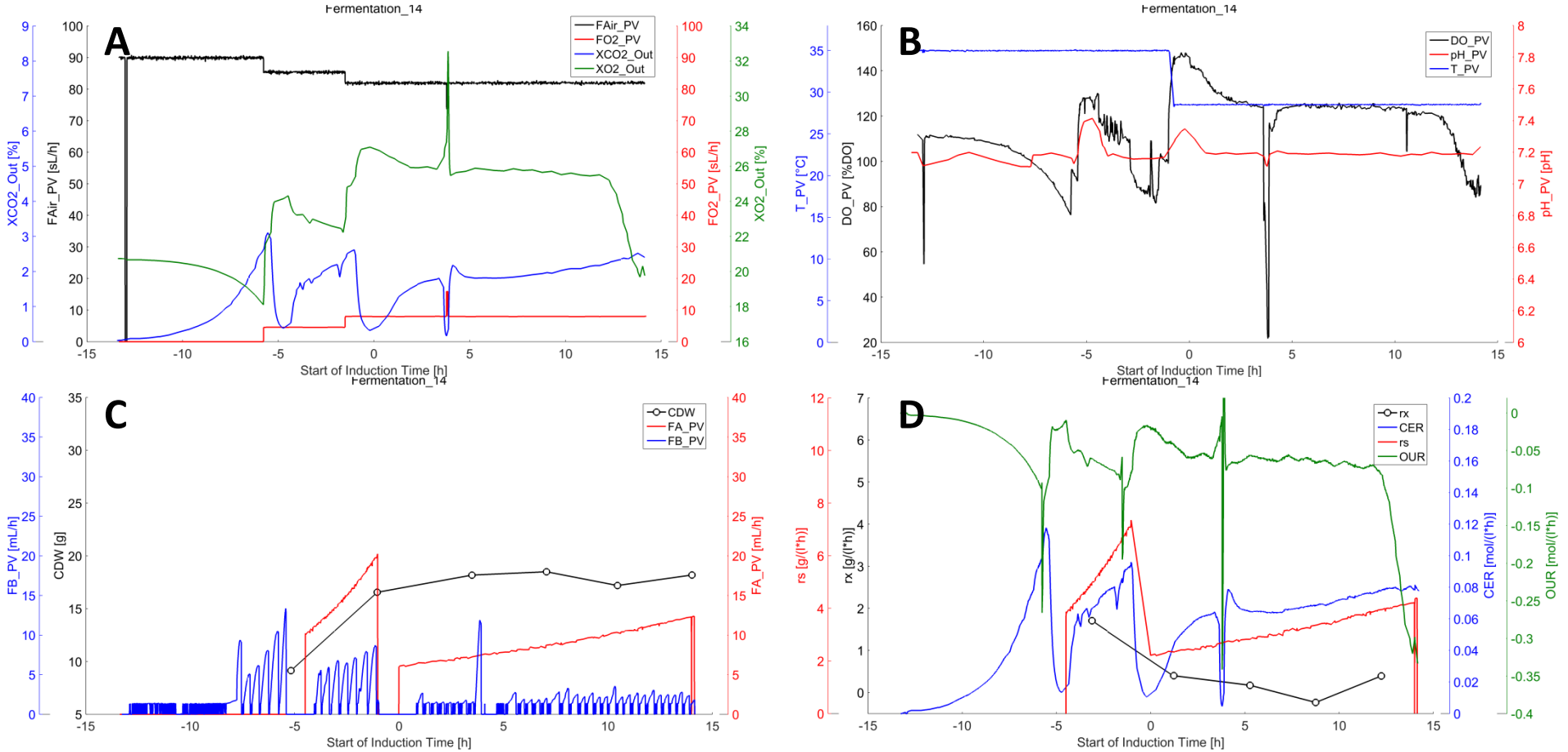

Fig. 41: Fermentation 14. (A) Air (black, Fair_PV) and oxygen (green, FO2_PV) inflow to the reactor and CO_2 (blue, XCO2_Out) and O_2 (green, XO2_Out) content in the off gas are shown in the graph at the top left corner. (B) Process parameters like temperature (blue, T_PV), pH (red, pH_PV) and dissolved oxygen (black, DO_PV) are plotted in the graph at the top right. (C) Liquid feeds like substrate feed (red, FA_PV) and base feed (blue, FB_PV) are plotted along with the cell dry weight (black, CDW) in the graph at the bottom left corner. (D) Calculated volumetric rates are shown in the graph at the bottom right corner. Volumetric growth rate (black, rx), volumetric substrate uptake rate (red, rs), volumetric carbon evolution rate (blue, CER) and volumetric oxygen uptake rate (green, OUR). All variables are plotted against the induction time. Hour represents the time of induction.

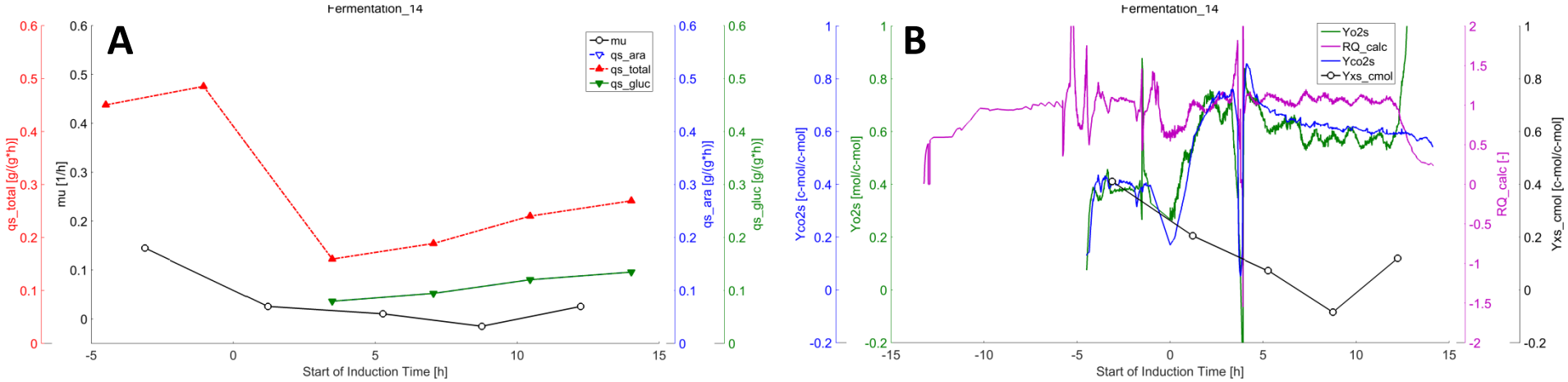

Fig. 42: Fermentation 14. (A) Specific conversion rates are shown in the graph on the left-hand side: specific growth rate (black, mu), specific total substrate rate (red, qs_total), specific arabinose uptake rate (blue, qs_ara) and specific glucose uptake rate (green, qs_gluc). (B) Yields on total substrate are displayed in the graph on the right-hand side: biomass yield (black, Yxs_cmol), CO_2 yield (blue, Yco2s), O_2 yield (green, Yo2s) and the respiratory coefficient (magenta, RQ_calc). All variables are plotted against the induction time. Hour represents the time of induction.

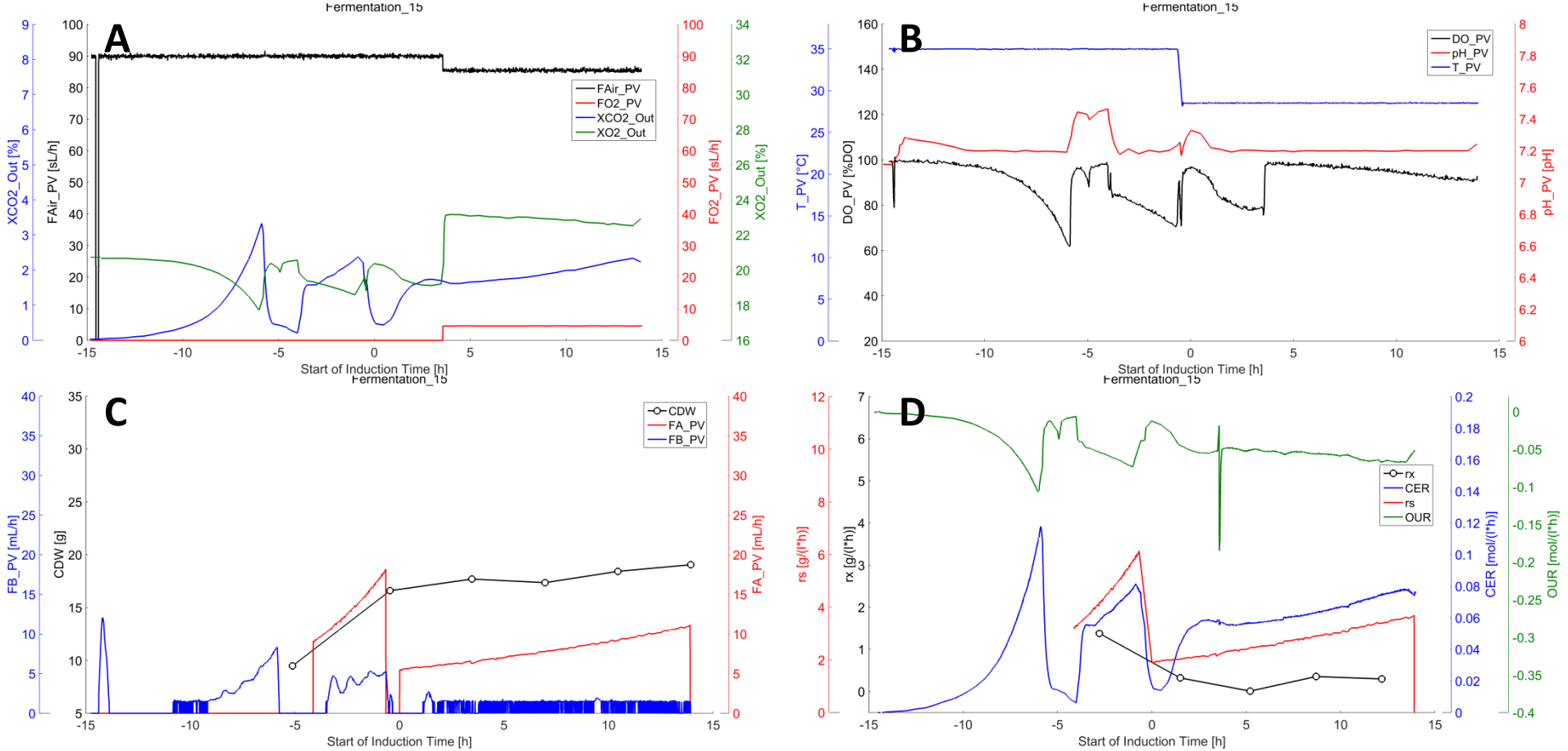

Fig. 43: Fermentation 15. (A) Air (black, Fair_PV) and oxygen (green, FO2_PV) inflow to the reactor and CO_2 (blue, XCO2_Out) and O_2 (green, XO2_Out) content in the off gas are shown in the graph at the top left corner. (B) Process parameters like temperature (blue, T_PV), pH (red, pH_PV) and dissolved oxygen (black, DO_PV) are plotted in the graph at the top right. (C) Liquid feeds like substrate feed (red, FA_PV) and base feed (blue, FB_PV) are plotted along with the cell dry weight (black, CDW) in the graph at the bottom left corner. (D) Calculated volumetric rates are shown in the graph at the bottom right corner. Volumetric growth rate (black, rx), volumetric substrate uptake rate (red, rs), volumetric carbon evolution rate (blue, CER) and volumetric oxygen uptake rate (green, OUR). All variables are plotted against the induction time. Hour represents the time of induction.

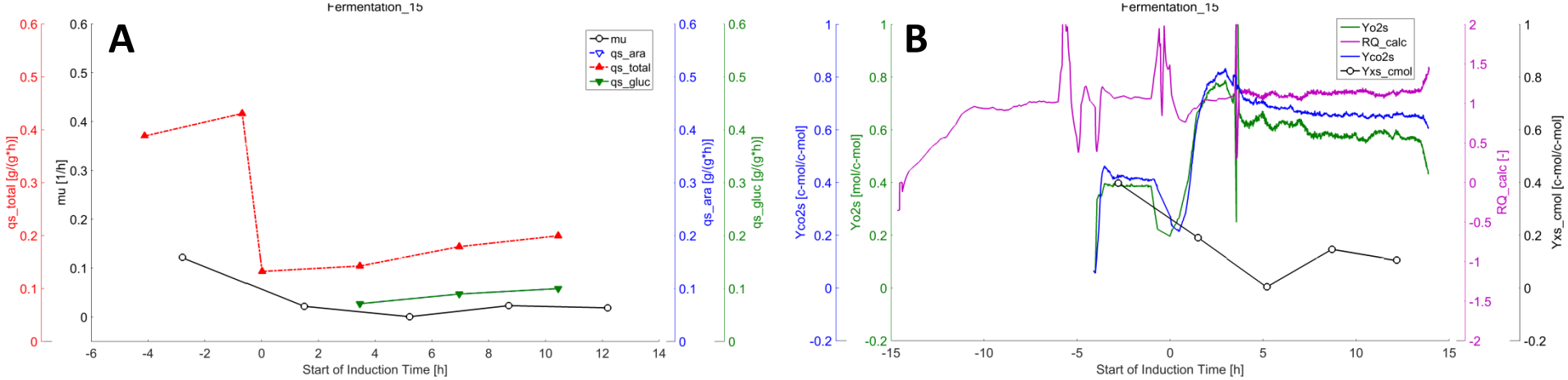

Fig. 44: Fermentation 15. (A) Specific conversion rates are shown in the graph on the left-hand side: specific growth rate (black, mu), specific total substrate rate (red, qs_total), specific arabinose uptake rate (blue, qs_ara) and specific glucose uptake rate (green, qs_gluc). (B) Yields on total substrate are displayed in the graph on the right-hand side: biomass yield (black, Yxs_cmol), CO_2 yield (blue, Yco2s), O_2 yield (green, Yo2s) and the respiratory coefficient (magenta, RQ_calc). All variables are plotted against the induction time. Hour represents the time of induction.

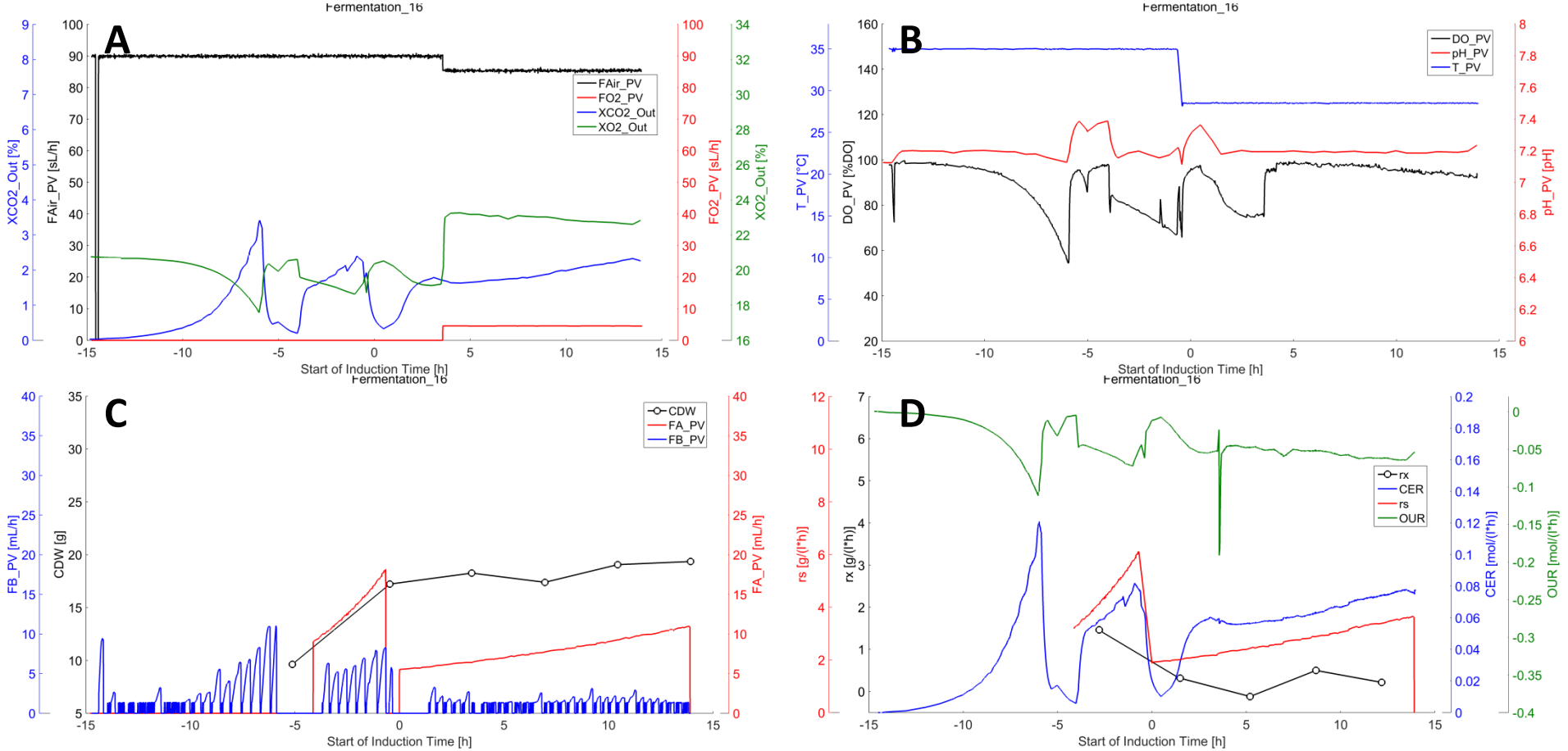

Fig. 45: Fermentation 16. (A) Air (black, Fair_PV) and oxygen (green, FO2_PV) inflow to the reactor and CO_2 (blue, XCO2_Out) and O_2 (green, XO2_Out) content in the off gas are shown in the graph at the top left corner. (B) Process parameters like temperature (blue, T_PV), pH (red, pH_PV) and dissolved oxygen (black, DO_PV) are plotted in the graph at the top right. (C) Liquid feeds like substrate feed (red, FA_PV) and base feed (blue, FB_PV) are plotted along with the cell dry weight (black, CDW) in the graph at the bottom left corner. (D) Calculated volumetric rates are shown in the graph at the bottom right corner. Volumetric growth rate (black, rx), volumetric substrate uptake rate (red, rs), volumetric carbon evolution rate (blue, CER) and volumetric oxygen uptake rate (green, OUR). All variables are plotted against the induction time. Hour represents the time of induction.

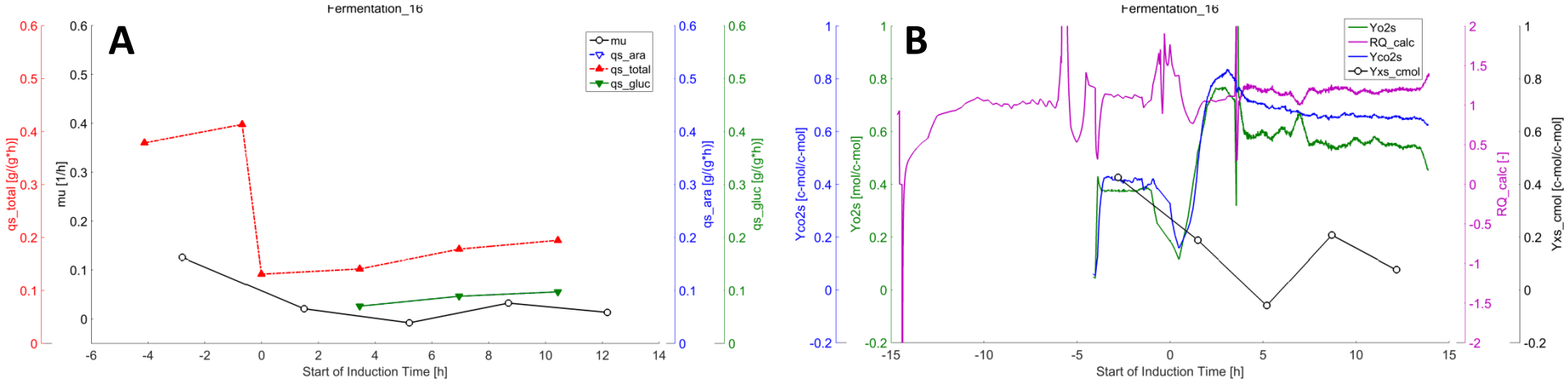

Fig. 46: Fermentation 16. (A) Specific conversion rates are shown in the graph on the left-hand side: specific growth rate (black, mu), specific total substrate rate (red, qs_total), specific arabinose uptake rate (blue, qs_ara) and specific glucose uptake rate (green, qs_gluc). (B) Yields on total substrate are displayed in the graph on the right-hand side: biomass yield (black, Yxs_cmol), CO_2 yield (blue, Yco2s), O_2 yield (green, Yo2s) and the respiratory coefficient (magenta, RQ_calc). All variables are plotted against the induction time. Hour represents the time of induction.

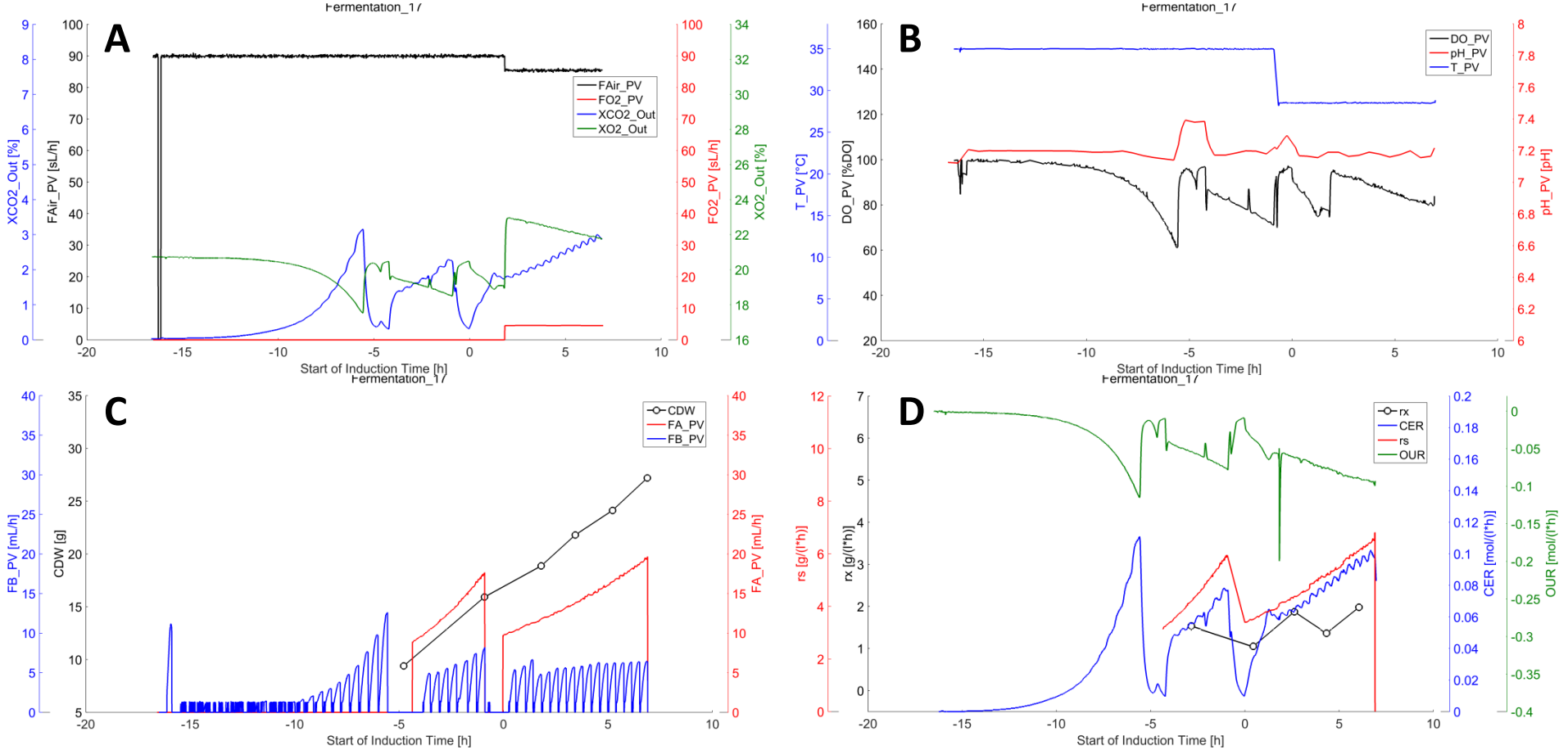

Fig. 47: Fermentation 17. (A) Air (black, Fair_PV) and oxygen (green, FO2_PV) inflow to the reactor and CO_2 (blue, XCO2_Out) and O_2 (green, XO2_Out) content in the off gas are shown in the graph at the top left corner. (B) Process parameters like temperature (blue, T_PV), pH (red, pH_PV) and dissolved oxygen (black, DO_PV) are plotted in the graph at the top right. (C) Liquid feeds like substrate feed (red, FA_PV) and base feed (blue, FB_PV) are plotted along with the cell dry weight (black, CDW) in the graph at the bottom left corner. (D) Calculated volumetric rates are shown in the graph at the bottom right corner. Volumetric growth rate (black, rx), volumetric substrate uptake rate (red, rs), volumetric carbon evolution rate (blue, CER) and volumetric oxygen uptake rate (green, OUR). All variables are plotted against the induction time. Hour represents the time of induction.

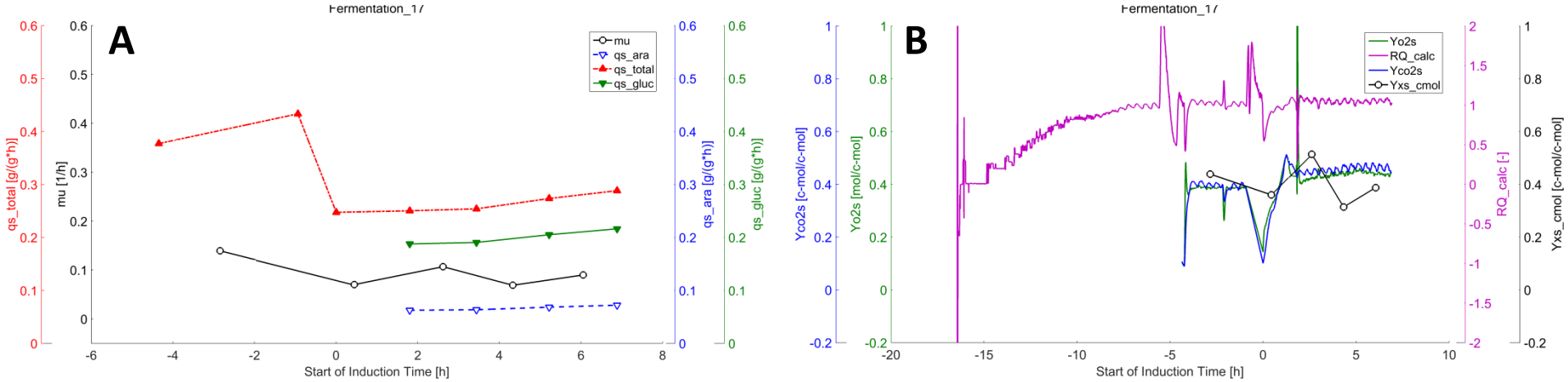

Fig. 48: Fermentation 17. (A) Specific conversion rates are shown in the graph on the left-hand side: specific growth rate (black, mu), specific total substrate rate (red, qs_total), specific arabinose uptake rate (blue, qs_ara) and specific glucose uptake rate (green, qs_gluc). (B) Yields on total substrate are displayed in the graph on the right-hand side: biomass yield (black, Yxs_cmol), CO_2 yield (blue, Yco2s), O_2 yield (green, Yo2s) and the respiratory coefficient (magenta, RQ_calc). All variables are plotted against the induction time. Hour represents the time of induction.

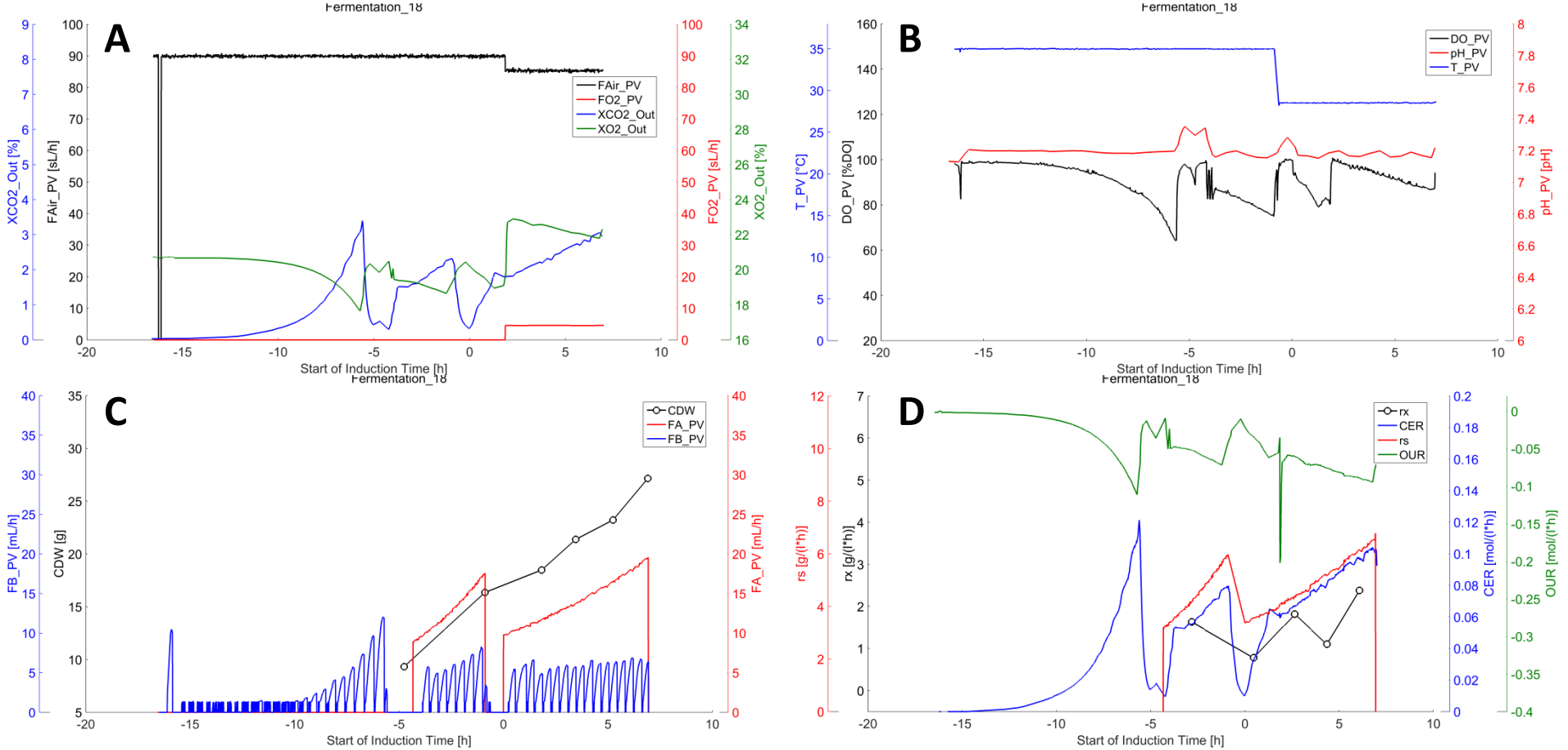

Fig. 49: Fermentation 18. (A) Air (black, Fair_PV) and oxygen (green, FO2_PV) inflow to the reactor and CO_2 (blue, XCO2_Out) and O_2 (green, XO2_Out) content in the off gas are shown in the graph at the top left corner. (B) Process parameters like temperature (blue, T_PV), pH (red, pH_PV) and dissolved oxygen (black, DO_PV) are plotted in the graph at the top right. (C) Liquid feeds like substrate feed (red, FA_PV) and base feed (blue, FB_PV) are plotted along with the cell dry weight (black, CDW) in the graph at the bottom left corner. (D) Calculated volumetric rates are shown in the graph at the bottom right corner. Volumetric growth rate (black, rx), volumetric substrate uptake rate (red, rs), volumetric carbon evolution rate (blue, CER) and volumetric oxygen uptake rate (green, OUR). All variables are plotted against the induction time. Hour represents the time of induction.

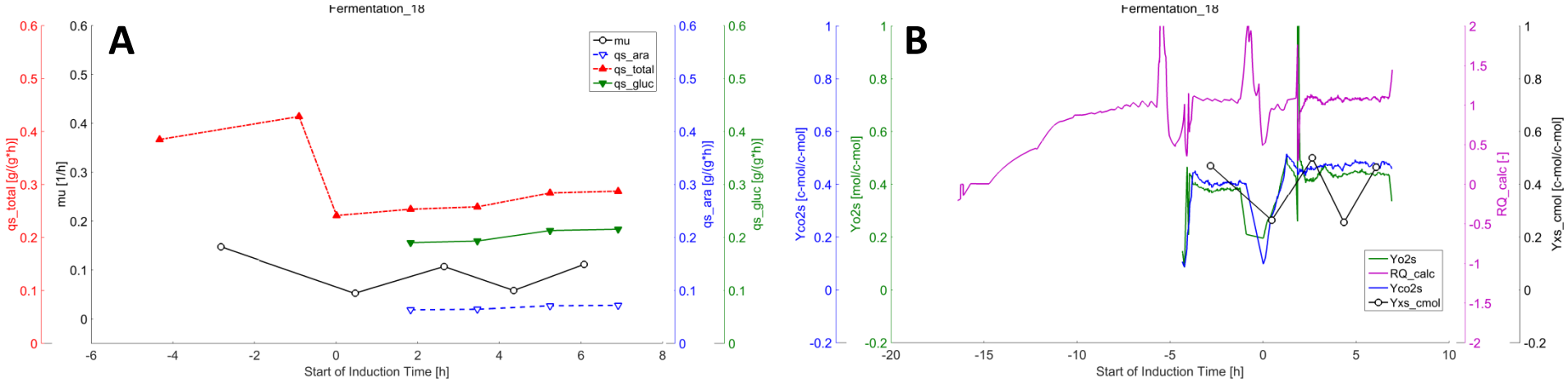

Fig. 50: Fermentation 18. (A) Specific conversion rates are shown in the graph on the left-hand side: specific growth rate (black, mu), specific total substrate rate (red, qs_total), specific arabinose uptake rate (blue, qs_ara) and specific glucose uptake rate (green, qs_gluc). (B) Yields on total substrate are displayed in the graph on the right-hand side: biomass yield (black, Yxs_cmol), CO_2 yield (blue, Yco2s), O_2 yield (green, Yo2s) and the respiratory coefficient (magenta, RQ_calc). All variables are plotted against the induction time. Hour represents the time of induction.

27 References

28 Conclusions and Outlook

Within this thesis expression tuning in *E.coli* was reviewed for the first time. Besides the discussion of biological and process technological aspects it was tried to give a concise definition for expression tuning as the purposeful adjustment of the recombinant gene transcription rate on cellular level. To allow expression tuning on cellular level the used promoter system must not respond in an all-or-none fashion, id est the culture must be induced uniformly without division into sub populations of producing and non-producing cells. In addition to that a constant inducer concentration within the cell needs to be maintained in order to keep them in an induced state.

To unfold the full potential of expression tuning the specific transcription level needs to be independently adjustable from the specific growth rate. Therefore two process technological strategies for transcription tuning have to be highlighted. Titration of a non-metabolisable inducer or the use of a metabolisable inducer as second substrate in a mixed-feed system both allow the control of the specific growth rate by adjusting the main substrate feed and the control of the transcription rate by altering the feed of inducer.

Following the extensive review transcription tuning was applied in the development of an upstream production process for VEGF-A_{165} in *E. coli* by using the araBAD mixed feed system on D-glucose and L-arabinose. In order to produce this disulfide bridge containing protein in soluble and active form the periplasm of *E. coli* with its oxidizing redox state served as a suitable folding environment. In order to gain mechanistic knowledge an innovative experimental design was used. Following QbD principles two factorial designs were planned, each for a different translocation mechanism. Due to the lack of a suitable quantification method the conducted experiments could not be evaluated. However it was shown that variation of expression levels did influence the product amount in the cytoplasm and the periplasm.

Although the gathered data could not be exploited to a full extent, expression tuning presented itself as an interesting alternative for varying the expression levels in process development using the same strain and the same promoter system. By only constructing one tunable strain in the beginning it was a very convenient way to adjust the expression rate by process technological means throughout the design of experiments.

Besides its demonstrated benefits for process development expression tuning can be beneficial for the manufacturing of recombinant products. By enabling on-line controllability of protein expression tuning is a promising candidate for tackling the issues of constant product quality and culture long term stability. Expression tuning therefore might pave the way for continuous production of biopharmaceuticals.