Application of the Enzyme Controlled Glucose Feeding for the Production of Recombinant Proteins

Starter cultures and Autoinduction

vorgelegt von

M.Sc.

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geb. in Kairo (Ägypten)

von der Fakultät III - Prozesswissenschaften

der Technischen Universität Berlin

zur Erlangung des akademischen Grades

Doktor der Naturwissenschaften

- Dr. rer. nat. -

genehmigte Dissertation

Promotionsausschuss:

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Gutachter: Prof. Dr. Peter Neubauer

Gutachter: Prof. Dr. Leif-Alexander Garbe

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Tag der wissenschaftlichen Aussprache: 18. Dezember 2017

Berlin 2018

The presented work was performed in the research group of Prof. Dr. Peter Neubauer (Chair of Bioprocess Engineering), Faculty III, Department of Biotechnology, Technische Universität Berlin.

Abstract

One of the prime objectives of any bioprocess development is to enhance the microbial growth and product yields. Correspondingly, for recombinant proteins it is the aim increase the yields of the soluble, active and correctly folded molecule. There are many factors that could influence the product yield like medium optimization and the performance of the bioprocess itself. The aim of this study is; (i) to investigate the enzymatic glucose feeding system as a pre-cultivation system for recombinant *E. coli*, (ii) To proof the scalability of this system as a main cultivation system for the production of a recombinant nucleoside phosphorylase, (iii) The application of autoinduction jointly with the enzyme based fed-batch technique with different induction protocols and (iv) to investigate the cell physiology upon exposure to a multi-substrate system during the diauxic growth.

Regarding the first point, robust pre-cultures of recombinant *E. coli* strains were obtained by fed-batch cultivations using the enzymatic glucose feeding system. According to the time plan of the expression experiment or bioreactor cultivation, potent short term (6-8 hours) or overnight pre-cultures were performed. Preparation of the pre-culture as a fed-batch process provides a well-controlled starter culture in a growing state instead of commonly applied overnight stationary phase cultures.

A scalability study for the enzymatic glucose feeding system (Enpresso B growth system) was performed with the optimized conditions using a nucleoside phosphorylase (NP) as a model protein. The results showed a more than three-fold increase in the protein activity and a 30% increase of the cell density from the well plate scale to the shake flask level. While about 25% increase was obtained by application of this system in the benchtop-bioreactor scale.

The effect of the IPTG on the expression of recombinant nucleoside phosphorylases was observed in autoinduced and single shot induced cultures. IPTG-autoinduced Enpresso cultures reached nearly the same nucleoside phosphorylase activity (≈ 3.0 - 3.5 U mL^{-1}) as obtained by single shot induced cultures in 15 hours shorter cultivations. However, the cell density in cultures with autoinduction was less than half of that of single shot induced cultures. Optimal concentrations for autoinduction were 70-100 µM IPTG or 2.0 g L^{-1} lactose, respectively.

Autoinduction is based on the de-repression of the *lac* promoter in glucose deficient conditions under diauxic regulation. A clear diauxic lag phase after the end of the batch phase (time of glucose consumption) was observed in *E. coli* diauxic growth. The length of this lag phase was around 30 minutes. By applying the diauxie cultivation at the benchtop-bioreactor scale, more than one lag phase was observed upon consumption of different substrates. Eventually, three phases seem to be relevant, related to the consumption of different substrates: (i) batch on glucose, (ii) consumption of lactose and metabolism of the glucose part, and finally (iii)

consumption of the galactose part. Additionally, acetate as the metabolic byproduct was consumed as a fourth substrate (i) in the diauxic lag phase and (ii) at the end of the cultivation.

Keywords *Escherichia coli*, recombinant protein, fed-batch cultivation, high cell density, EnBase, Autoinduction, Diauxic growth, pre-culture, scale-up, *lac* operon.

Zusammenfassung

Die Erhöhung des mikrobiellen Wachstums und des Produktionsertrages gehören zu den wichtigsten Zielen jeder Bioprozessentwicklung. , Entsprechend ist es für rekombinante Proteine das Ziel, die Ausbeuten des löslichen, aktiven und korrekt gefalteten Moleküls zu erhöhen. Es gibt viele Faktoren, die den Produktionsertrag beeinflussen können, wie die Optimierung von Medien und die Leistung des Bioprozesses. Die Ziele dieser Studie sind folgendermaßen zusammenzufassen: (i) Erforschung des enzymatischen Glukose-Zufütterungssystems als ein Vorkultivierungs-System für rekombinanten *E. coli*, (ii) Nachweis der Skalierbarkeit dieses Systems als Hauptkultivierungs-System für die Herstellung einer rekombinanten Nukleosidphosphorylase, (iii) Die Anwendung der Autoinduktion gemeinsam mit der Enzym-basierten Fed-Batch Technik mit verschiedenen Induktionsprotokollen und (iv) Erforschung der Zellphysiologie nach Exposition gegenüber einem Multi-Substrat-System während des diauxischen Wachstums.

Das erste Ziel betreffend, wurden robuste Vorkulturen eines rekombinanten *E. coli* Stammes in Fed-Batch Kultivierungen unter Verwendung des Glukose-Freisetzungssystems generiert. Je nach Zeitpunkt des Expressionsexperiments oder Bioreaktor-kultivierung, potente Kurzzeit- (6-8 Stunden) oder Über Nacht-Vorkulturen durchgeführt wurden. Die Vorbereitung der Vorkultur als Fed-Batch-Prozess bietet eine gut kontrollierte Starterkultur in einem wachsenden Zustand anstelle von üblicherweise angewandten über Nacht stationären Phasenkulturen.

Eine Skalierbarkeitsstudie für das enzymatischen Glukose-Zufütterungssystems (Enpresso B Wachstums-System) wurde unter optimierten Bedingungen Verwendung der Nukleosidphosphorylase (NP) als Modellprotein durchgeführt. Die Ergebnisse zeigten eine mehr als dreifache Anstieg der Proteinaktivität beziehungsweise eine 30% Zelldichte vom Well-Platten-Maßstab bis zum Schüttelkolben-Maßstab. Während die Anwendung dieses Systems in dem Auftisch-Bioreaktor-Maßstab einen Anstieg von etwa 25% erreichte.

Die Wirkung des IPTG auf die Expression rekombinanter Nukleosidphosphorylasen in Autoinduktions- und Single-Shot-induzierten Kulturen beobachtet wurde. IPTG-autoinduzierte Enpresso Kulturen fast dieselbe Nukleosidphosphorylase-Aktivität (etwa 3,0 - 3,5 U mL^{-1}) erreichten, wie die durch Single-Shot-induzierten Kulturen, die eine 15 Stunden kürzere Kultivierungszeit aufwiesen. Jedoch war die Zelldichte, die durch Autoinduktion erreicht wurde, weniger als die Hälfte von Single-Shot-induzierten Kulturen. Optimale Konzentrationen für die Autoinduktion waren jeweils 70-100 µM IPTG bzw. 2,0 g L^{-1} Lactose.

Die Autoinduktion basiert auf der De-Repression des *lac*-Promotors in glukose-defizienten Zustände unter diauxischer Regulation. Eine deutliche diauxischer Lag-Phase nach dem Ende der Batch-Phase (Zeit des Glukoseverbrauchs) wurde bei einem diauxische Wachstum von *E. coli*

beobachtet. Diese Zeitdauer dieser Lag-Phase betrug etwa 30 Minuten. Durch Anwendung der Diauxie-Kultivierung in dem Bioreaktor-Maßstab wurde mehr als eine Lag-Phase beim Verbrauch unterschiedlicher Substrate beobachtet. Schließlich waren drei Phasen scheinen relevant zu sein, bezogen auf den Verbrauch verschiedener Substrate: (i) Batch-Phase auf Glukose, (ii) Verbrauch von Laktose und Metabolismus des Glukose-Teils und schließlich (iii) Verbrauch des Galactose-Teils. Zusätzlich wurde Acetat, ein metabolisches Nebenprodukt, als viertes Substrat (i) in der diauxischen Lag-Phase und (ii) am Ende der Kultivierung verbraucht.

Schlüsselwörter *Escherichia coli*, rekombinantes Protein, Fed-Batch-Kultivierung, hohe Zelldichte, EnBase, Autoinduktion, Diauxisches Wachstum, Vorkultur, Scale-up, *lac*-Operon.

Acknowledgements

This work has been done at the Department of the Bioprocess Engineering of the Technical University of Berlin in Germany. I am so grateful for being one of the BVT team for almost four years.

First of all, I want to express my deep appreciation and gratitude for my supervisor, Professor Peter Neubauer for his scientific care, supervision and even the spiritual support throughout the whole study. During the four years he gave me a lot and important ideas and advices which absolutely gave me more knowledge and impacted me a lot through my study.

Especially I feel grateful to Dr. Antje Neubauer for her grateful supervision during the study. She helped me a lot in understanding of the cell physiology; the valuable information about the EnBase technology. I would like to thank her also for her continuous and instructive advices.

Additionally, I would like to thank BioNukleo UG team for providing the recombinant strain of the study and the valuable information about the recombinant protein.
I would kindly acknowledge also the support by the AUTOBIO project funded by the German Federal Ministry of Education and Research (BMBF) within the Framework Concept “Research for Tomorrow’s Production” which is managed by the Project Management Agency Karlsruhe (PTKA).
I would also like to gratefully acknowledge the German Academic Exchange Service (DAAD) for the financial support for four years.
I would like to send all my appreciation and respect for all my colleagues at the Bioprocess Engineering lab. Irmgard Maue-Mohn, Brigitte Burckhardt and Thomas Högl for their technical support. Sabine Lühr-Müller for help with the documentations. Florian Glauche for his great support and help during the practical work. Sarah Kamel and Heba Yehia for their support & encouragement and removal of the homesickness. My office-mates; Christian Reitz, Klaus Pellicer Alborch and Ongey Elvis Legala for the nice office atmosphere with a lot of life discussions and friendship atmosphere. Dr. Diana Lopez, Dr. Howard Diego Ramirez Malule and Anna-Maria Marba for their kind and spiritual support. Sebastian Hans and Emmanuel Anane for continuous help in solving my software-related problems. Dr. Nicolas Cruz-Bournazou, Dr. Stefan Junne, Anika Bokisch, Anja Lemoine, Andri Hutari, Juan Antonio and all other colleagues for creating a pleasant working environment.

A special thanks to my very nice friend Sarah El-Helw for the English proofreading of the thesis in a very short time. All my gratitude for my DAAD colleagues and my former classmates for their motivation and support.
Last but not least I warmly thank all my beloved family. My parents who are behind my strength and everything good surrounds me. My warm appreciation for my husband, Dr. Mohamed Sayed Sofy for his continuous support and encouragement.

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List of Abbreviations

µ	Growth rate
Ac	Adenylate cyclase
AI	Autoinduction
araB	Arabinose promoter
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CO_{2ex}%	CO_2 concentration in the exhaust gas (produced CO_2)
Crp	cAMP receptor protein
DCW	Dry cell weight
DWP	Deepwell plate
E. coli	*Escherichia coli*
EnpressoB/ Enpresso B (D)	Pre-culture in Enpresso B & main culture in Enpresso B (D)
Enpressp BD	Enpresso B Defined
G6P	Glucose-6-phosphate
GTP	Guanosine triphosphate
IPTG	Isopropylthiogalactoside
KLa	Volumetric oxygen transfer coefficient
*lac*I	Lac repressor
lac operon	Lactose operon
*lac*Y	Lactose permease
*lac*Z	β-galactosidase
LB	Luria Bertani
LB/ Enpresso B (D)	Pre-culture in LB & main culture in Enpresso B (D)
MSM	Mineral salts medium
MWP	Multiwell plate

NP	Nucleoside phosphorylase
O_{2ex}%	O_2 Concentration in the exhaust gas (consumed O_2)
OD_{600}	Optical density at 600 nm
Ovn	overnight
pH	Concentration of H^+ ions
PH_0	Initial pH
pO_2 [%]	Partial oxygen concentration [%]
PTS	Phosphotransferase system
PyNP	Pyrimidine nucleoside phosphorylase
QCO_2	CO_2 formation rate
QO_2	Oxygen uptake rate
Reagent A	Glucoamylase enzyme (biocatalyst)
RNAP	RNA polymerase
rpm	Revolutions per minute
SBM	Substrate Booster mixture
SD	Standard deviation
SDR	Sensor Dish Reader
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel
SSI	Single shot induction
TCA	Tricarboxylic acid cycle
UYF	Ultra-yield flask
X	Biomass concentration
σS	Stress sigma factor (S)

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1 Background

Proteins are the building blocks of life. Recombinant proteins are those produced in industry with the aid of protein engineering. Both native and recombinant proteins have major importance in different aspects of life like biopharmaceutical, food, agriculture, detergents or polymer and plastic industry.

The demand for new recombinant proteins used for therapeutic application is growing with time. The impact of recombinant techniques on the industry of biopharmaceuticals is clear. For instance, diabetics are now more comfortable finding quite high-grade insulin in the market. Patients with granulomatous disease could now live normally by taking a doses of gamma interferon (Demain and Vaishnav, 2009). Recently, researchers paid more attention towards studying the cell physiology and the optimal cultivation conditions as an important step in this process optimization. Generally, the process of the recombinant protein production is divided into two main consecutive phases, growth phase and protein expression phase. In the growth phase of the cultivations the cells are grown to a certain optical density at which the second phase of the recombinant protein expression is started.

Up to now, *Escherichia coli* is an important expression host and it is considered as a work-horse organism characterized by fast growth kinetics, high cell densities, and availability of efficient tools for preparing recombinant expression strains. Cultivation of *E. coli* for recombinant protein production can be performed as batch, fed-batch or continuous cultivations. However, fed-batch mode of cultivation is the most common mode for protein production at the industrial level. The preference to fed-batch mode is due to the possibility to control the bacterial growth rate, the robustness of the production processes, and the possibility to perform the cultivation without an excessive acetate production.

To start a fed-batch bioprocess for high cell density cultivation, a reproducible starter culture (pre-culture) is needed. Pre-cultures are usually performed in a batch cultivation mode. Batch processes are often accompanied with side effects like the oxygen limitation and low cell yields. In this study, the so-called EnBase® is applied as a pre-cultivation technique, that is a fed-batch which is based on the feeding of a concentrated glucose solution to the bioreactor, here the glucose is continuously released by a biocatalyst from a glucose polymer. Additionally, the impact of the length of the starter culture on the lag phase of the cells and their behavior in the fed-batch cultivation were investigated.

The cultivation conditions and the expression conditions affect the yield of the recombinant protein. Induction time point and the length of the induction phase are important factors that affects the production of a recombinant protein. Many studies have been conducted during the last few years for optimization of the induction process. Autoinduction is a widely used reliable, handy and convenient protocol for the production of recombinant proteins. It regulates the protein expression in *E. coli* without need for monitoring the culture growth or addition of the inducer during the cultivation. It is based on the de-repression of the *lac* promoter under conditions of glucose depletion based on the carbon catabolite repression. Diauxic regulation is activated when the cells are exposed to a mixture of two sugars. One of the sugars (usually glucose) is highly preferred, while the second sugar (e.g. lactose) is not metabolized until glucose is consumed (Fischer *et al.*, 1998; Studier, 2005). Since autoinduction can be applied for recombinant protein production both in research scale (in shaken cultures) and production scale (large shake flasks or bioreactors), the applicability of the high cell density providing EnBase technology for autoinduction was studied.

As a model recombinant production system, expression of the thermostable nucleoside phosphorylase of *Thermus thermophilus* (Tt) in *E. coli* was studied. Nucleoside phosphorylases "NP's" are versatile biocatalysts suitable for the synthesis of nucleoside analogs which are used e.g. as antiviral agents and for cancer treatment (Szeker *et al.*, 2012).

1.1 Research objectives

The research objectives are summarized into four main research questions (RQ);

RQ1: What are the differences between batch and fed-batch pre-cultures? What is the influence of the pre-cultivation technique on the cell growth and recombinant protein production in fed-batch main cultivations? What is the influence of the pre-culture length on the cell density and protein expression?

RQ2: Is the fed-batch like Enpresso B growth system scalable over the different scales (from the milliter scale shaken cultures up to two-liter scale bioreactor cultivation) for production of a recombinant thermophilic nucleoside phosphorylase as a model recombinant protein?

RQ3: How can the different ways of autoinduction systems be combined with the fed-batch like Enpresso growth system. What do they attribute to the cell growth and the protein production?

RQ4: Considering the diauxic regulation, what are the main characteristics of the *E. coli* physiology during the diauxic growth and the diauxic lag phase in cultivations performed with the Enpresso growth system?

2 Review of the Literature

Recombinant protein production

The process of the recombinant protein production is divided mainly into two important phases. The process starts by inoculation the cultivation medium with the recombinant strain. In the "*growth phase*" the culture is let to grow until a desired cell density suitable for the induction is reached. This is followed by the "*protein expression phase*", in which the culture is induced for the recombinant protein production. The yield of the final product is affected by the production synthesis capacity of the cells and the capacity of the protein folding machinery. Therefore, the environmental conditions of the pre-induction have a major effect on the product yield and quality. For instance, there are some products which are synthesized over few hours only and consequently need a very high product formation rate (Neubauer and Winter, 2001). However, because every protein is different the protein production and purification protocol must be worked out individually (Graslund, 2008). This review addresses the different approaches for production of different target proteins.

2.1 Growth phase

The microbial cells can be grown in the laboratory in tubes or multiwell plate (µL / mL scale), shake flasks (100 to 1000 mL scale) and bioreactors which are used for larger scales of industrial processes under well controlled conditions. Accordingly, there are different cultivation modes which are common for every type of these cultivation vessels.

2.1.1 Cultivation modes

Recombinant strains can be cultivated in different ways. The type of the cultivation depends on the type of the microbial system, purpose of the cultivation and the type of the product of interest. Batch, Fed-batch and Continuous processes are the main types of cultivations. Each type of cultivation has its own benefits and application areas. For instance, fed-batch processes are commonly used for industrial purposes like achieving higher cell densities or higher protein yields. In contrast, today continuous process in view of recombinant processes are usually applied for studying the growth and product formation kinetics. The most widely used cultivation type is however the batch process, which is applied to the majority of shaken cultures in research laboratories.

- **Batch process**

The batch mode of cultivation is the easiest way to perform microbial cultivations. In this system, all nutrients are added to the cultivation system from the beginning. However, it is difficult to control the growth during the process. The conditions can be changing over time of the process due to many factors like changes in the concentration of dissolved oxygen or pH level. This latter can easily change by the production of overflow metabolites like acetate in *E. coli*.

- **Continuous process**

Continuous processes are typical for the microbial processes in nature like lakes or rivers. In this process, the feed with the whole medium is added to the reactor, meanwhile the reactor content is withdrawn continuously with the same feed rate to keep the volume in the reactor constant. One substrate component in the feed can be added at a rate which is lower than the maximum rate of consumption. Continuous processes can be controlled by different ways; accordingly, there are four major types of the continuous process. They are chemostat, turbidostat, pH-auxostat and nutristat. The chemostat is the most common type of the continuous cultivations in research laboratories as it enables to obtain information about the cellular behavior at different growth rates (Enfors, 2011).

- **Fed-batch process**

Fed-batch process is the most commonly used mode for recombinant protein production in bioreactors. It is a batch process in which one growth limiting nutrient, mostly glucose, is added in a way that affects the growth rate and the rate of respiration but causes a minimal change in the culture volume (Enfors, 2011). Moreover, this energy source is usually added according to the desired rate of consumption in order to attain a maximum yield of target protein per cell.

The major obstacle for achieving high cell density cultures of *E. coli* is the too high initial substrate concentration. The uncontrolled usage of the substrate may cause the production of the inhibitory byproduct, acetate, when the substrate inflow overwhelms the respiratory capacity. This typically happens in batch operational mode which is commonly used in small-scale cultivations like in shake flask cultures (Weuster-Botz, Altenbach-Rehm and Arnold, 2001), but also still is applied in some industrial bioprocesses. The control of the growth limiting nutrient in a fed-batch process can reduce the metabolic overflow from pyruvate to acetate which results in severe pH change and inhibition of cell growth (Neubauer and Winter, 2001). Additionally, by controlled glucose inflow in fed-batch cultivation the oxygen consumption rate and the bacterial growth rate (μ) can be easily controlled and the oxygen depletion at higher cell densities is avoided. For these reasons, fed-batch mode is the standard technology for achieving high cell density cultures and higher product yield.

There are different strategies for fed-batch cultivations (Neubauer and Winter, 2001; Enfors, 2011). Exponential feeding is most commonly used after an initial batch phase. Thereby the feed rate is commonly controlled by an exponential function to keep the specific growth rate (μ) constant. A constant continuous feeding rate is normally used as the third phase after the critical oxygen transfer rate of the bioreactor has been reached with an exponential feeding. However due to its simplicity it also is sometimes applied directly after the batch phase. Constant feeding is addressed in this study through the enzymatic glucose feeding mechanism introduced before by (Panula-Perälä *et al.*, 2008) and known as enzyme based substrate delivery (EnBase technology).

2.1.2 Fed-batch cultivations and overflow metabolism in *E. coli*

E. coli is a facultative anaerobic microorganism. Glucose is first processed via glycolysis (EMP pathway). Under oxygen sufficient conditions, the intermediates of glycolysis will be processed via citric acid cycle (TCA), while under the oxygen limited conditions, the energy required for the cell metabolism will be generated by fermentative metabolism and the glycolytic intermediates will be channeled into different short chain organic acids (mixed acid fermentation, Figure 2-1). In the mixed acid fermentation, phosphoenolpyruvate is converted to succinate or over pyruvate to lactate, formate, acetate, ethanol, CO_2 and H_2 (Sawers and Jarsch, 1996). Therefore, concentration of the TCA intermediates required for the protein biosynthesis and growth will decrease. The total net energy obtained through the fermentative pathway is much lower than the energy obtained through the aerobic metabolic pathway and thus, either the rate of glucose consumption is increased or growth is slowed down.

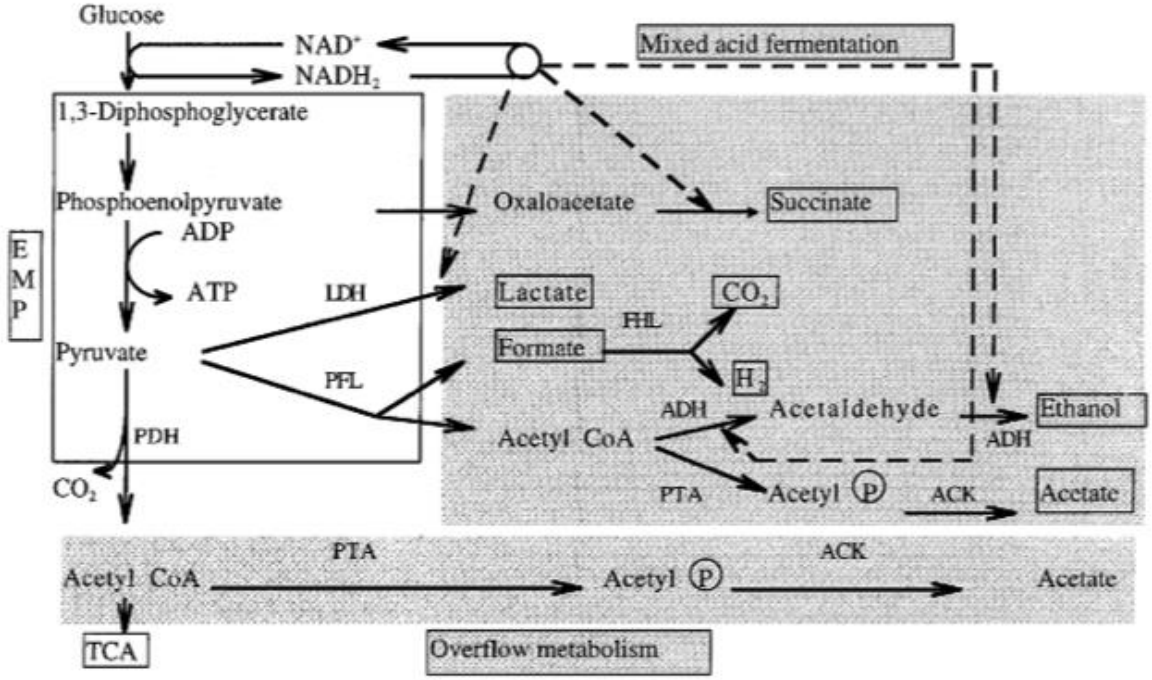


Figure 2-1 Schematic routes of the glucose in glycolysis (Embden–Meyerhof–Parnas; EMP pathway), Fermentative pathway (mixed acid fermentation) and the overflow metabolism as presented by (Xu, Jahic and Enfors, 1999)***. TCA – Tricarboxylic acid cycle; PDH – Pyruvate dehydrogenase; PTA – Phosphate acetyltransferase; ACK – Acetate kinase; LDH – Lactate dehydrogenase; PFL – Pyruvate formate lyase; ADH – Alcohol dehydrogenase.***

Under glucose excess after a combined uptake and phosphorylation by the phosphotransferase (PTS) system, most of the glucose-6-phosphate goes through glycolysis and finally yields pyruvate and acetyl-CoA. In *E. coli*, acetate also can be formed aerobically if the flux through the glycolysis is too high, either by decarboxylation of pyruvate by pyruvate oxidase (proxB) or from Acetyl-CoA (Xu, Jahic and Enfors, 1999). Acetate as well as other central metabolic intermediates like pyruvate, glucose-6-phosphate and α-ketoglutarate can be secreted to the culture medium, if they are accumulated (Paczia *et al.*, 2012). After the glucose is consumed, the cells start to re-assimilate the formed acetate (Luli and Strohl, 1990). It was reported before that *E. coli* BL21 are more active in acetate uptake, TCA cycle and glyoxylate shunt and produce very little acetate, even in high glucose concentration, than *E. coli* MJ 109. (Phue *et al.*, 2005).

Accordingly, glucose limited fed-batch cultivations limit the flux of glucose through the glycolysis by a controlled availability of the glucose and thus are preferred to reduce the overflow metabolism and to control the growth rate (Luli and Strohl, 1990).

2.1.3 Enzyme based substrate delivery (EnBase technology)

The EnBase technology is an enzyme controlled fed-batch technique introduced by (Panula-Perälä *et al.*, 2008). It forms the basis of the Enpresso Growth System (Enpresso GmbH, Berlin, former BioSilta). The original system applied enzymatic degradation of a glucose polymer (starch) by the aid of a biocatalyst (e.g. glucoamylase). Once the enzyme degrades the starch molecules, the glucose is released into the medium at a rate defined by the enzyme dose.

The continuous feeding of the growth limiting substrate (glucose) with a feed rate that limits the substrate uptake rate affect directly the growth and respiration rates. The respiration activity is closely related to the metabolic flux through the glycolysis by the reduction of NAD^+ to $NADH^+ + H^+$. Therefore, the glucose feeding rate is also closely related to the consumption of the oxygen and thereby controls the cell metabolism.

In a bioreactor scale, fed-batch is realized by mechanical pumps to control the feed rate into the cultivation (Figure 2-2). However, it is challenging to set up a mechanical pump in shake flasks or multiwell plates (MWP's). Therefore, the controlled substrate release gave the chance to control the feed rate without any mechanical pumps. The concentration of the glucose released in the medium which depend on the concentration of the enzyme added enabled researchers to apply fed-batch type of cultivations even in small scales like the 48-Bioreactor (2mag bioreactor) and even to 96 multiwell plates (Knepper *et al.*, 2014).

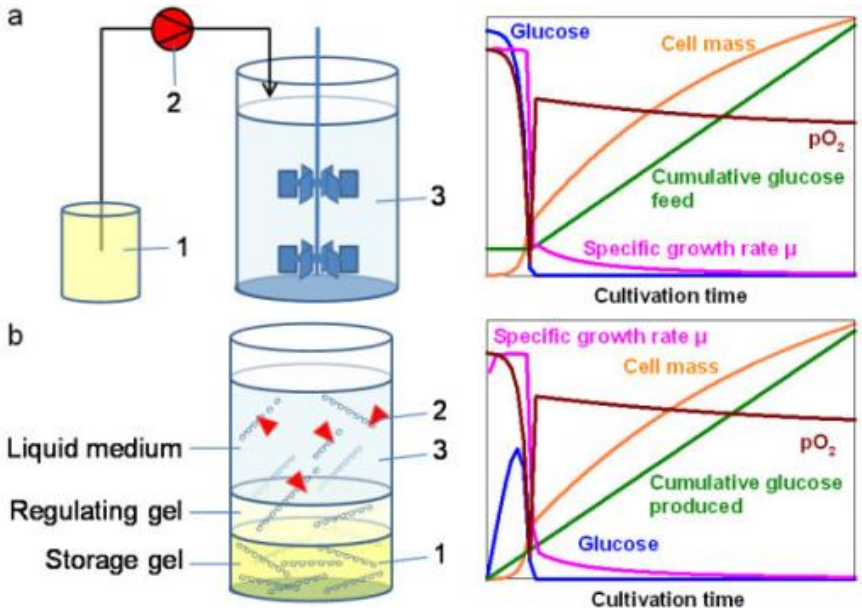


Figure 2-2 Substrate limited fed-batch cultivation in bioreactor scale (a); Enzyme based substrate delivery system (EnBase) (b) as illustrated by (Panula-Perälä *et al.*, 2008)***.*** *In the system (a): the substrate in a feed reservoir (1) enters via a mechanical pump (2) to the bioreactor (3). In the system (b): the substrate (1) is supplied via a specific concentration of the biocatalyst (2) to the cell culture (3). The right side shows a time course of the cultivation parameters during the fed-batch cultivations in both systems. In the system (a): the fed-batch cultivation starts with a high glucose concentration which decreases with time. In the system (b): the glucose concentration increases with time by the action of the biocatalyst. At the end of the batch phase; both systems will have a very low concentration of glucose.*

Also, some other controlled glucose release systems have been introduced. Jeude et al. introduced the FeedBeads system, in which glucose or other substrate is immobilized into a silicone matrix (Jeude *et al.*, 2006). Once the silicon discs are added to the aqueous solution, glucose diffuses out from the silicone material into the medium. The rate of glucose release in this system depends on the matrix, particle size of the glucose, hydrophobicity, concentration gradient between the matrix and the surrounding solution. The release rate is however not linear, and fast diffusion in the beginning may lead to substrate accumulation.
Bähr et al. have presented a system in which a concentrated feed solution is added into a reservoir tube which release the nutrient by diffusion. The feed rate is adjusted by changing the concentration of the feed solution (Bähr *et al.*, 2012).

Supplying a chemically defined medium with buffering components is necessary to maintain the optimal pH of the microbial culture in shake flask experiments. While in bioreactor cultivations, the optimal pH is usually controlled by the addition of acid or base through a mechanical pump. However, using pumps for pH control in shake flasks limit the parallel use of many shake flasks. Alternatively, a self-sustainable pH is optimized in the EnBase system through supplying the culture with a balanced mixture of organic and inorganic ammonia compounds during the

glucose limitation phase (Krause, Neubauer and Neubauer, 2016). In mineral salt medium, the consumption of ammonium ions is the main reason of medium acidification aside from the formation of acetate. In contrast in complex media, under carbon limitation conditions, bacteria start to use complex nitrogenous compounds (such as amino acids, peptones, yeast extract) as a secondary carbon source. Since the cells are not in the need of the nitrogen (which is ample in the medium composition), ammonia is released from oxidative deamination of the organic compound and released into the medium. This provides a robust mechanism for simple shaken cultivations. These two mechanisms provide in the EnBase a robust pH control that is applicable for different recombinant bioprocesses aiming the production of high yield of 'challenging' therapeutic and non-therapeutic recombinant proteins. This has been proven over different research areas like the following examples showed in Table 2.1.

Table 2.1 *Outline of different research areas with different target products produced by EnBase system*

Objective	Product
Enzymes	*Pseudomonas aeruginosa* lipoxygenase (PA-LOX) (Banthiya *et al.*, 2015) Sortases: SrtC1-2b, Srt2-2b, SrtA (Lazzarin *et al.*, 2015) Nitrilases (Veselá *et al.*, 2016) Rat lactate dehydrogenase (Nowicki *et al.*, 2015) Zebrafish ALOX2 (lipoxygenase) (Susan Adel, Dagmar Heydeck, Hartmut Kuhn, 2015) Zinc-dependent metalloprotease Zmp1 (Rubino *et al.*, 2016) α-amylase AmyM from *Geobacillus stearothermophilus* (Ploss *et al.*, 2016)
Antibodies, antibody fragments	Zinc transporter 8 autoantibodies (ZnT8A) (Skärstrand *et al.*, 2015) Different nanobody variants (Ta *et al.*, 2015) 10 scFv and 10 Fab fragments (Gaciarz *et al.*, 2016) DNA binding protein LiaR (Davlieva *et al.*, 2015) DNA binding transcriptional regulator NadR (Neisseria adhesion A regulator) (Liguori *et al.*, 2016)
Disulfide bonded proteins	AppA, a phytase (four disulfide bonds) (Matos *et al.*, 2014) Human growth hormone hGH (58) (Matos *et al.*, 2014) Human-derived glycosyltransferase GalNAcT2 (five disulfide bonds) (Lauber *et al.*, 2015)
Bioactive peptides	Non-ribosomal peptide valinomycin (Li *et al.*, 2014; Li, Jaitzig, Lu, *et al.*, 2015; Li, Jaitzig, Theuer, *et al.*, 2015) Ribosomal peptide microviridin (Gatte-Picchi *et al.*, 2014)

2.1.4 Batch and Fed-batch modes in the pre-cultivation

Pre-cultures are usually performed as a batch process although it is known to have several side effects like catabolite repression and oxygen limitation and low cell densities. Therefore, a trend towards pre-cultivation in fed-batch process has been seen. For example, a fed-batch pre-cultivation technique has been formerly introduced by Pham et al. (Pham, Larsson and Enfors, 1999). In this proposed study, 10% of the pre-cultivation medium is inoculated from a normal shake flask culture. The rest of the medium (90%) was added to the pre-culture either through constant or exponential feeding.
The inoculum age affects the vitality and fitness of the cells. This impact is clear when the cells are transferred to the main culture, as it affects the length of the lag phase and the final product yield. A recent study by Jõer and Tenson (Jõers and Tenson, 2016) showed that the cells that have last ended their metabolism in the pre-culture are those who start earliest to recover when they are exposed to a fresh medium and consequently show the shortest lag phase. Additionally, they observed that this "metabolic memory" can persist for several days. The growth resumption process depends on the type of the carbon source. For instance, glucose can promote a rapid growth resumption. The long lag phase commonly happens due to the presence of a high number of dead cells or as a result of medium change when cultivating the pre-culture and the main culture in two different media. Therefore, it is important to check the best harvest time of the pre-culture at different conditions of the cultures.

The study of Luidalepp et al. (Luidalepp *et al.*, 2011) showed that most of the cells which are transferred from stationary phase can resume growth quickly in a new medium. However, a few of these cells "persisters" remain in a non-growing state for a long time. The proportion of these persisters depends on the inoculum age, the type of the new fresh medium and the type of the chosen antibiotic. Additionally, exposure of wild type *E. coli* cells to a long stationary phase leads to the generation of more persister cells which can survive antibiotic treatment and resume growth after removal of the drug (Lewis, 2010).

The aeration level of a cultivation system is also important for achieving an efficient pre-culture. A batch pre-cultivation is not ideal because of overflow metabolism and oxygen limitation due to uncontrolled growth and high respiration rate. In contrast, with EnBase the glucose feeding rate can be adjusted to match the oxygen transfer rate of the cultivation system. The enzyme based glucose delivery system (EnBase) was applied in this study for preparation of a fed-batch type of pre-cultures while the batch pre-cultivations were demonstrated by a conventional pre-cultivation using a complex Lysogeny Broth medium (LB medium).

2.1.5 Process development for the recombinant proteins production

Process optimization and scale-up are required to get high volumetric and specific production of a target product. One of first well-documented bioprocesses for production of the natural bio-products was the industrial penicillin production at the beginning of the 1940s (Schmidt, 2005). Many industrial bioprocesses use plasmid vectors for recombinant protein production. The plasmids with a high copy number are often preferred for the production of recombinant proteins. This is because the gene dosage depends mainly on the plasmid copy number and the high gene dosage leads to higher expression levels (Friehs, 2004). Eventually, assuming that each daughter cells should get at least one plasmid during the cell division the culture can be segregationally stable. Nevertheless, since plasmid-free cells can have a reduced metabolic burden, development of plasmid free cells is possible. They often show a higher specific growth rate (μ), especially during the production phase, i.e. after induction, and thus can easily overgrow the plasmid carrying cells which leads in turn in a significant low productivity. This happens particularly if these plasmid free cells appear early in the cultivation (Friehs, 2004).

Plasmid stability can be improved by adding antibiotic to the culture thus generating a selective pressure. By this, only the cells having a developed resistance to this antibiotic by the resistance gene incorporated to the plasmid can grow (Dong-Cheol Lee and Hak-Sung Kim, 1996). The plasmid free cells will either die or stop growing. However, using of beta-lactam antibiotics like ampicillin for plasmid stabilization is problematic (Kim *et al.*, 1998; Friehs, 2004). The resistance is achieved by the expression of ß-lactamase (*bla*) gene of the plasmid. Once ß-lactamase is produced it is transported to the periplasm and further may be released into the medium where it can degrade ampicillin (Georgiou, Shuler and Wilson, 1988). Since this problem is present already in the starter culture, it is highly advisable to wash the biomass of the pre-culture before inoculation of the main cultures, if ampicillin is used for plasmid stabilization (Kim *et al.*, 1998).

As release of ß-lactamase especially happens in the stationary phase cultures, growing cultures should be used as inoculum, aside from other reasons, which were discussed above.

The optimization process of the target protein expression starts usually with small shake flasks before going to the lab scale bioreactors (Figure 2-3).

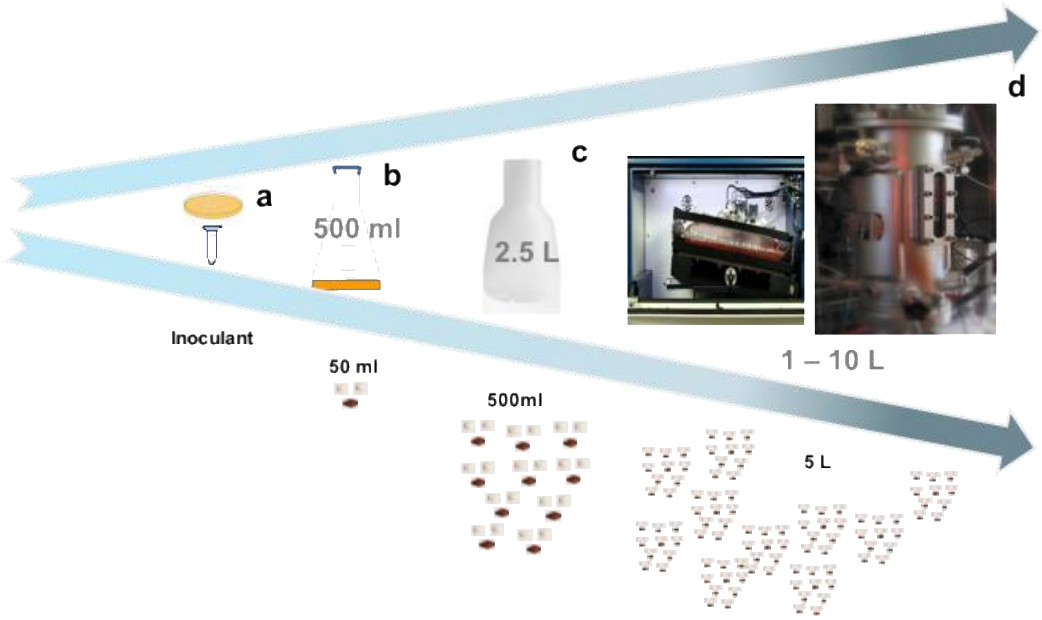


Figure 2-3 Scale-up bioprocess of NP production in E. coli BL21 [pKS2: TtPyNP] using EnBase technology. *a. vials of glycerol stocks or inoculum on the plate; b. cultivations in 50 mL scale in Erlenmeyer shake flasks; c. cultivations in small batch of 500 mL in 2.5 L Ultra yield flasks; d. bench-top bioreactor scale in 2 L stirred tank bioreactor.*

Traditionally, the sensors and online monitoring systems of the cultivation are available only for the bioreactors. It is challenging to monitor the cultures in a small volume because small volume makes connection of online monitoring electrodes difficult. Several studies have recently showed successful miniaturization of bioreactors. For instance, PreSens monitoring systems offer the possibility for online monitoring of the growth kinetics. Consequently this facilitates the optimization of the growth and protein expression (Diederichs *et al.*, 2014; Glauche *et al.*, 2015). Aside of this, there are different modern cultivation systems which have been recently introduced such as bioREACTOR 48 (2mag AG, München, Germany), Micro-24 MicroReactor (Pall Corporation, Port Washington, WI) or BioLector (m2p-labs GmbH, Baesweiler, Germany) (Knepper *et al.*, 2014). These new cultivation systems introduce the technology of miniaturization, parallelization and sensing together. This enables a better understanding of the metabolic state of the microbial cultures inside the vessel, and makes the scale-up process more robust.

Overexpression of the recombinant proteins has a vast effect on the microbial metabolism and makes the bioprocess optimization challenging. Therefore, different strategies should be applied to manipulate the cellular metabolism in order to enhance the recombinant protein production. Selection of the suitable strain or strain engineering, process development, application of the computational tools are different approaches that can be applied to enhance the recombinant protein production.

❖ **Strain engineering approaches**

The choice of the suitable strain for the bioprocess is highly important. For instance, acetate formation is strain dependent (Phue *et al.*, 2005). For example, *E. coli* BL21 produces less acetate levels and reacts more efficiently towards acetate accumulation than *E. coli* JM109 by reducing its specific glucose uptake. (Phue and Shiloach, 2004). It is reported by Phue et al. (Phue *et al.*, 2005) that acetate concentration above 40 mM (2.4 g L^{-1}) affect the growth and possibly the recombinant protein production.

Strain engineering means the modification of the genomic DNA to provide more stable constructs than those obtained via the cloning of the plasmid vectors. Gene complementation is one of the approaches that have been recently introduced for optimization of the recombinant protein expression. It facilitates the generation of stable expression strains which are not dependent on antibiotic selection. For example, in the amino acid-auxotrophy gene complementation approach, the antibiotic resistance markers can be replaced by amino acid auxotrophy complementation in auxotrophic strains. The auxotrophic strains are transformed with the plasmid that contains the gene complementing the auxotrophy. By this, the new transformed cells can grow in minimal media without amino acids supplementation (Vidal *et al.*, 2008).

Dynamic knockout of *E. coli* central metabolism aims to change the metabolic fluxes of the host in order to generate more effective cell factories. For example, Zhang et al. (Zhang *et al.*, 2015) applied this approach to identify the outer membrane proteins of *E. coli* K-12 in relevance to kanamycin resistance. Kanamycin acts through the porin channels and interact with the ribosomal protein in gram negative bacteria, inhibit the protein synthesis and increase the translation errors. They found that the deletion of the gene *tolC* (Δ*tolC*-Km) leads to two-fold decrease in the minimum inhibition concentration of kanamycin while deletion of *mipA* (Δ*mipA*-Km) leads to a four-fold increase in the minimum inhibition concentration of kanamycin.

❖ **Process development (change in culture conditions)**

Process optimization can be accomplished through optimization of the cultivation medium by supplying the medium with some building blocks like amino acids or nitrogenous compounds like yeast extract or peptone which are common sources of nucleotide bases required for protein and biomass synthesis. The cultivation medium has an impact on the *E. coli* metabolic pathway. Širkus and Neubauer introduced the application of reducing conditions for enhancement the productivity of a correctly folded ribonuclease/angiogenin inhibitors (RI) in *E. coli* K12 using EnBase fed-batch cultivation. Application of dithiothreitol (DTT) as reducing agent improved the yield of the RI in both cytpolasmic and periplasmic compartments in *E. coli* K12 (Šiurkus and

Neubauer, 2011). Another recent example was published by Wandrey et al. who presented a new approach for induction of flavin mononucleotide-based fluorescent reporter protein (FbFP) using photocaged IPTG (cIPTG). This was applied in microtiter plates based cultivation system (BioLector) using *E. coli* Tuner(DE3)/pRhotHi-2-*lac*I- EcFbFP with T7-RNA polymerase dependent *E. coli* expression system. The microtiter plate was exposed to a high-power UV-A irradiation. Upon this, IPTG is released and induce the product formation. The amount of the released IPTG can be changed in each well gradually by changing the duration of the UV-exposure, irradiance and the concentration of photocaged IPTG, added at the beginning, in an individual manner. The maximum FbFP production formation was obtained at the very early induction times, 6-8 s of UV-A irradiation, or at IPTG concentration of 60-80 μM (Wandrey *et al.*, 2016).

A recent study using *Saccharomyces cerevisiae* AH22 was presented by Glauche et al. (Glauche *et al.*, 2017). In this study, the secretion of heterologous endo-polygalacturonase (EGP) using *S. cerevisiae* AH22 was characterized in parallel miniaturized cultures using microwell plates with enzyme-based-fed-batch medium. By changing the enzymatic glucose release, different growth rates (μ) was obtained. The growth-dependent product formation profile was consequently analyzed.

E. coli strains with altered levels of alcohol dehydrogenase ''ADH'' or pyruvate decarboxylase ''pdc'' (like *E. coli* KO11 with *Zymomonas mobilis* pdc) show a reduced metabolic flux from pyruvate to the fermentative pathways when they are cultivated in a defined medium if compared with the complex medium (Underwood *et al.*, 2002). Moreover, the metabolic flow of the carbon into α-ketoglutarate in the tricarboxylic acid cycle will be restricted which reduces the growth yield. This study also reported that the detrimental effect of this metabolic engineering was reduced by addition of pyruvate to the mineral salts medium containing 1% corn steep liquor and 10% xylose (Underwood *et al.*, 2002). Therefore, the process optimization can be achieved by supplementation of some specific nutrients whether to promote the protein synthesis or to promote certain metabolic pathway.

Starvation-inducible promoters based on alkaline phosphatase gene (*phoA*) have been applied to the induction of the target genes (Wanner, 1993; Champion *et al.*, 2001). The target genes under the control of *phoA* promoter are induced when phosphate is depleted in the cultivation medium. Huber et al. (Huber *et al.*, 2011) studied the effect of the induction time along with phosphate limitation on the specific productivity of the T7 expression system *E.coli* BL21(DE3) pRhotHi-2-EcFbFP which produces a fluorescence protein EcFbFP. They claimed for 21% to 31% enhanced productivity of EcFbFP upon the phosphate limitation in the cultivation medium. The idea behind this approach is reduction in the cell division or biomass formation by the phosphate limitation while the protein synthesis is not hindered. This is especially beneficial in high throughput cultivations wherein the cultivation volume is constant and cannot be changed and the biomass cannot increase due to limited oxygen transfer rates.

Cultivation mode also affects the cellular responses towards the metabolic stresses of the host cells that might be initiated during the cultivation like the nutrient limitation (Carneiro, Ferreira and Rocha, 2013). For instance, high cell density cultures (HCDC) have been preferred for the recombinant bioprocesses with *E. coli* (Choi, Keum and Lee, 2006). However, HCDC may suffer from several problems like limitation of substrate or oxygen. These limitations induce metabolic stresses and consequently expression of stress responsive proteins which compete for the translation machinery of the host and for energy and metabolic sources which results in productivity loss.

❖ **Computational tools**

Metabolic or stoichiometric models are often used to identify the optimum cultivation parameters or a specific reaction in order to improve the final yield of the target product. Genetic modifications can be applied with the computational and modelling tools in many biotechnological processes. For example, (Cruz Bournazou *et al.*, 2016) introduced an efficient computer-aided bioprocess development. They applied an online optimal re-design of parallel dynamic processes, and estimated the parameter set for kinetic models for *E. coli* fed-batch processes with to aim to perform the optimization with a minimal number of experiments.
(Chemler *et al.*, 2010) used a constraint-based metabolic model to identify combinations of knockout candidates to maximize the growth associated products as well as the production rate of NADPH which is extensively needed in protein biosynthesis.
(Sarkandy *et al.*, 2010) used a stoichiometric model to identify the most-needed amino acids to enhance the production of interleukin-2 (IL-2). By predicting and supplementing the needed amino acids, leucine, aspartic acid and glycine, the final yield of IL-2 increased by two-fold.

2.2 Protein expression phase

Protein expression phase is the time of generating a specific protein. Most commonly used expression systems (e.g. the T7-RNA polymerase / pET system) contains components of the *lac* operon, relies on the inducible T7 RNA polymerase (RNAP) because it results in expression of high yields of recombinant proteins. The coding sequence of the RNAP is inserted to the bacterial chromosome under the control of the inducible *lac*UV5 promoter and the *lac* repressor (*lac*I) blocks the expression of the T7 RNAP coding sequence. While T7 RNAP produced after induction specifically transcripts the coding sequence of the target protein which is inserted to the expression plasmid under the control of T7 promoter (Briand *et al.*, 2016).
Leaking expression (basal protein expression) is a phenomenon happening when a low protein expression occurred during the growth and prior to the induction. This leaking expression might cause a bacterial growth inhibition if the expressed protein was toxic, but also due to the metabolic load of the extra protein synthesis.

2.2.1 Basal protein expression

Basal expression is the expression of a heterologous protein as a result of a background expression in the absence of an inducer. In expression systems based on *lac* promoter this is often associated with a low level of the repressor protein (*lac*I). The basal expression, though usually very low, can have a strong impact on the growth of the culture and the normal proliferation of the strain as well as on plasmid stability. There are many approaches to minimize this unintended expression: 1. Introduction of *lac*I^q which is a mutant of the repressor protein with a higher expression level (Calos, 1978) , 2. Expression of the inhibitor of T7 RNA polymerase (T7 lysozyme). T7 lysozyme binds to RNAP and inhibits the transcription from T7 promoter and consequently controls the unintended expression of heterologous proteins under T7 promoter (Neubauer and Winter, 2001; Studier, 2005). The rhamnose inducible promoter (rhaP$_{BAD}$) is also applicable for the tight regulation of the recombinant protein expression in *E. coli*. Rhamnose in this case acts as an inducer and control the rhamnose regulon (Haldimann, Daniels and Wanner, 1998), 3. Supplying the medium with glucose concentration between 0.2 - 1% is helpful for controlling the basal expression level of any promoter inducible with sugars or sugar analogues. The growth of the host should go well after basal expression control; however if protein expressed is still toxic the growth will be stopped (Rosano and Ceccarelli, 2014).

2.2.2 Induction of the recombinant protein expression

The major goal of any bioprocess is obtaining correctly folded, functional recombinant protein of interest in high yields. Induction of the protein expression at the suitable time point in the cultivation process is very important. However, the physiological conditions of the culture at the time of induction has a great impact on the metabolic response towards a successful protein expression.

For instance, if the induction is done at the mid-log growth phase, cells can provide the sufficient energy and basic requirements that are needed for the protein synthesis. If the induction is done at the late-log growth phase or at stationary phase the cells will not be in the same catabolic capacity, and recombinant protein yield may be poor. Moreover, stress proteins that are usually present during the stationary phase like proteases will impair the final yield of the target protein (Carneiro, Ferreira and Rocha, 2013). Therefore, it is quite important to fine-tune the conditions for the recombinant protein expression.

The *lac* operon provides all the main components required for the induction of the recombinant protein expression in *E. coli*. (i) *lac* operator (*lac*O); which represents the binding site for the *lac* repressor, (ii) *lac* promoter (*lac*P) which represents the binding site for the RNA polymerase (for the transcription of the gene into molecules of messenger RNA (mRNA)) and (iii) *lac* repressor

(*lac*I) which binds to the *lac*O in the absence of lactose and blocks the binding of the RNA polymerase to the lac promoter (*lac*P). (Mayer *et al.*, 2014).

The induction process can be applied with chemical inducers like IPTG; nutrients like lactose or galactose (Gombert and Kilikian, 1998; Studier, 2005; Xu *et al.*, 2012) or even by shifts in some physical/chemical factors of the bioprocess like temperature for thermo-inducible *lac* expression system (Yabuta, Onai-Miura and Ohsuye, 1995). Also, a combination of different inducers can be used for expression.

While there are many studies and reviews which refer to the use of the *lac* promoter, an interesting approach is the study by (Su, Hong and Wu, 2015) for expression of *Thermobifida fusca* cutinase in *E. coli*. This study showed that the best induction conditions were obtained with a combination of IPTG and lactose for induction at dry cell weight of 13 g L^{-1}. In the case of this publication IPTG was added at once while lactose was fed to the cultivation at a rate of 0.5 g L^{-1} h^{-1}.

The study by (Herai *et al.*, 2004) used a different regulatory system "P_{nitA}-NitR" which was developed for gene expression in *streptomycetes*. This system is based on the expression mechanism of *Rhodococcus rhodochrous* J1 nitrilase which is induced by ε-caprolactam. Fine-tuning of gene expression and expression of different heterologous proteins have been demonstrated using this system which is characterized by a low level of basal expression and hyper-inducible expression.

Balzaer et al. (Balzer, Kucharova and Megerle, 2013) compared different regulator/promoter systems (XylS/*Pm* (wild-type), XylS/*Pm* ML1-17 (a *Pm* variant), *lac*I/P_{T7lac}, *lac*I/P_{trc} and *Ara*C/P_{BAD}) by controlling the design of the replicon backbones (RK2- or pMB1- origin of replication). They obtained high expression levels which correlated the replicon copy number, and combination *lac*I/P_{T7lac} gave more transcripts than the other cassettes used in this study.

2.2.2.1 Alternative gene regulation systems for recombinant protein production in E. coli **(Stress-response promoters)**

Synthetic promoter systems which are easily induced by environmental conditions of the culture and show low or no basal expression when the promoters are repressed are extremely advantageous for recombinant protein expression.

Some earlier studies have addressed gene regulation using synthetic promoters that can be easily induced by specific metabolic conditions, e.g. inorganic phosphate starvation (Wanner, 1993); pH regulation (Tolentino, G.J., Meng, S.Y., George, N. Bennett, G.N., and San, 1992); high medium osmolarity (Herbst, Kneip and Bremer, 1994); low oxygen levels (Sawers and Jarsch, 1996); and oxygen limitation conditions (Lee, Cho and Lee, 1996).

Schroeckh et al. used a modified *E. coli* glnAP2 promoter system which is induced when the ammonium concentration in the medium below 1 mM (nitrogen limitation) (Schroeckh *et al.*, 1996). This promoter is especially useful in the expression of proteins which are targeted to the periplasm in order to achieve oxidative conditions for correct formation of disulfide bonds. Such protein includes Fab fragments and KI-streptokinases, which are easily produced as incorrectly folded proteins when very strong promoters are used. This modified *E. coli* glnAP2 promoter system overcomes such problems.

Another approach was proposed by (Bulter *et al.*, 2004). It is based on the construction of a gene-metabolic circuit to create quasi artificial quorum sensor. The idea is based on the use of acetate accumulation in the medium as a signal for gene expression by a two-component system consisting of acetate kinase and the nitrogen-regulation system.

While the use of starvation promoters was already addressed in the 1990s by A. Matin and others (Reeve, Bockman and Matin, 1984; Blum *et al.*, 1990), synthetic stationary phase/ stress promoters were applied by a Miksch et al. (Miksch, Bettenworth, Friehs, Flaschel, *et al.*, 2005) who evaluated them with green fluorescence protein (GFP) as a reporter protein. The induction process of these promoters is happening during the entrance into the stationary phase and took around 1.4 to 2.7 hours. They reported that these synthetic promoters showed a three to four-fold higher activity than the natural promoters.

2.2.2.2 State of the art in induction strategies

Due to major limitations coming from using the conventional induction protocols, the research is always going further to address these limitations. For example, a study published in 2016 (Briand *et al.*, 2016) reported a new system called Self-inducible Expression system (SILEX) which is principally based on the autoinduction principle. They engineered genetically *E. coli* BL21 (DE3) containing SILEX plasmid that encodes the human heat shock protein 70 (hHsp70) and another plasmid that encodes the target protein (six diverse proteins were chosen in this study as follows: *Richardella dulci ca* miraculin (MCL), *Xanthomonas campestris* methionine sulfoxide reductase B (MsrB), *E. coli* purine nucleoside phosphorylase (PNP), *E. coli* thioredoxin 1 (Trx1), *H. sapiens* glutathione transferase A1 (GSTA1) and the N-terminal domain of *H. sapiens* taste receptor type 1 member 1 T1R1 (T1R1)). The process starts by interaction between the slowly expressed hHsp70 and an endogenous protein from the host organism (GAPDH, glyceraldehyde 3-phosphate dehydrogenase), which improves the host cell potential for efficient expression. Accumulation of the inducer (lactose) results in removal of the *lac* repressor protein and subsequently enhances hHsp70 expression by autoinduction and at the same time induces the recombinant protein expression. The authors reported that the yields of the tested proteins

obtained by the SILEX system were at the same level obtained by classical IPTG induction without any addition of external inducers or medium components.

Other studies by (Tsao *et al.*, 2010; Nocadello and Swennen, 2012) succeeded to couple the gene expression with the cell density using cell-to-cell communication (Quorum sensing; QS). They designed a new expression system for expression of different recombinant proteins expression in *E. coli* under the control of *lux* regulon genes of *Vibrio fischeri's* QS system.

Tandem repetitive promoters (TRP) is a recent strategy of constructing promoter clusters with a multi-copy promoter in tandem in order to improve the gene expression (Wu *et al.*, 2016). In this study, *ybaC* gene encoded the target enzyme acetyl esterase (EA) was cloned from *E. coli* RB3 and the expression vector pKK223-3-*ybaC* was constructed. The vector was then introduced into *E. coli* JM110 to obtain recombinant (1) *E. coli* JM110 (pKK223- 3-*ybaC*) (EcKy). In parallel, core-*tac*-promoter (C*tac*) was inserted into the expression vector pKK223-3-*ybaC* to generate a new vector pKTRP-*ybaC*. This new TRP vector was introduced into new *E. coli* JM110 cells to obtain recombinant (2) *E. coli* JM110 (pKTRP-*ybaC*) (EcTy).
The parental strain (*E. coli* RB3) and the two recombinant *E. coli* JM110 (EcKy and EcTy) were cultivated in LB medium. The highest EA activity was obtained from EcTy which was 131-folds of that from the parental strain. The transformant with the tandem repetitive promoters showed also the highest level of the transcription. Moreover, the thermal stability of EA was improved EcKy and EcTy compared to the crude enzyme from the parental strain.
Similarly, expression of the green fluorescence protein (GFP) in the engineered strain (with a 5 copies tac-promoter) was enhanced 4.4-fold in comparison to the strain containing only one copy of the promoter (Li *et al.*, 2012).

Weuster-Botz et al. (Schmideder, Cremer and Weuster-Botz, 2016) presented a new induction strategy based on a gradual induction for scale-up from the milliliter scale to the liter scale fed-batch process. In this study, a miniaturized bioreactor system was used for performing a continuous operation of 8 parallel chemostats, 10 mL each, for the production of recombinant photoactivatable mCherry (PAmCherry) with *E. coli* BL21 (DE3) under the control of the T7 RNA polymerase expression system. The induction started by a single addition of 1 mM IPTG and then different induction strategies were performed like reducing the IPTG concentration gradually to 100 µM and continuous addition of IPTG.

Compared to induction with a single dose of 1000 µM IPTG, they obtained a 21% higher protein yield by the dynamic increase of IPTG concentration to 100 µM. The best-performed induction strategy was transferred from mL-continuous process to a liter scale fed-batch process. By this, the feasible reaction conditions for fed-batch processes were identified in parallel, small-scale continuous cultivations.

A large number of biosensors based on stress-related genes has been developed in the last few years. A promoter that controls the stress-related genes is cloned into a plasmid. This plasmid is then transferred to a genetically modified organism which carries a reporter gene. The plasmid will be responsible for controlling the expression of this reporter gene. The system will act as a biosensor for different stresses (Polizzi and Kontoravdi, 2015). For example, a biosensor based on nitrile reductase promoter has been used to detect the oxygen levels in *E. coli* bioreactor cultivations (Garcia *et al.*, 2009). In another example, the biosensors had a dual function in GFP expression under bacterial membrane stresses. They act as an indicator for the GFP leakage into the culture broth and as an indicator for the membrane integrity (Brognaux *et al.*, 2013).

At the level of the gene expression, a study for Delvigne et al. monitored the expression of rpoS gene, which is connected to the stress response in *E. coli*, by the synthesis of the reporter green fluorescent protein (GFP). They addressed an increase in the GFP level during the transition from batch to fed-batch phase. A significant decrease in the rpoS gene level was also observed in the bioreactors in which the mixing conditions are bad (Delvigne *et al.*, 2009).

2.2.2.3 Autoinduction and Diauxie

As extensively discussed in the sections above, the major goal of any fermentation process is to increase the production of the target protein and many factors affect the yield of the protein per cell and the cell productivity. Induction time is one of the crucial factors that has impact on the expression system.
Autoinduction is based on de-repression of the *lac* promoter in glucose deficiency conditions as a result of diauxic regulation. Diauxic regulation is activated when the cells are exposed to a mixture of two growth-limiting substrates as originally described in the PhD thesis of Jacques Monod (Monod, 1942). Cells show a different substrate consumption patterns (Fischer *et al.*, 1998; Studier, 2005). Lactose is often used as an inducer for autoinduction of *lac* derived promoters. Alternatively, autoinduction can be applied for different carbon sources like arabinose when using the *araB* promoter from *Salmonella typhimurium* (Cagnon, Valverde and Masson, 1991). In both cases, mutant strains in the catabolism of these sugars are sometimes used to ensure that lactose or arabinose will act only as inducers and not as carbon sources (Neubauer and Winter, 2001).

In the beginning of the diauxie, the glucose acts as a substrate for the growth initiation. It is transported into the cells via the phosphotransferase system for sugar uptake (PTS). Enzyme E III (EIII, one of the PTS components is specific for glucose) is dephosphorylated (EIIId) and inhibits the transport of any sugars other than glucose. This dephosphorylated form of PTS enzyme III binds to the lactose permease and inhibits the lactose transport; which is called carbohydrate-mediated inducer exclusion. During this phase, the expression of the target protein is repressed while undisturbed cell growth with high cell densities is achieved (Figure 2-4 (A)). During the

growth of the cells on glucose, the uptake and utilization of lactose is prevented as a result of inducer exclusion and the expression of *lac* operon components, namely lactose permease (*lac*Y) and β-galactosidase (*lac*Z) which are responsible for lactose uptake and utilization respectively, is reduced (Figure 2-5).

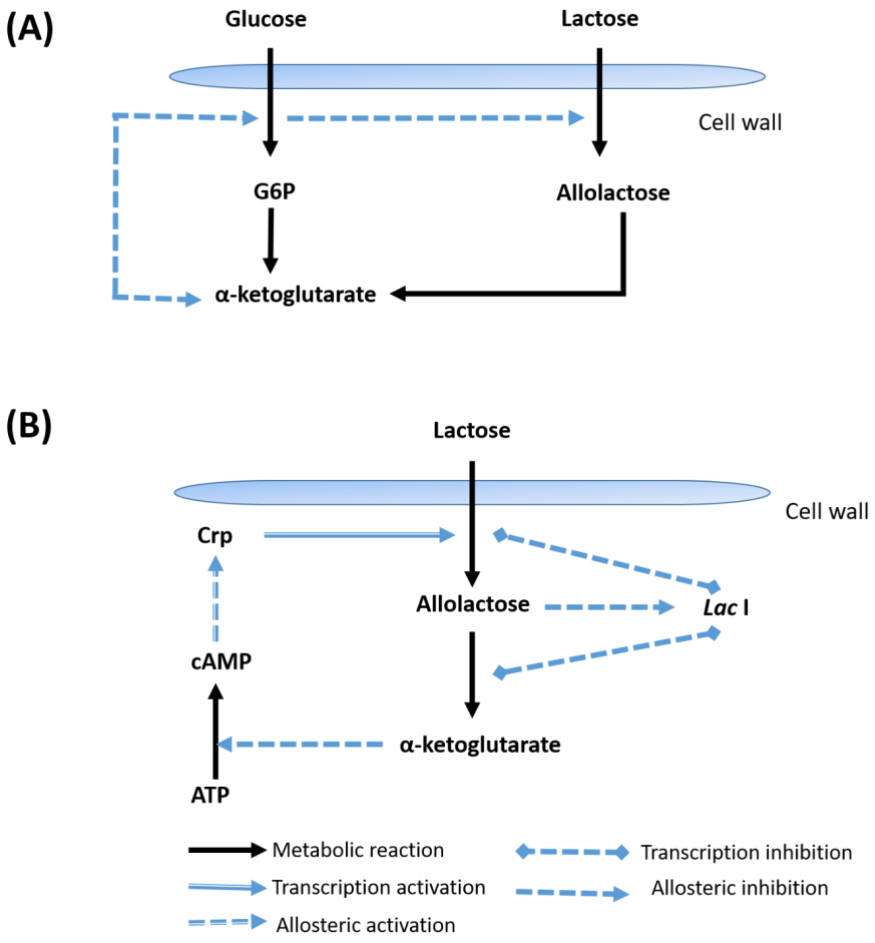


***Figure 2-4 Crp-mediated regulatory circle of sugar uptake in the diauxic growth of E. coli*. (A)** Sugar uptake regulation; **(B)** Control loop of the sugar uptake.

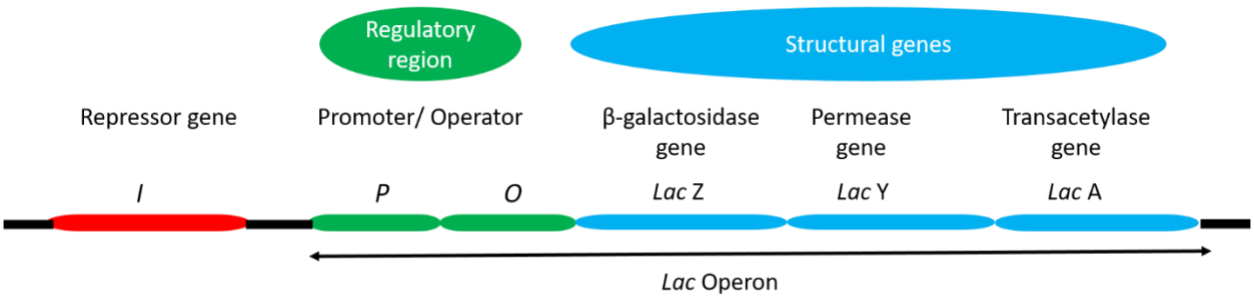


Figure 2-5 The structure of the lac operon

After glucose exhaustion, the de-repression starts with a transient growth arrest called diauxic lag phase. The phosphorylated EIII will be dominant and activates the enzyme adenylate cyclase (Ac). This enzyme stimulates the synthesis of intracellular messenger 'cyclic adenosine monophosphate' (cAMP) which in turn activates the transcription factor (cAMP receptor protein; Crp). cAMP-Crp complex has the regulation function of the lactose uptake via expression of the genes which are involved in the lactose catabolism (Deutscher, Francke and Postma, 2006; Bettenbrock *et al.*, 2007; Shimada *et al.*, 2011). Another study for (Chubukov *et al.*, 2014) pointed out that α-ketoglutarate and related α-ketoacids prohibit the synthesis of the enzyme adenylate cyclase (Ac) and in turn affect the Crp-mediated activation of the carbon-uptake genes (Figure 2-4 (B)).

This diauxic lag phase is considered as a stress phase during which the *lac*Y and *lac*Z genes start to be induced for lactose uptake and consumption. As a stress response, the general stress sigma factor of *E. coli* σ^S, *rpoS* gene product is strongly induced under glucose limitation and, interestingly also in the diauxic lag phase to change the transcriptional program to one that promotes survival. *RpoS* gene induction is followed by activation of different σ^S-dependent genes which may lead to change in cell morphology and physiology (Fischer *et al.*, 1998). The stringent response is another level of response at which the level of cellular amount of (p)ppGpp increases as a result of starvation of energy source or amino acids, which is followed by a series of metabolic changes. The accumulation of (p)ppGpp is accomplished through the synthesis of the *relA* genes, (p)ppGpp synthetase. It also requires GTP, ribosome, mRNA and uncharged tRNA bound to the ribosome (Figure 2-6). The stringent response is attained not only by (p)ppGpp accumulation but also by slow degradation of (p)ppGpp by the *spoT* genes (Steinchen and Bange, 2016). However, interplay between the σS response, the stringent response and the cAMP response has not been investigated in details. Therefore, the diauxic growth system is not fully understood at the proteomic level.

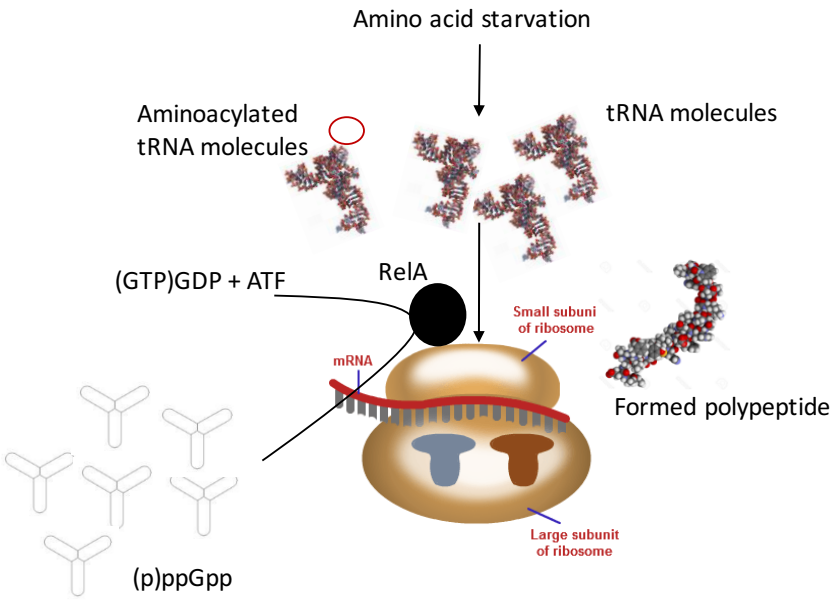


Figure 2-6 Scheme for synthesis of (p)ppGpp molecule under amino acid starvation

Once the cells restart growth on lactose, the expression level of these genes starts to reduce again (Fischer *et al.*, 1998). Lactose is then isomerized by action of β-galactosidase (encoded by *lac*Z) into allolactose which binds to the repressor protein *lac*I and cause the induction of heterologous protein expression. However, lactose concentration in the autoinduction medium should be limited because a high concentration of lactose may result in higher growth rates and oxygen limitation problems.

Expression strains applicable for autoinduction should contain a specific transporter for the sugar, or the other requirements according to the inducing sugar should be fulfilled. For instance, lactose uptake requires lactose permease (*lac*Y gene product) while β-galactosidase (*lac*Z) is needed for breaking the bonbon between glucose and galactose. In another example, induction by galactose requires a strain that lacks galactosekinase; otherwise galactose will be soon metabolized by the cells. IPTG is generally thought to enter the cells by passive diffusion. However, it has shown that when present in very low concentration, IPTG will enter the cell by the lactose permease activity. Consequently, with suitable concentration, IPTG can be used for autoinduction (Khlebnikov and Keasling, 2002; Fernández-Castané *et al.*, 2012).

Glycerol, arabinose or galactose can act as supporting carbon sources that do not prevent the use of lactose during the protein production phase, but support the protein yield and cell density (Studier, 2005). Glycerol is a good choice since it does not initiate catabolite repression on lactose consumption. Galactose can also act as an inducer for recombinant protein expression. Lactose is enzymatically hydrolyzed to glucose and galactose. Glucose is used as a carbon source while

galactose first is discharged into the medium and only consumed after the availability of glucose is limited but is an inducer at the same time (Xu *et al.*, 2012).

Lactose could be also used as an inducer in fed-batch cultivations in which the growth is initiated by continuous supply of glucose as the growth limiting factor (Pei *et al.*, 2010). In fed-batch cultivations the glucose concentration in the medium is very low though the continuous supply of glucose. Its availability may result in down-regulation of lactose uptake, but the intake of lactose is not completely prevented (Ukkonen, Mayer, *et al.*, 2013). In connection to this, (Ukkonen, Mayer, *et al.*, 2013) showed that continuous feeding of glucose during the protein expression in diauxic shift is possible. By this lactose will act as an inducer and will not be efficiently catabolized.

Four different *E. coli* genotypes were investigated for their growth behavior in diauxic growth cultivations with different glucose and lactose concentrations of 3.0 and 5.0 g L^{-1}. Three out of four strains are *E. coli* K-12 strains (BW25113, MG1655 and W3110) while the fourth is *E. coli* B strain (BL21). Figure 2-7 showed the derivatives of the *E. coli* genotypes used in the diauxie study, as it was presented by **(Bachmann, 1996)** .

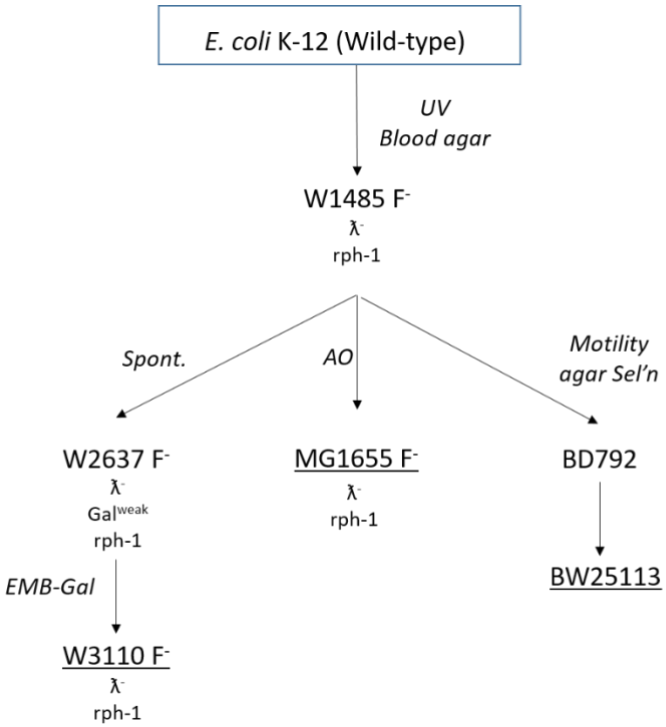


Figure 2-7 Derivatives and genotypes of Escherichia coli K-12

2.2.2.4 Advantage of autoinduction over conventional induction protocol

Autoinduction has several advantages over conventional induction protocols. By supplying the different cultures with the same carbon sources, parallel induction of the cultures without the need for addition of an external inducer can be easily accomplished (Diederichs *et al.*, 2014). Growth of the cells until saturation (growth at the maximum specific growth rate; μ) before starting the protein expression ensures initiation of a seed culture with high fractions of fully inducible cells (Studier, 2014). All the parallel cultivations will be then induced at the same cell density, allowing comparative studies with minimal manual input.
However, precautions must be taken in case of using strains expressing proteins which cause stress on the cells. In that case, growth until saturation may increase the risk that the culture is overgrown by cells that have lost the plasmids (Dubendorf and Studier, 1991).

By induction of the target protein using autoinduction medium with slower growth/ expression rates, many protein expression related problems could be avoided. For instance this smooth induction decreases aggregation and thus the accumulation of inclusion bodies (Studier, 2005).
One more apparent advantage for the autoinduction is the automatic transition from uninduced state of the cells to the induced cultures under the control of metabolic activity of the cells. This will reduce the manual handling of the experiment which perfectly fits in high throughput applications (Blommel *et al.*, 2007). However, autoinduction has a few drawbacks. For instance, it cannot be used with host strains that have deletions in the lac operon genes.

2.2.2.5 Factors affecting the efficiency of the autoinduction

Many factors interfere with the efficiency and the success of the autoinduction process, yield of the target recombinant protein and the biomass. Some of these factors are chemical like composition and concentrations of the complex medium or medium components which is pivotal on the protein expression. Some factors are physical, like the process parameters which may be difficult to change, because they may interfere with the parameters required for the optimal bacterial growth. Some of these factors are addressed here.

a) Chemical and structural factors.

Composition of the complex medium affects to a high extent on the cell growth, cell saturation and unintended induction in some cases (Studier, 2005).

- Complex media contain normally amino acids and small peptides. They are contained by enzymatic digests of nitrogenous compounds of the medium like tryptone, peptone or yeast extract. Lactose may be present in these enzymatic digests and since it is an inducer of T7 expression system, minor concentrations of lactose in these digests can promote higher level of induction especially in lower rates of aeration (Studier, 2005).

- Glucose concentration in the complex media is also crucial for the autoinduction. Mainly, glucose represses the induction process. Therefore, glucose concentration should be chosen to be depleted by mid to late log phase of the cultivation and then autoinduction starts actively. However, (Studier, 2014) pointed out that finding the concentration of glucose that prevents the induction process without affecting the culture pH at saturation is difficult.
- The strength of induction under the control of *lac* operon is also cAMP-CRP sensitive. High level of cAMP leads to higher expression of *lac*Y (lactose permease) and consequently higher lactose uptake and higher titers of protein expression under glucose limitation conditions (Deutscher, Francke and Postma, 2006; Bettenbrock *et al.*, 2007; Shimada *et al.*, 2011).
- Yeast extract enhances autoinduction of phage T7 RNA polymerase (RNAP) and expression of recombinant protein in *E. coli* BL21 in the long-term cultivations (Jia *et al.*, 2011). Baracat-Pereira et al. also suggested that a low molecular weight effector is present in the yeast extract and responsible for maintenance a high level of cAMP in the fungal cells of *Pecnicillium griseoroseum*. They characterized this effector as an organic, thermostable, polar substance activated by heat, soluble in the nucleotide fraction during the extraction of the yeast extract. They observed an six-fold increase in the cAMP level in the first minute of the yeast extract addition to the culture (Baracat-Pereira *et al.*, 1999).
- Yeast extract supports also the cell growth. However, the concentration of yeast extract in the complex medium should be optimized. High content of yeast extract can lead to a drop in cell viability, plasmid instability through autoinduction of proteins and unintended induction in bioreactor cultivations (Jia *et al.*, 2011).
- Galactose results from the hydrolysis of some complex components like yeast extract. Galactose cause unintended induction in *E. coli* BL21 strains because they lack galactose kinase and cannot metabolize it. This leads to galactose accumulation to a limit that can induce the *lac* operon (Studier *et al.*, 2009; Xu *et al.*, 2012).
- Availability of allolactose during autoinduction for expression systems under *lac* operon control promotes protein expression (Hoffman *et al.*, 1995). Blommel et al. reported that using a plasmid with a high copy number may produce 200-fold higher levels of *lac* repressor compared to the concentration present in wild type cells (Blommel *et al.*, 2007).

b) Physical factors.

- Cultivation time has an impact on the success of the autoinduction and protein expression. In some cases long-term cultivation until saturation phase is necessary for autoinduction in order to reach high cell densities (Studier, 2005).
- Autoinduction works over wide range of temperature between (18-37°C) (Studier, 2014). This gives autoinduction an advantage since some proteins are soluble if expressed at low temperatures, while growth saturation is easily reachable at higher temperatures.
- Aeration is an important factor that affects the induction process especially when using lactose as an inducer for the protein expression. Oxygenation state of the autoinduction culture affects the order of the carbon sources uptake (Blommel *et al.*, 2007). Under oxygen limited conditions, the lactose consumption is preferred (earlier lactose uptake and protein expression), while under oxygen sufficient conditions, the lactose consumption is delayed.
- The impact of oxygenation state of the culture on the level of protein expression is very crucial especially in high throughput applications (Duetz and Witholt, 2004). Since the oxygen consumption rate can differ between different clones, oxygen saturation levels in different wells can vary in cultivations performed in multiwall plates (Ukkonen, Mayer, *et al.*, 2013).

2.3 Thermophilic Nucleoside phosphorylase "NP's" as a model recombinant protein of the study

Nucleoside phosphorylases (NP's) are a group of ubiquitous enzymes that have been found in bacteria, eukaryota, Archaea and dsDNA viruses. They catalyze the reversible phosphorolysis of nucleosides in prokaryotes and eukaryotes (Almendros, Berenguer and Sinisterra, 2012). A number of NP's have been also reported to be used as biocatalysts for synthesis of modified nucleosides by combining an acceptor nucleobase and pentose-1-phosphate. These modified nucleosides can be considered as group of antiviral agents and anticancer drugs (Szeker *et al.*, 2012). Nucleoside analogues can be synthesized chemically or enzymatically. However, the enzymatic synthesis of these analogues has many advantages over the chemical method. It involves mild reaction conditions and is an environment friend method without the need to use extra reagents or organic solvents (Zhou *et al.*, 2015).

NP's are classified into two main classes according to their substrate specificity. First class is Purine NPs (PNP's; EC 2.4.2.1) and the second class is pyrimidine NP's (PyNP's; EC 2.4.2.2). PyNP's are further divided into three categories: uridine phosphorylases (UP), thymidine phosphorylases (TP) and general-purpose PyNP's which accept both uridine and thymidine. There are some other classifications according to their molecular mass and structure (Szeker *et al.*, 2012).

Expression of heterologous thermos stable enzymes from thermophiles in *E. coli* is attractive in different applications. However, biosynthesis of these thermozymes in mesophilic *E. coli* is challenging. One of these challenges is the formation of stable secondary structure at 5' end of mRNA. These challenges affect the protein expression levels as well as the folding process. Therefore, different approaches haven been introduced to overcome these problems like (a) raising the cultivation temperature above the normal temperature used for recombinant protein expression (Szeker *et al.*, 2010), (b) substitution of the base pairs in the 5' coding sequence of the target gene with a sequence encoding the hexahistidine tag (Szeker *et al.*, 2010, 2012).

TtPyNP, the model protein of this study, is one a PyNP (UP) which belongs to a class of enzymes known as thermozymes. It has been isolated from the thermophilic strain *Thermophilus thermophilus* HB27. This group of thermozymes is resistant to high temperature (up to 80°C), pressure and organic solvents (Konisky, Michels and Clark, 1995). They can be expressed in *E. coli* at high levels and easily purified by heat treatment (Zhou *et al.*, 2013).

3. Material and Methods

3.1 Bacterial strains and expression vector

The strain used for recombinant NP production in this study was *E. coli* BL21 (Novagen®). TtPyNP gene with Hexahistidin-Tag at 5' -end (GenBank accession number AAS81754.1) was cloned in expression vector pKS2 (Szeker *et al.*, 2012) and then it was transformed into the *E. coli* BL21 cells. The vector used is characterized by an IPTG- inducible *lac* promoter derivative and an ampicillin resistance cassette.

Additionally, wild *E. coli* strains BW25113, W3110 (ATCC 27325), BL21 (New England BioLabs) and MG1655 (DSM 18039) were used in this study for the diauxy-part.

3.2 List of Materials

Table 3.2-1 *List of Devices*

Device	Name	Manufacturer
24 Deep well plates	OxoDish OD24 HydroDish HD24	PreSens-Precision Sensing GmbH, Regensburg, Germany
Sensor Dish Reader (SDR) **Shake Flask Reader (SFR-Vario)**		PreSens-Precision Sensing GmbH, Regensburg, Germany
Microplate reader	Synergy™ MX	BioTek Instruments GmbH, Bad Friedrichshall, Germany
Microplate	96 well F-bottom plate	Greiner Bio-One GmbH, Frickenhausen, Germany
Shaking incubator	Lab Term LT-X	Adolf Kühner AG, Birsfelden, Suisse
Shake flasks	Ultra-Yield Flask (UYF)	Thomson, San Diego, USA
	Shake flask with integrated sensors	PreSens-Precision Sensing GmbH, Regensburg, Germany
Shake flask sealing	AirOtop® enhanced flask seal™	Thomson, San Diego, USA

Sterile filter (0.45 µm and 0.2 µm)	Rotalibo® Spritzenfilter	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Photometer	Ultraspec Plus	GE Healthcare, München
Thermo Shaker	Dry Bath-ThermoCell Mixing Block	BIOER Technology, Beijing, China
Electrophoresis power		PharmaciaBiotech
pH meter	FE20/EL20	Mettler-Toledo GmbH, Gießen, Germany
Centrifuge		Hitachi Koki CT15E, Japan
Off-gas analysis system	BlueInOne$_{ferm}$-Sensor	BlueSens GmbH
2L bioreactor	KLF 2000	Bioengineering AG, Switzerland

Table 3.2-2 *List of softwares*

Software	Use	Manufacturer
Gen5™ 1.11.5	Synergy™ MX control	BioTek Instruments GmbH, Bad Friedrichshall, Germany
SDR version v4.0.0	Sensor Dish reader control	PreSens-Precision Sensing GmbH, Regensburg, Germany
SigmaPlot 12	Graphs and figures	Systat Software GmbH, Erkrath, Germany

Table 3.2-3 *List of chemicals*

Chemical	Name	Manufacturer
EnBase® Medium	EnPresso™ B und EnPresso™ B defined Medium	Biosilta, Oy, Finland
Glucose monohydrate	Dextrose monohydrate	Roquette Frères, Lestrem, France
Glucose Hexokinase FS kit		Diagnostic Systems GmbH, Holzheim, Germany
Lactose/ D-Galactose kit		Boehringer Mannheim / R-Biopharm
Protein assay kit	BCA Protein Quantitation Kit	BioVision, Inc.
BugBuster™ Protein Extraction Reagent		Novagen® GmBH

Benzonase® Nuclease	Purity > 99%	Novagen® GmBH
NaCl		VWR ProLabo®, USA
Ampicillin		VWR ProLabo®, USA
NaOH	NaOH ≥ 99 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Glycerol	Glycerin, water-free	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
All other chemicals were obtained from Sigma-Aldrich (Steinheim, Germany).		

3.3 Cultivation media

Four types of media were used in this study according to the aim of the experiment. Description of these media is presented below.

3.3.1 Enzyme based substrate auto-delivery system (EnBase)

Two different types of fed-batch like growth systems used in this study; EnPresso B and EnPresso B defined with a defined chemical composition (defined medium). Both media are based on the EnBase technology introduced by (Panula-Perälä *et al.*, 2008) and have been initially commercialized by the company BioSilta Oy (Finland, now Enpresso GmbH, Berlin, Germany).

The main composition of EnPresso media is mineral salts, thiamine and trace elements, additionally, a mixture of yeast extract and peptone is present in EnPresso B in small concentrations, but no complex additives are present in EnPresso B defined. A soluble starch-derived polysaccharide is involved into the media as a source of the glucose. The medium was prepared by dissolving two EnPresso B or EnPresso B defined medium tablets, respectively (*prepared according to the manufacturer´s description*) into 50 mL of deionized water.
Glucose is biocatalytically released from the soluble polysaccharide substrate through addition of Reagent A (which according to manufacturer declaration is a mixture of glucose-releasing enzymes, assumable glucoamylases) in the beginning of the cultivation and at the time of induction as an additional dose. In the beginning of the cultivation, 1.5 U L^{-1} of reagent A is added to EnPresso B cultures and 3.0 U L^{-1} to EnPresso B defined cultures. 0.1 mL L^{-1} of Antifoam 204 was added to prevent foaming and 100 mg L^{-1} of Ampicillin was added for plasmid stability.

After overnight cultivation (15-18 hours), the cultures were supplemented with an extra solution (Substrate Booster Mixture; SBM). At the same time, cultures were simultaneously induced for product formation with 100 µM IPTG. The cultures were run for 12 hours after induction.

EnPresso culture pass through three main phases as it is simplified in Figure 3-1. *Growth phase* in which the glucose feed rate and thus the growth rate can be controlled by adjustment of the reagent A concentration added into the medium. *Induction phase* and medium supplementation comes after overnight cultivation at the time when the culture has a higher biomass concentration. The growth rate increases at this stage due to addition of new medium additives and additional reagent A. The *Protein expression phase* is started after induction. At this phase, the applied concentration of 100 µM IPTG is suboptimal to provide a slower protein production rate to support formation of a correctly folded protein.

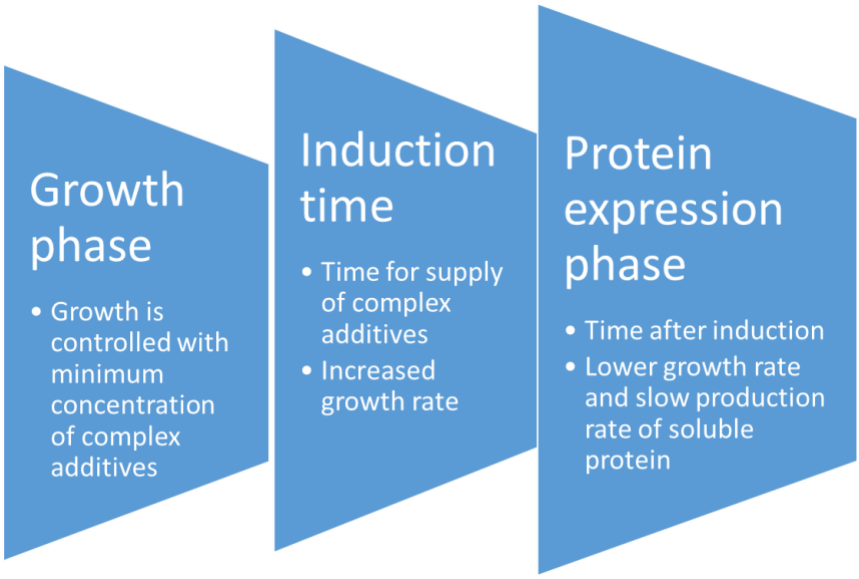


Figure 3-1 *Scheme for the different cultivation phases of the EnBase cultivation system*

❖ **Preparation of Substrate Booster Mixture solution (SBM)**

A stock solution was prepared with 4.8 g/40 mL yeast extract, 2.4 g/40 mL tryptone, 2 g/40 mL glucose polymer in EnPresso Booster mix powder and 6 g /40 mL glucose polymer (EnPump). The mixed solution was then autoclaved at 121°C for 15 minutes. Finally, 300 µL of the substrate mixture solution was added to each culture of 3 mL cultivation volume. The final concentration per culture were; 20 g/L glucose polymer, 12 g/L yeast extract, 6 g/L tryptone. Similarly, 15 g/L and 10 g/L of the substrate booster mixture solution were prepared as it is shown in Appendix.

3.3.2 Autoinduction media

For autoinduction EnPresso B growth medium tablets were solved in 50 mL deionized water. At the time of inoculation also either IPTG (stock solution of 0.1 M) or lactose (stock solution of 20 g L^{-1}) were added at different final concentrations as presented in Table 3.3-1.

Table 3.3-1 *Different chemical inducers used in this study with corresponding induction type and concentrations*

Induction source	Type of induction	Concentrations
IPTG	AI	10, 20, 40, 70, 100 and 250 µM
	SSI	10, 20, 40, 100, 250 and 1000 µM
Complex nitrogenous supplements	AI	10, 15 and 20 g L^{-1}
Lactose	AI	0.1, 0.2, 0.5, 2.0, 5.0 and 10 g L^{-1}

AI- autoinduction, SSI- single shot induction

3.3.3 Reference medium

LB broth (Lysogeny Broth) was used in this study as a reference medium. It consists of (per liter): peptone 10 g; yeast extract 5 g; NaCl 5 g. Additionally, 0.1 mL L^{-1} Antifoam 204 was used to prevent foaming and 100 mg L^{-1} ampicillin was also added to the media to maintain plasmid stability.

3.3.4 Diauxie medium

Diauxic growth was performed in this study using mineral salt medium (MSM) with 3, 5 g L^{-1} glucose and 3, 5 g L^{-1} lactose. The composition of mineral salts medium is summarized in Table 3.3-2 & Table 3.3-3.

Table 3.3-2 *Chemical composition of MSM-Macroelements*

Component	***Concentration (g L^{-1})***
Na_2SO_4	2
$(NH_4)_2SO_4$	2.5
NH_4Cl	0.5
K_2HPO_4	14.6
NaH_2PO_4 x $2H_2O$	4
$(NH_4)_2$-H-Citrat	1
Antifoam (204)	0.1 mL L^{-1}
Thiamin	0.1
$MgSO_4$ (1M)	2 mL L^{-1}
Trace elements solution	2 mL L^{-1}

Table 3.3-3 *Chemical composition of MSM-Trace elements*

Trace elements solution	***Concentration (g L^{-1})***
$CaCl_2$ x $2H_2O$	0.5
$ZnSO_4$ x $7H_2O$	0.18
$MnSO_4$ x H_2O	0.1
Na_2-EDTA	20.1
$FeCl_3$ x $6H_2O$	16.7
$CuSO_4$ x $5H_2O$	0.16
$CoCl_2$ x $6H_2O$	0.18

$Na_2SeO_3 \times 5H_2O$	0.085
Na_2MoO_4	0.14
$Ni(NO_3)_2 \times 6H_2O$	0.725

Glucose, $MgSO_4$ (1 M) and trace elements solutions were separately autoclaved and added to the rest of the medium separately before inoculation. Thiamin (Stock solution of 50 g L^{-1}) was sterilized by filtration using 0.45 µm sterile filter and then added to the medium before inoculation (2 mL L^{-1}) to give a final concentration of 0.1 g L^{-1}. Thiamin solution was kept at 4°C because of its thermal instability.

3.4 Cultivation conditions

All bacterial cultures were inoculated with cells from pre-made glycerol stocks. For preparation of the glycerol stocks the cells were streaked on LB-agar plates with 100 mg L^{-1} ampicillin (for plasmid stability in recombinant *E. coli* strain). After overnight cultivation at 37°C the cells were harvested and washed off with 5 mL LB medium containing 20% glycerol, and separated in separate Eppendorf vials with 100 µL each. The glycerol stocks were stored afterwards at -80°C.

Pre-cultures were done in EnPresso B medium and/or LB as a reference medium. Pre-cultures were inoculated directly from the glycerol stocks with start OD_{600} of 0.05 by formula [(0.05 * pre-culture volume)/ OD of the glycerol stock], incubated at 30°C on an orbital shaker with 50 mm offset and shaking speed of 250 rpm for 6 hours (otherwise according to the design of the experiments). The pre-cultures were used afterwards as inoculum for fed-batch like cultivations in EnPresso B and/or EnPresso B defined medium. Diauxic cultivations were done as batch cultivations in MSM media as mentioned before in 3.3.1.

3.4.1 Multiwell plate (MWP) cultivations

❖ **Cultures with online oxygen and pH monitoring**

For online monitoring of dissolved oxygen, cultures were prepared in 24-square deepwell plates (24 DWP) with integrated optical oxygen sensors (OxoDish OD24; PreSens GmbH, Regensburg, Germany) with a working volume of 3 mL per well. The sensors consist of fluorescent, oxygen sensitive dye.

OxoDishes were combined with System Duetz clamp system and covered with oxygen permeable sandwich covers (EnzyScreen). The plates were placed afterwards on to a Sensor Dish Reader (SDR; PreSens GmbH, Regensburg, Germany). The plate and the reader were fixed on an orbital shaker with 50 mm offset and shaking speed of 220 rpm. Dissolved oxygen concentration pO_2 [%] was monitored online over the whole cultivation period in 1 minute intervals. The signals of the pO_2 were sent to the computer and visualized with the software SDR_v4.0.0 (PreSens GmbH, Regensburg, Germany).

❖ **Cultures with online pH monitoring**

For online monitoring of pH, cultures were prepared in 24-round well plates with integrated optical pH sensors (HydroDish HD24; PreSens GmbH, Regensburg, Germany) with a working volume of 1 mL per well.
HydroDishes were prepared, covered, coupled with the same system of the OxoDishes. pH was monitored during the whole cultivation in 1 minute intervals. The signals of the pH measurements were sent to the computer through SDR_v4.0.0 (PreSens GmbH, Regensburg, Germany).

3.4.2 Shake flask cultivations

For flask-scale expression, two different shake flask scales were used in this study. 50 mL EnPresso B cultures were grown in 250 mL Ultra Yield flasks (UYF; Thomson Instruments) and 500 mL of EnPresso B cultures were grown in 2.5 L Ultra Yield flasks (UYF; Thomson Instruments). Both cultures were inoculated with a pre-culture with a cell density of 0.15 by following the formula [(0.15* culture volume)/ OD of the pre-culture], covered with adhesive airporous membranes (AirOtop; Thomson Instruments). Cultivations were run at 30°C at a shaking speed of 250 rpm and the offset of the orbital shaker was 25 mm.

❖ **Shake flask cultures with multi-monitoring systems (Vario system)**

A combination of oxygen, pH and biomass were monitored simultaneously in the shake flask cultivations through Shake Flask Reader (SFR-vario; PreSens GmbH, Regensburg, Germany). The device optics can read out pre-calibrated oxygen and pH sensor spots which integrated into ready-to-use cultivation vessels (PreSens GmbH, Regensburg, Germany).
Diauxic cultures were done in the ready to use Presens shake flasks (Corning 250 mL flasks with a 50 mL working volume). The culture was inoculated with an OD_{600} of 0.15 and run for 24 hours. During the cultivation pO_2 [%], pH and biomass were online monitored in 1 minute intervals.

3.4.3 Bioreactor cultivations

Fermenter cultivations were performed in a 3 Liter Stirred tank bioreactor Type KLF 2000 (Bioengineering AG, Switzerland) with 2 Rushton turbines with a cultivation volume of 2 L. The gas flow rate was adjusted to 1.15 L min^{-1} at the beginning, then was increased gradually up to 2 L min^{-1}. For exhaust gas analysis; the fractions of out-gases (O_2 [vol.-%] & CO_2 [vol.-%]) were measured during the cultivation every 30 seconds using a BlueInOne$_{ferm}$-Sensor (BlueSens GmbH). Additionally, pO_2; pH and the temperature were monitored during the fermentation. The pH was controlled in diauxic cultivations with the aid of the pH controller of the bioreactor by using a 25% ammonia solution.

3.4.3.1 Bioreactor cultivation with EnPresso B growth system

Cultivation medium used for the 2 L EnBase cultivation in bioreactor was EnBase based on EnPresso B medium. For this purpose, four bags of a new released EnPresso B 500 kit (Enpresso GmbH, Germany) were used for the preparation of 2 L cultivation media. The inoculum was prepared as shown in Figure 3-2. Additionally, 2 mL of ampicillin was added manually and aseptically to 2 L cultivation (final concentration of 100 mg L^{-1}). Sterile antifoam 204 (Sigma) was added manually also upon foam initiation. Otherwise, the cultivation protocol was run as described in 3.3.1.

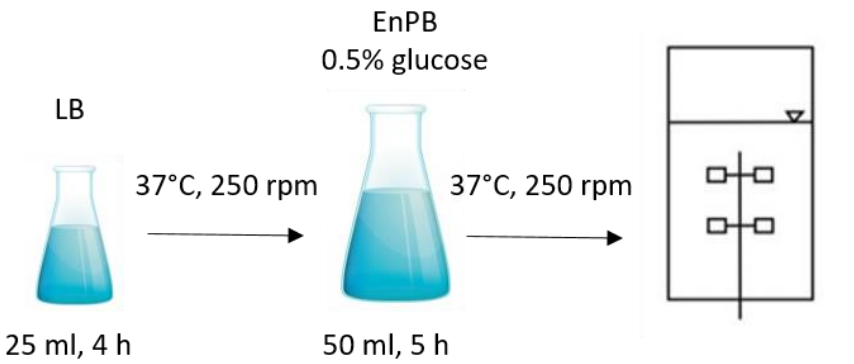


Figure 3-2 *Scheme for preparation of the pre-culture of the bioreactor cultivations*

3.4.3.2 Bioreactor cultivation under diauxic-growth

Cultivations for diauxic growth in 2 L cultivations were performed in mineral salt medium supplemented with 4.0 g L^{-1} glucose and 4.0 g L^{-1} lactose as described in 3.3.4. The inoculum was prepared as shown in Figure 3-2. The cultivation was run for 12 hours and samples were taken at different times during the cultivation. Fractions of oxygen and carbon dioxide in the exhaust gas of the bioreactor cultivation was determined by BlueInOne$_{ferm}$-Sensor system.

3.5 Analysis

3.5.1 Sampling

In multiwell plates cultivations, the sample volume was between 200-300 µL. Samples were stored on ice, part of the samples was taken for OD_{600} measurement and the protein samples were taken afterwards. For protein analysis, culture samples were normalized to OD_{600} of 5 (sample volume = 5/OD of the sample), collected and pelleted by centrifugation for 10 min at 4°C and 15.000 xg (Hitachi Koki CT15E, Japan). Supernatant was discarded and pellets were frozen at -20°C for later analysis.

3.5.2 Optical density measurement

For MWP cultivations, cell growth was monitored by measuring the optical density of the samples at 600 nm using a 200 µL F-shape 96 well plate (Greiner Bio-One GmbH, Frickenhausen, Germany) and Synergy™ Mx plate reader (BioTek Instruments). The plate reader was operated by the software Gen5™ 1.11.5. Samples were diluted with 0.9% $NaCl_{aq}$ until the extinction is within the range of validity of the Beer–Lambert law (0.4-0.5) with 0.9% $NaCl_{aq}$ as reference. Samples were prepared in duplicates for double measurements.

Additionally, a correction factor (between the photometer and plate reader measurements) was manually constructed by making a calibration curve with different OD_{600} measurements from both (
Figure *3-3*). The slope was equal to 2.6 which considered as a correction factor of the plate reader measurements.

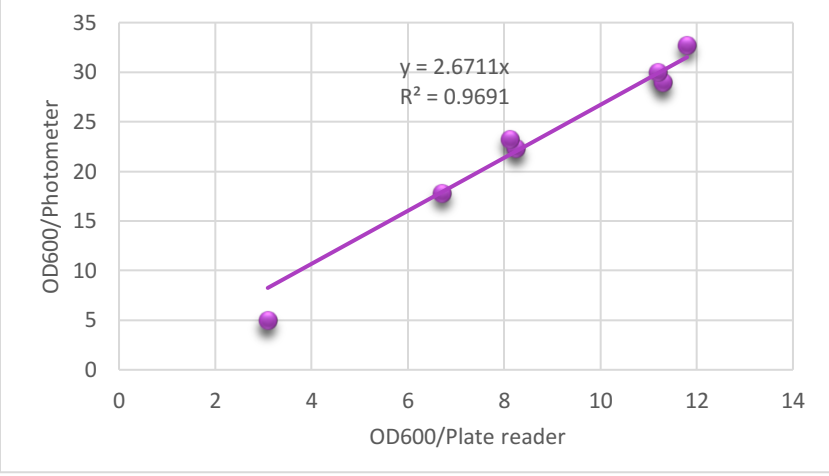


Figure 3-3 *Calibration curve of the cell growth measurments photometrically and with the plate reader*

For shake flask and bioreactor cultivations, cell growth was monitored by measuring the optical density at 600 nm using cuvettes of 1 mL volume and Ultraspec Plus Spectrophotometer (GE Healthcare, München). Samples were diluted with 0.9% $NaCl_{aq}$ with 0.9% $NaCl_{aq}$ as reference. Samples were prepared in duplicates for double measurements.
Additionally, dry cell weight (DCW) was measured in bioreactor samples. For this reason, 1.5 mL clean vials were previously dried and pre-weighted and used afterwards for DCW measurements. 1 mL of the culture sample was taken, centrifuged for 10 min at 4°C and 21500 xg. The supernatant was removed into new clean vials and stored at -20°C for sugar analysis and the pellet was re-suspended into 1 mL of 0.9% $NaCl_{aq}$ and centrifuged again. The washed pellets were dried finally in an oven at 75°C until the next day (or until getting a constant weight).

3.5.3 Glucose analytics

Stored supernatants were thawed and used for measurements of the extracellular glucose. Glucose was measured with the enzymatic Glucose Hexokinase FS kit (DiaSys; Diagnostic Systems GmbH, Holzheim, Germany). The enzymatic assay is based on two enzymatic reactions. Firstly, glucose into the sample is converted to glucose-6-phosphate in the presence of hexokinase and ATP consumption (1). Glucose-6-phosphate is then converted to gluconate-6-phopshate with the aid of glucose-6-phosphate dehydrogenase with generating of NADH (2). The increase in NADH measured photometrically at 340 nm.

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{Glucose-6-Phosphate} + \text{ADP} \qquad (1)$$

$$\text{G6P} + \text{NADP}^{+} \xrightarrow{\text{G6PDH}} \text{6-Phosphogluconate} + \text{NADPH} \qquad (2)$$

The glucose enzymatic assays were performed in high throughput scale in F-shape 96 wellplate (Greiner Bio-One GmbH, Frickenhausen, Germany). The assay starts by mixing 4 parts of reagent 1 of the enzymatic kit (R1) with 1 part of reagent 2 (R2). 190 µL was pipetted in each well for each sample, glucose standard and blank, followed by addition of 10 µL of the sample or the standard. Samples and glucose standards were incubated for 15 minutes at room temperature. The light absorption was measured at 340 nm with using Synergy™ Mx plate reader (BioTek Instruments). The glucose concentration was finally calculated with the aid of the glucose calibration curve (Figure 3-4).

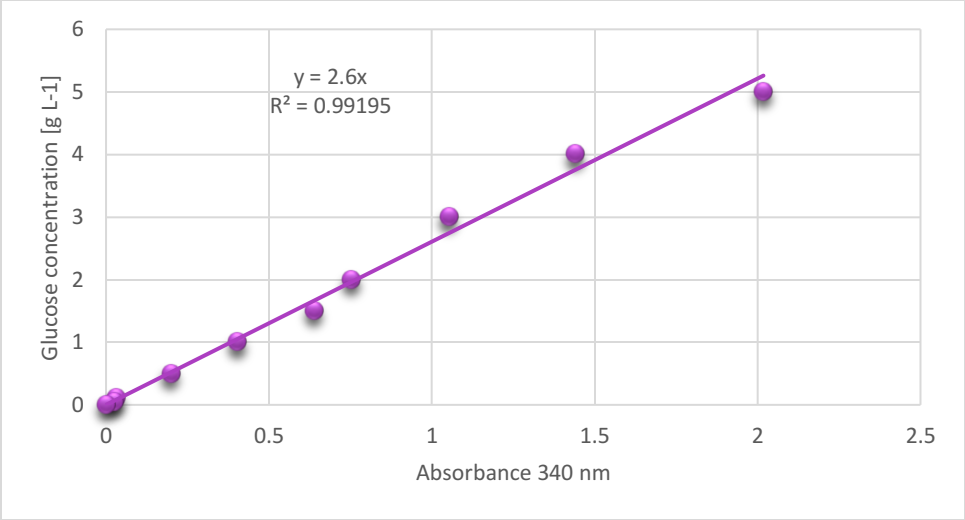


Figure 3-4 *Calibration curve of the the glucose measurements*

3.5.4 Lactose and galactose analytics

Lactose was measured from the culture supernatant like the glucose assay using Lactose/ D-Galactose kit (Boehringer Mamnnheim / R-Biopharm, Cat. No. 10 176 303 035). The enzymatic assay is based on two enzymatic reactions. Firstly, the lactose into the samples is hydrolyzed to D-glucose and D-galactose in the presence of β-galactosidase and water (1). D-galactose is then oxidized to D-galactonic acid in the presence of β-galactose dehydrogenase (Gal-DH) (2). The increase in NADH measured photometrically at 340 nm. The amount of NADH is stoichiometric to the amount of lactose.

$$\text{Lactose} + H_2O \xrightarrow{\beta\text{-galactosidase}} \text{D-glucose} + \text{D-galactose} \qquad (1)$$

$$\text{D-galactose} + NAD^+ \xrightarrow{\text{Gal-DH}} \text{D-galactonic acid} + NADH + H^+ \qquad (2)$$

The lactose/ galactose enzymatic assays were performed also in F-shape 96 wellplate (Greiner Bio-One GmbH, Frickenhausen, Germany). The enzymatic assay kit contains 4 different solutions. The procedure of the enzymatic assay in 96 wellplate scale is summarized in Table 3.5-1.

Table 3.5-1 *Procedure of the enzymatic analysis of lactose & galactose in 96-wellplate*

Pipette into wells	Lactose		Galactose	
	Blank	**Sample**	**Blank**	**Sample**
Solution 1	10 µL	10 µL	10 µL	10 µL
Solution 2	2.5 µL	2.5 µL	-	-
Sample	-	5 µL resuspend	-	5 µL resuspend
Cover plate with Parafilm. Incubate for 20 min at 20-25°C.				
Solution 3	50 µL	50 µL	50	50
bi-dest H2O	100 µL resuspend	95 µL resuspend	102.5 µL resuspend	97.5 µL resuspend
Cover plate with Parafilm. Shake for 1 min. Incubate for 1 min.				
Read absorbances of the solutions (A1).				
Solution 4	2.5 µL	2.5 µL	2.5 µL	2.5 µL
Cover plate with Parafilm. Shake for 15 min.				
Read absorbances of the solutions (A2).				
Cover plate with Parafilm. Shake for 20 min.				
Read absorbances of the solutions (A2).				
Cover plate with Parafilm. Shake for 5 min.				
Read absorbances of the solutions (A2).				

The light absorption was measured at 340 nm with using Synergy™ Mx plate reader (BioTek Instruments). The calibration curves of lactose and galactose are shown in Figure 3-5 & Figure 3-6 respectively.

❖ **Calculation**

For calculation of the lactose concentration, the following equation was followed;

$$c=(V*MG)/(\varepsilon*d*v*1000)*\Delta A \ [g\ L^{-1}]$$

V = 0.165 mL final volume
v = 0.005 mL sample volume
MW = molecular weight of lactose (342.29 g mol^{-1})
MW = molecular weight of galactose (180.16 g mol^{-1})
ε = 6.3 L/mmol x cm extinction coefficient of NADH at 340 nm
d = 0.418 cm

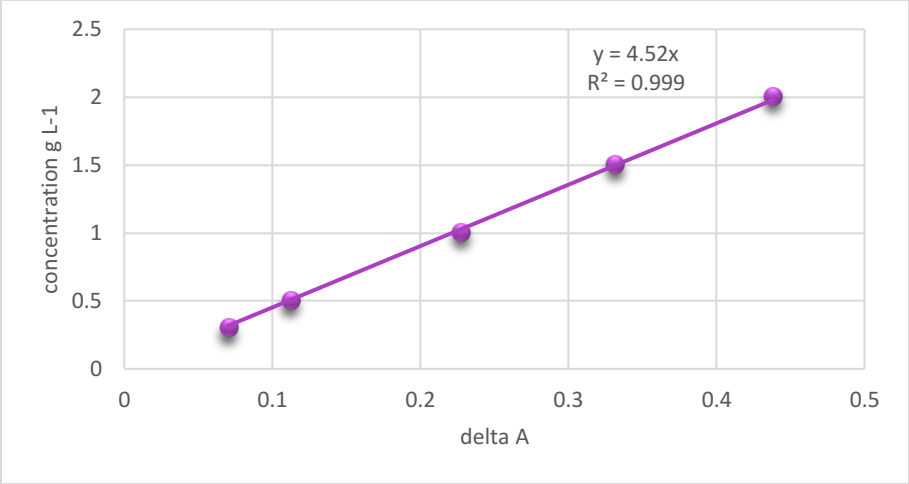


Figure 3-5 *Calibration curve of the the lactose measurements*

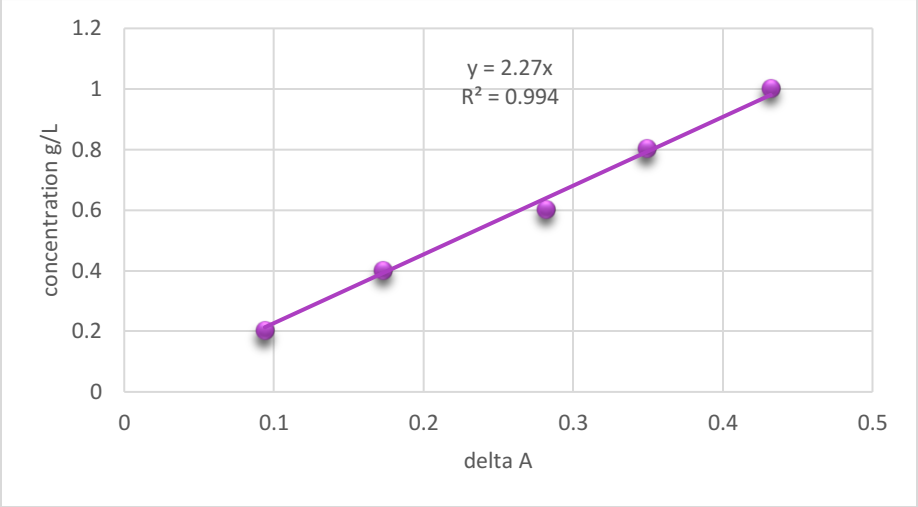


Figure 3-6 *Calibration curve of the galactose measurements*

3.5.5 Protein purification and analysis

Stored pellets were thawed on ice and then re-suspended in 300 µL of cell lysis solution [1 mL of Bugbuster (Novagen®, #70584-4), 1 µL lysozyme (50 mg mL^{-1}; Fulka, 62971-50G-F) and 1 µL Benzonase (Novagen®, #70746-3)] for 30 minutes at room temperature. Target protein (TtPyNP, 46.81 kDa) is thermostable and therefore it was purified by heating the samples at 80°C for 15 minutes at 300 rpm using Dry Bath-ThermoCell Mixing Block (BIOER Technology, Beijing, China). Heated samples were centrifuged at 16.000 x g for 15 min at 4°C to remove cell debris. Soluble protein fractions were analyzed from the lysate after removal of the insoluble part by centrifugation. Thermally purified protein in the cell lysate was visualized on reducing SDS-PAGE gels stained with Coomassie Brilliant Blue.

3.5.6 Protein analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Soluble protein fractions in the cell lysate and insoluble fractions in the pellets were visualized on reducing SDS-PAGE gels. The SDS-PAGE gels and solutions were prepared as described in Table 3.5-2 & Table 3.5-3. Two protein markers were used in this study to determine the size of the target protein bands (a) Spectra™ Multicolor Broad Range Protein Ladder with 10 different protein sizes with a size range of 10-260 kDa (Figure 3-7 (a)) and (b) ROTI®-Mark Tricolor with protein range of 10-245 kDa (Figure 3-7 (b)). The protein samples were mixed with the loading dye (Coomassie Brilliant Blue) (V/V) and heated for 5 min at 95°C. Then, 7 to 10 µL protein sample and protein marker were applied into the gel pockets. After the samples were loaded, a voltage of 60 V was applied for 30 min until the samples reach the running gel and then the voltage was increased to 120 V for 1-1.5 hours. The gels were then washed with 20-30 mL deionized water and heated in microwave (600W) for 10 sec. The washing steps was repeated twice before immersing the gels into the coloring solution with stirring for 4 h. Finally, gels were washed again as described and prepared for scanning.

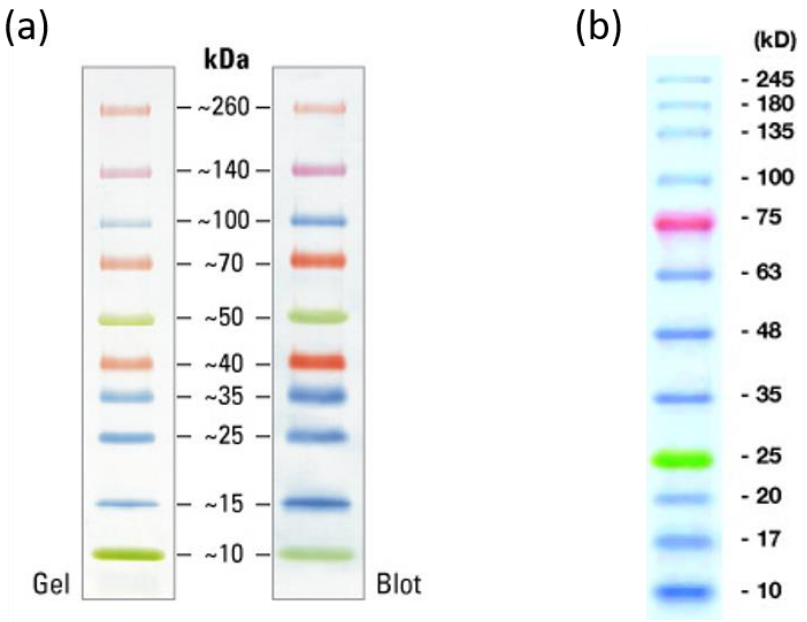


Figure 3-7 Protein markers. *(a) Spectra™ Multicolor Broad Range Protein Ladder; (b) ROTI®-Mark Tricolor*

Table 3.5-2 *Chemical composition of SDS-PAGE gels (in mL)*

	12% Running gel (for 5 mL)	***5% Stacking gel (for 1 mL)***
H_2O	1.6	0.68
30% acrylamide mix	2	0.17
1 M Tris buffer pH 6.8	-	0.13
1.5 M Tris buffer pH 8.8	1.3	-
10% SDS	0.05	0.01
10% ammonium persulfate	0.05	0.01
TEMED	0.002	0.002

Table 3.5-3 *SDS-PAGE solutions*

SDS-PAGE Solutions	
Loading dye	200 µL DDT+ Loading dye
Running buffer (per 1 L)	❖ 10 mL of 10% SDS 100 mL of 10X Tris/glycine ❖ Fill to 1 L with distilled H_2O
10X Tris/glycine	❖ Dissolve 15.15 g + 72 g glycine for 500 mL distilled H_2O ❖ Fill it to 1 L with dis. H_2O
Coomassie Brilliant Blue dye (Coloring solution)	❖ Dissolve 60-80 mg of G250 Comassie Brilliant Blue per 1 L bidest H_2O for 2-3 h ❖ Add HCl until a concentration of 35 mM (3 mL per 1 L)

3.5.7 Protein activity assay

The activity assay of the thermally purified protein (TtPyNP) was run in 50 mM potassium phosphate buffer of pH 7.0 with 1 mM of uridine as a substrate. 200 µL of substrate solution was pre-heated for 2 min before addition of 5 µL of the enzyme per 200 µL reaction volume. The reaction was run at 80°C and 300 rpm for 2 min. The reaction was stopped with a 10 M of NaOH. The optical density of the samples (including the reaction product; uracil) was measured at 290 nm using a 200 µL F-shape 96 wellplate (Greiner Bio-One GmbH, Frickenhausen, Germany) and Synergy Mx plate reader (BioTek Instruments). One unit activity of the enzyme is defined as the amount of the enzyme catalyzed in the conversion of 1 µM of the substrate (uridine) to the end product (uracil) in one minute under the reaction conditions.

- **Activity calculation**

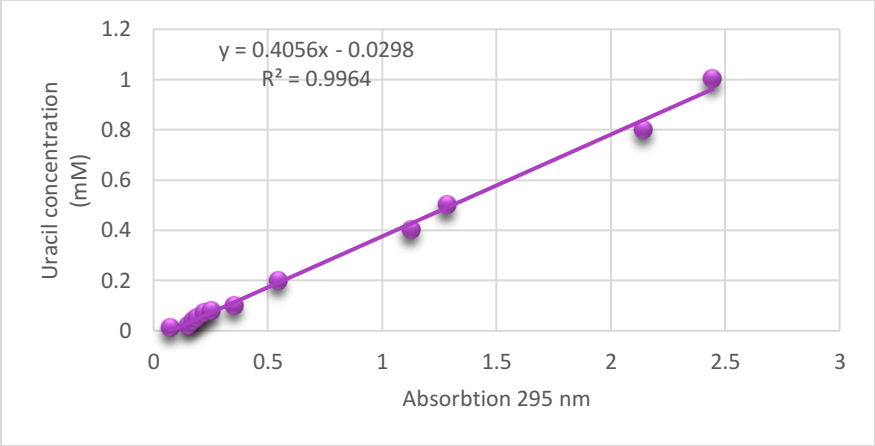


Figure 3-8 *Calibration curve of the NP activity measurements*

The calculation of the unit activity is started from the trend line equation of the calibration curve (Figure 3-8). It was run as follows:

Uracil concentration [mM] = (Slope*Ab_{295}) - Y intercept

Conversion [%] = [Product] / $[Substrate]_{initial}$ x 100 %

Enzyme [U] = (Conversion [%] * Substrate concentration [mM] *Reaction volume [mL]) / Reaction time [min])

Enzyme U/OD5 = Enzyme [U] * (Volume of cell lysate [µL] / Enzyme volume in the reaction [µL])

Specific enzyme activity [U/OD_{600}] = (U/OD5) / 5

Volumetric enzyme activity [U/mL] = (U/OD_{600}) / OD_{600}

4. Results

There are different factors that affect the success of a bioprocess as well as the yield of the recombinant protein as mentioned before. Starter culture, mode of the cultivation, culture conditions, cultivation medium, induction time are examples of these factors. Therefore, a detailed study for these factors will be introduced in this part.

4.1 Investigation of the pre-cultivation conditions

Fed-batch process is still preferred for high cell density cultivations (HCDC) and for metabolic control as well. It is challenging to apply the fed-batch process to the smaller laboratory cultivation systems like shake flasks and microwell plates. Therefore, EnPresso growth system is used in this study to apply the controlled glucose feed to the small scale, shaken cultures. By then, lower growth rates with a proper folding of the target protein are obtained.

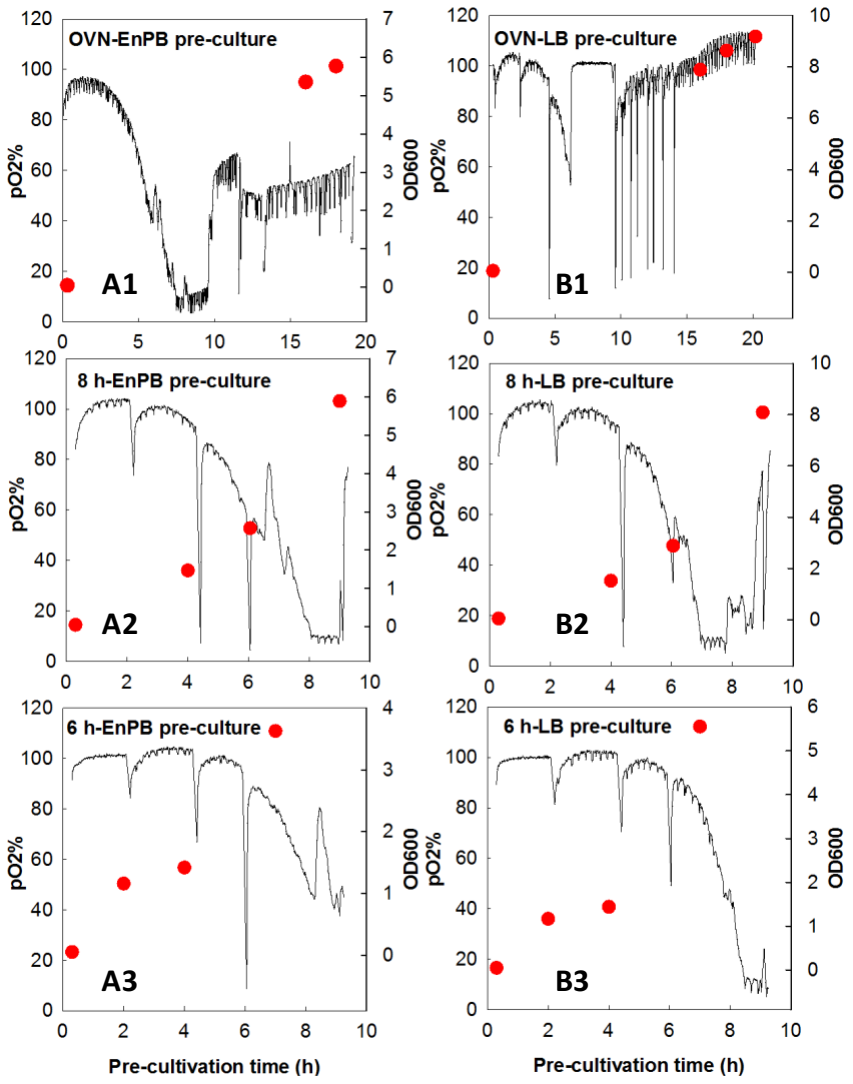
E. coli cultures have been prepared in this part in multiwell plates for a successful miniaturization and parallelization. It is important to monitor the dissolved oxygen (pO_2) as a crucial parameter for the microbial cultures. On one hand, the exponentially growing cells are in high demand to the dissolved oxygen while the oxygen consumption may overwhelm the oxygen transfer rate in the liquid culture. On the other hand, oxygen limitation has many adverse effects on the cell growth and the product formation. To overcome these problems, an integrated pO_2 online monitoring system to the MWPs in form of 24 deepwell OxoDishes are used here for at-line pO_2 [%] monitoring during the cultivations.

4.1.1 Pre-cultivation time

The aim of this part of work was to investigate (1) the impact of the pre-culture age on the length of the lag phase of the cells when they are transferred to a new fresh medium in the main culture. Therefore, pre-cultures were harvested at different time points (after 8 hours, 6 hours and overnight cultivation) and used then as inoculums for fed-batch main cultivations. (2) The impact of preparation of the pre-cultures of recombinant *E. coli* BL21 as batch process (in LB medium) and fed-batch process (EnPresso B growth system) in terms of the cell growth and protein production.

The pO_2 [%]and OD_{600} profiles of different pre-cultures with different pre-culture's lengths are shown in Figure 4-1. At the time of inoculation of the main cultures, the overnight pre-cultures were taken in fed-batch phase; the 8 hours pre-cultures were taken in the beginning of the fed-

batch phase and the 6 hour pre-cultures were taken in a batch phase. The cell growth was exponential with the time of all the pre-cultures, while the final OD_{600} values of the different pre-cultures were ranging between 6 and 8 at the time of harvest. Yet, the 6 hours pre-cultures showed the lowest cell densities (OD_{600} ≈ 4 -5).



	Pre-cultivation method	
Pre-cultivation time:	Fed-batch (Enpresso B)	Batch (LB medium)
Overnight	Fig. A1	Fig. B1
8 h	Fig. A2	Fig. B2
6 h	Fig. A3	Fig. B3

Figure 4-1 Batch & fed-batch pre-cultures of E. coli BL21 TtPyNP. *pO_2 [%] (represented by solid line) and OD_{600} values (represented by rounded symbols) of 3 mL Enpresso B cultures (on the left) and LB pre-cultures (on the right) in 24 deepwell OxoDish. Figures of each Enpresso B culture are clearly summarized in the attached table.*

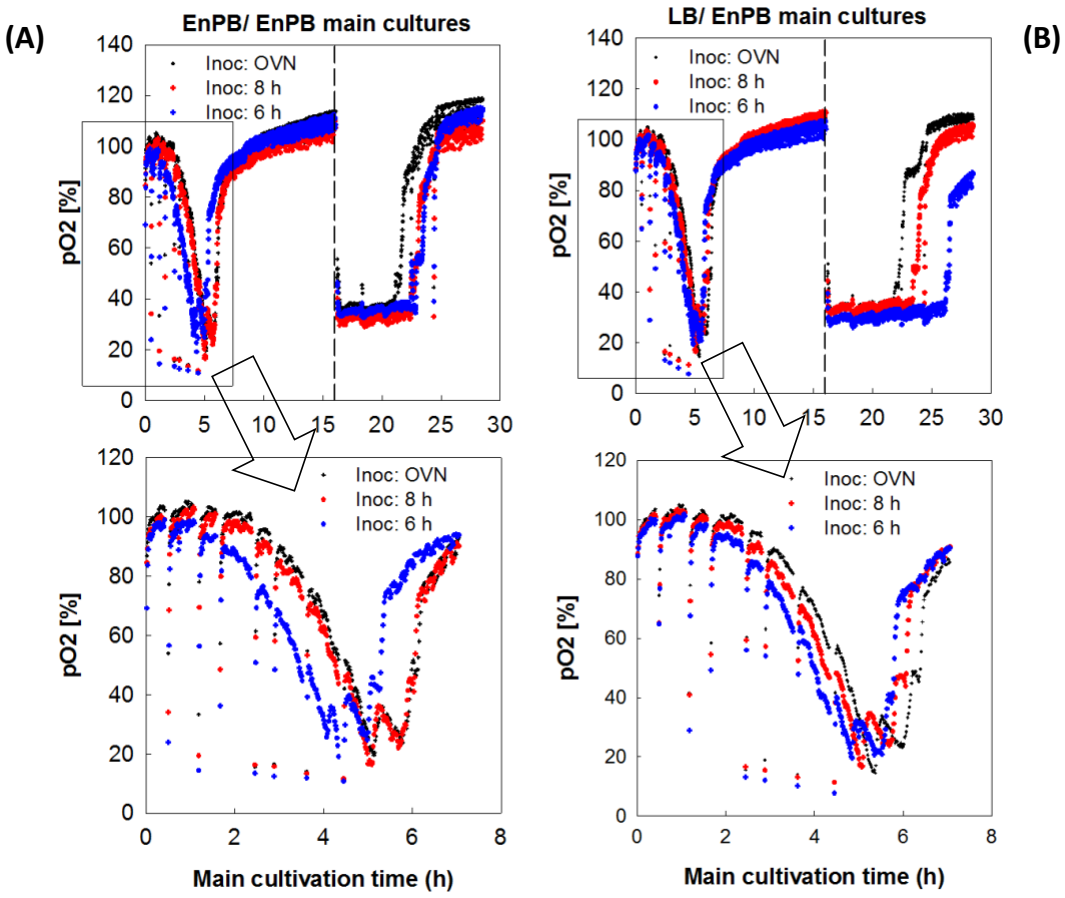


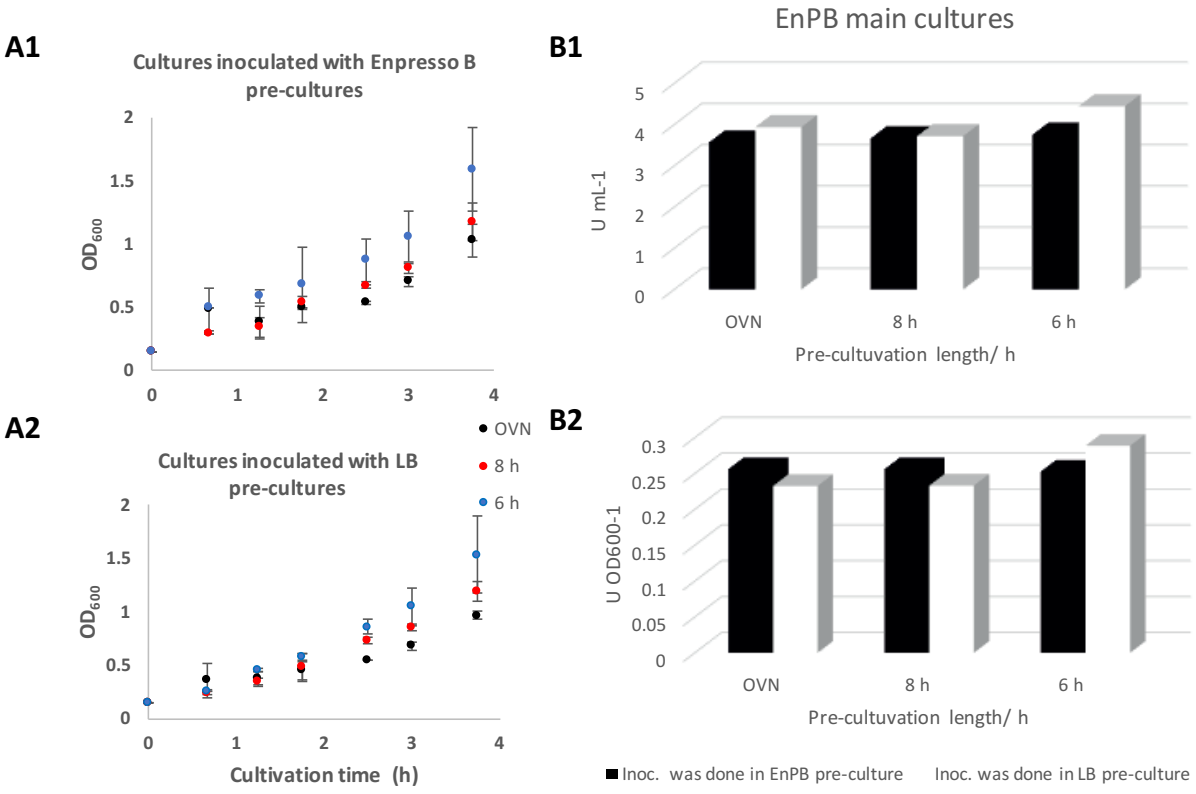
Figure 4-2 Fed-batch like Enpresso B main cultures with different batch and fed-batch pre-cultures. *pO_2 [%] profiles of fed-batch like Enpresso B main cultures originated from different pre-cultures (Enpresso B pre-cultures "Enpresso B/Enpresso B"* ***(A)*** *and LB pre-cultures "LB/Enpresso B"* ***(B)****). Detailed pO_2 [%] profiles of the batch phase part of the Enpresso B main cultivation (which marked with circle into the (A & B) graphs) are shown in separate graphs. Induction time is indicated by a vertical dashed line.*

Enpresso B main cultivations show two distinct growth phases (i) the growth phase (cultivation prior to induction, which is shown by the dashed line): With cultures inoculated with Enpresso B 6 hours pre-culture showed a shorter batch phase (≈ four hours) than the cultures inoculated with 8 hours and OVN pre-cultures (≈ 6 hours) (Figure 4-2A). On the other hand, cultures inoculated with LB-cultures (Figure 4-2B) did not exhibit a significant difference in the length of the batch phase with the different inoculum lengths. Additionally, LB pre-culture provided a longer batch phase than an Enpresso B pre-culture. Cells of the Enpresso B pre-culture showed also a shorter lag phase (≈ one hour) until the exponential growth was initiated with exponential oxygen consumption. While the cells of the LB pre-culture took around two hours until the cells start adaptation and exponential growth. This extended lag phase can be a result of a long stress time due to starvation initiated in the batch pre-culture. On the other hand, the growth of

Enpresso B pre-cultures in fed-batch like conditions, i.e.; slow and continuous glucose supply, provided an active inoculum for the main culture. The inoculum size has an influence also on the length of the lag phase due to the increase in the variability lag time of the individual cells. To avoid such problems, all Enpresso B main cultures have been inoculated here with the same inoculant concentration (OD_{600} of 0.15). Also, the medium change during the pre- and main cultivations would probably affect the length of the lag phase. Therefore, cultivating the cells into the same media of their pre-cultures would minimize the time required for adaptation to the new environment.

(ii) The protein expression phase (time after induction). The cells grew under the fed-batch conditions after the batch phase, which lasted roughly six hours. This fed-batch phase, characterized by low respiration rate, lasted roughly nine hours (Figure 4-2 (A&B)). In order to enhance the activity of the bacteria for protein production, at the time of culture induction the cultures were supplemented with an additional dose of the biocatalyst. Additionally, nitrogenous organic compounds (Enpresso booster) were added. These nitrogenous compounds act as a buffering system to neutralize the medium pH to the optimal range for both the bacterial growth and to a desired level of the activity of added biocatalyst.

The *Enpresso B main cultures inoculated with LB pre-cultures* of different lengths showed very different behaviors. Although all the Enpresso B main cultures started with inoculum concentration of OD_{600} = 0.15, they also showed different cell densities. Although that main cultures inoculated with overnight pre-culture exhibited lower cell densities than the main cultures inoculated with 8 & 6 hours pre-cultures; they started the fed-batch earlier (approximately 6 hours after induction& booster addition), followed by cultures with 8 hours and 6 hours inocula. In contrast, the *cultures inoculated with an Enpresso B pre-cultures* (Figure 4-2 (A)) did not show significant differences. The main cultures with an overnight inoculum started the fed-batch phase after approximately 4 hours of the induction followed by the two other cultures.



C

1	Inoc.: OVN EnPB
2	Inoc.: 8 h- EnPB
3	Inoc.: 6 h- EnPB
4	Inoc.: OVN- LB
5	Inoc.: 8 h- LB
6	Inoc.: 6 h- LB

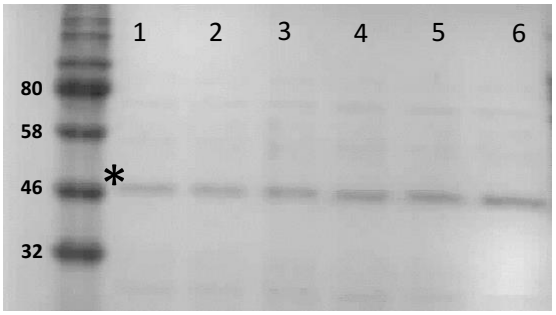


Figure 4-3. Impact of the pre-culture method on growth and recombinant protein production. *OD_{600} values of fed-batch like Enpresso B main cultures originated from different inocula (Enpresso B **(A1)** & LB **(A2)**) during their batch phase. The error bars represent the Standard deviation of 3 parallel cultures; **(B)** TtPyNP activity (per volume (B1)) and specific activity (per cell (B2)); **(C)** TtPyNP expression in Enpresso B main cultures after 12 hours of induction (soluble fractions of heated samples loaded on SDS-PAGE). The definition of samples is added in an attached table.*

The main cultures with 6 hours inocula (LB & Enpresso B) were growing to higher OD_{600} values compared to 8 hours or overnight inocula (Figure 4-3).

The impact of the different inocula on the activities of the expressed protein is not significant. Data shows that the protein activities (volumetric and specific) have not been greatly affected by the type or the length of the pre-culture used. This could be interpreted by the expression of the

protein during the second phase of the cultivation which means after the all the cultures have been reached their fed-batch phase (stationary phase at the time of induction). This observation was confirmed by the similar level of the protein expression shown by SDS-PAGE.

4.2 Optimization of the enzyme based substrate delivery system for recombinant protein production

The aim of this part was to study and investigate the optimum conditions of the enzyme based fed-batch cultivations for recombinant protein production in order to scale it up.

4.2.1 Enpresso B growth systems

For this evaluation, the pre-cultures are harvested after 6 hours. At the time of harvest, the cells in the LB pre-culture had just finished their batch phase and had already started the stationary phase with a final OD_{600} of 4.5. In contrast, the cells in the Enpresso B pre-culture was still growing in the batch phase. Finally, the pre-cultures were normalized to OD_{600} = 0.15 for inoculation of the main cultures.

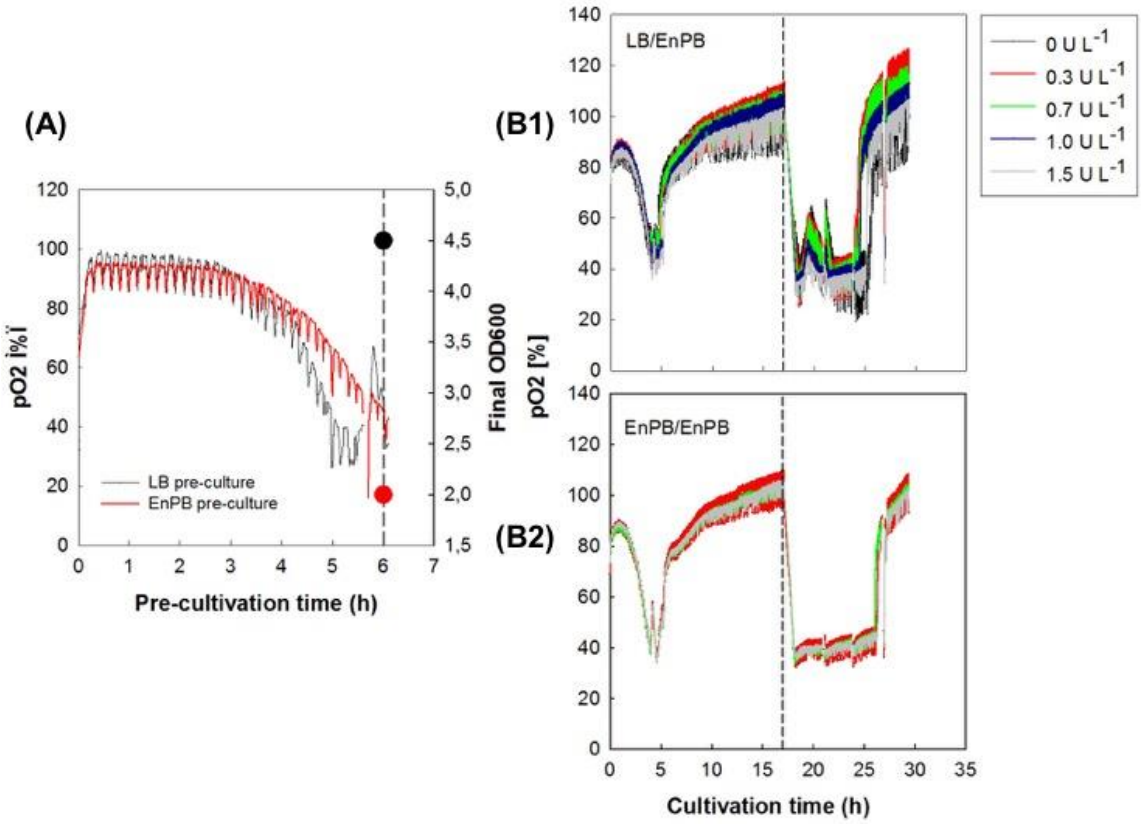


Figure 4-4 Characteristics of the main cultures treated with different glucose release rates (Reagent A dosing) and inoculated by Enpresso B or LB pre-cultures. (A) *pO_2 [%] of 3 mL Enpresso B and LB pre-cultures (solid line) and final OD_{600} of the pre-cultures (rounded circle). Pre-culture harvest time is indicated by vertical dashed line;* ***(B)*** *pO_2 [%] values of fed-batch like Enpresso B main cultures originated from LB pre-cultures "LB/Enpresso B"* ***(B1)*** *& from Enpresso B pre-cultures "Enpresso B/Enpresso B"* ***(B2)*** *with different concentrations of reagent A added at the induction time which is indicated by a vertical dashed line.*

The length of batch phases in the main culture were similar in both inoculation systems (LB or Enpresso B), lasting about 5 hours. To investigate the impact of the additional dose of the reagent A at the time of culture induction, different enzyme (reagent A) concentrations (0.3, 0.7, 1.0 and 1.5 U L^{-1} and 0 U L^{-1} as negative control) were added after 17 hours cultivation time. The influence of addition of reagent A on the Enpresso B main cultures is shown in Figure 4-4 (B1 & B2). pO_2 fluctuated in the LB/Enpresso B culture during the first 3 hours after the induction/ boosting time. This can be considered the time needed for adaptation of the cells to resume their exponential growth. Interestingly, this large fluctuation was not present in the cultures inoculated with Enpresso B cultivation - the cells resumed their batch phase right after induction/ boosting time without pO_2 fluctuation. Furthermore, addition of more Reagent A (glucose-releasing reagent) increased oxygen consumption in both cultures.

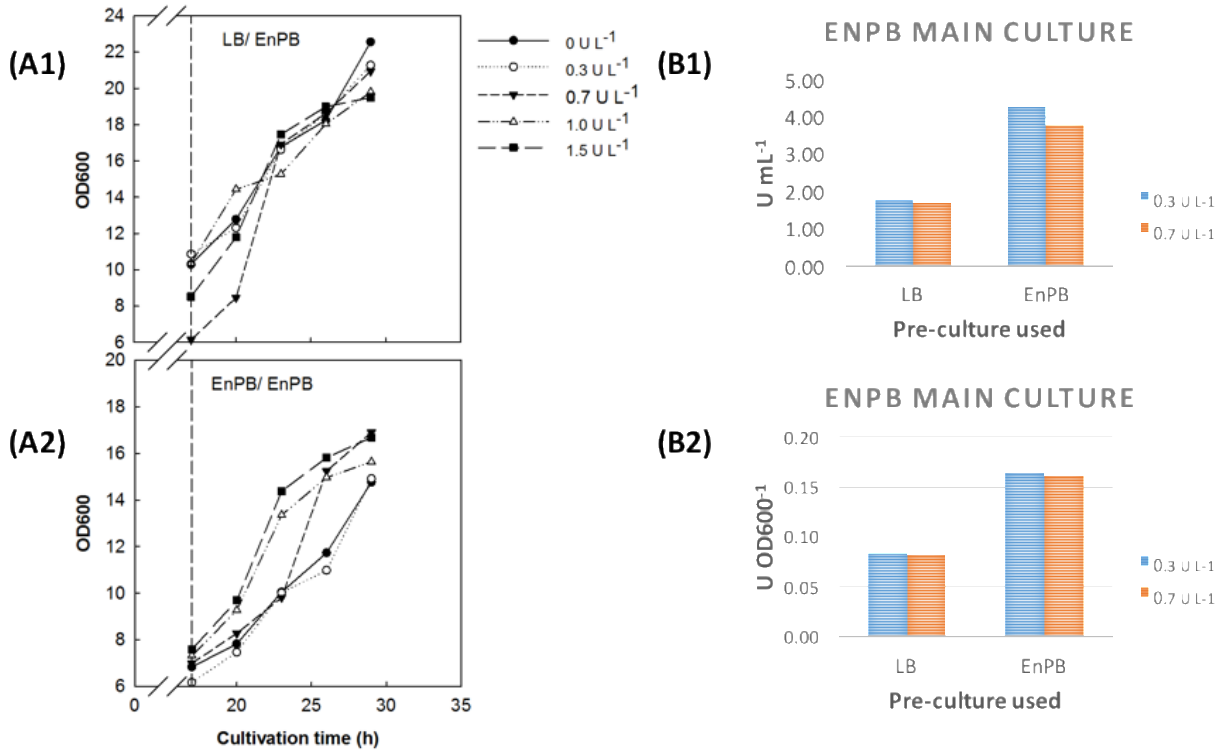


Figure 4-5 Impact of the glucose release rate on yield of biomass and recombinant protein. (A) *OD_{600} values after induction of the Enpresso B main cultures originated from different inocula (LB **(A1)** & Enpresso B **(A2)**); **(B)** TtPyNP activity (per culture volume **(B1)**) and specific activity (per cell **(B2)**).*

The values of OD_{600} were measured every 3 hours after the time of addition of the biocatalyst and induction to see the impact of reagent A on the biomass. The cultures had different cell densities after the enzyme addition (Figure 4-5), but already six hours after the enzyme addition the biomass values were again quite similar. Therefore, the fluctuation which happened just after the enzyme addition might be similar to the adaptation time seen in the pO_2 graphs of LB/Enpresso B cultures in Figure 4-4 (B1). The cultures inoculated with Enpresso B pre-culture behaved differently (Figure 4-5 (A2). The cells continued the growth differently and proportionally to the different enzyme concentration.

In terms of the activity of the expressed protein, cultures with 0.3 and 0.7 U L^{-1} Reagent A dosing were chosen for analysis of the recombinant product's enzyme activities since they were seen to be the cultures having favorable oxygenation level and thus expected to have best conditions for protein folding. Indeed, these cultures expressed higher titers of an active NP (both volumetric and specific activities) than the LB/Enpresso B cultures regardless of the concentration of the reagent A added at the induction time (Figure 4-5).

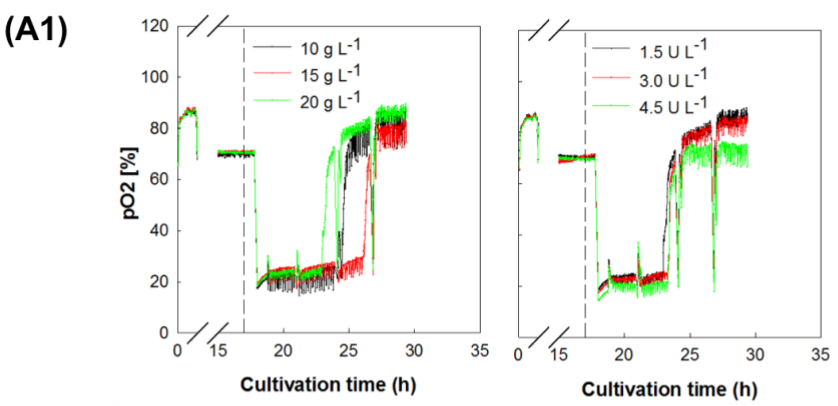
❖ Supplementation of Enpresso B cultures with additional glucose polymer

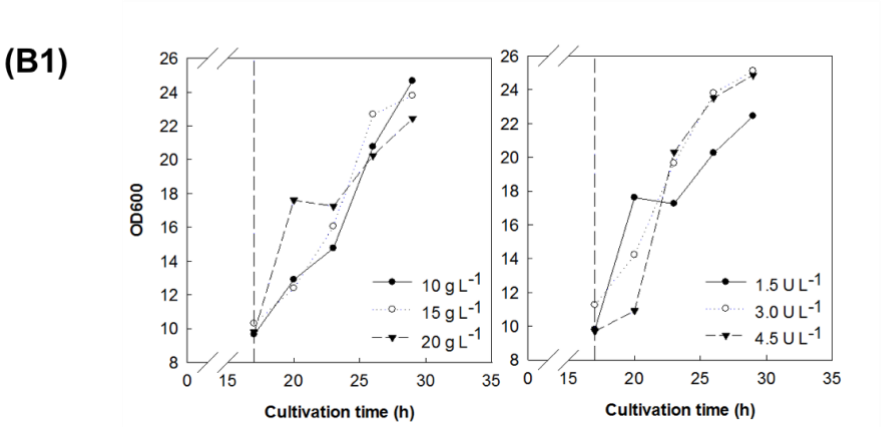
The Enpresso B main cultures were supplied with an additional dose of the glucose polymer simultaneously with the additional dose of the enzyme added at the time of induction. This was done in order to see whether higher substrate content could enhance the growth and protein yields. The glucose polymer substrate was mixed with the Enpresso B booster in a form of substrate booster mixture (SBM) to get concentrations of the additional polysaccharide of 10, 15 and 20 gL^{-1}. This mixture was fed to the cultures along with additional Reagent A (*1.5 U* L^{-1}). In parallel, the use of reagent A in doses of 1.5, 3.0, 4.5 U L^{-1} was investigated in combination with the highest polysaccharide concentration (*20 g* L^{-1}) (Table 4.2-1). All the cultures were inoculated with 6 hours Enpresso B pre-culture.

Table 4.2-1 *Change of supplementations at the time of induction of Enpresso B cultivation*

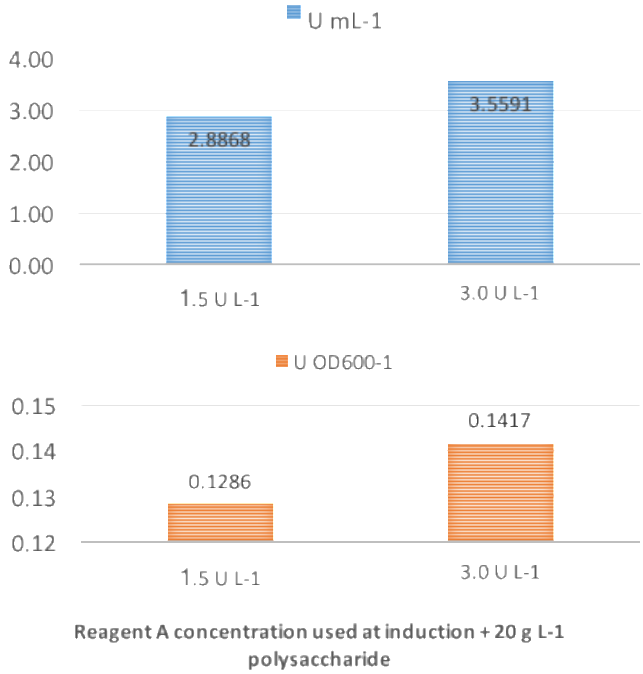
#1	#2
Different glucose polymer concentrations	Different enzyme concentration (higher doses)
1. 10, 15 and 20 g L^{-1} 2. Fixed enzyme concentration of 1.5 U L^{-1}	1. 1.5, 3.0 and 4.5 U L^{-1} 2. Fixed polysaccharide concentration of 20 g L^{-1}

The addition of the polysaccharide to the cultivation medium at the induction time accelerated the cell growth (Figure 4-6 (A1)). It could be seen from the pO_2 graphs that the cells resumed the growth right after the supplementation of the enzyme & SBM. The cultures supplied with 20 g L^{-1} polysaccharide had finished their batch phase earlier than the other cultures (approximately seven hours after induction/ supplementation time). This is probably due to the high osmolarity that could have been initiated by the high substrate concentration which had an impact on the growth of the cells. On the contrary, the length of the batch phases (after induction) of all the cultures were quite similar (Figure 4-6 (A2)). On the other hand, the cultures supplied with 20 g L^{-1} SBM behaved differently from the other cultures (Figure 4-6 (B1)). OD_{600} values were quite different at the first 8 hours after the induction. However, they reached the same OD_{600} values at the end of the cultivation. In Figure 4-6 (B2), it was found that supplying the cultures with 20 g L^{-1} needed to be coupled with high concentration of the reagent A (like 3.0 or 4.5 U L^{-1}). Generally, the final biomass of Enpresso B cultures was enhanced by 1.5-fold by addition of additional dose of the glucose polysaccharide simultaneously with the dose of the enzyme at the time of induction.





(C)



Reagent A concentration used at induction + 20 g L-1 polysaccharide

(D)

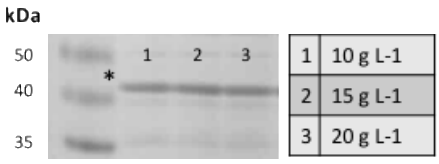


Figure 4-6 Effect of glucose releasing Reagent A dosing and glucose polymer substrate concentration. ***(A)*** *pO_2 [%] profile and OD_{600} values of Enpresso B main cultures with different polysaccharide concentrations added with 1.5 U L^{-1} reagent A at the time of induction "marked by vertical dashed line"* ***(A1 &B1)*** *and with different enzyme concentrations with 20 g L^{-1} polysaccharide added at the time of induction* ***(A2 &B2)****;* ***(C)*** *Final TtPyNP activity (volumetric and specific activities) of fed-batch like Enpresso B main culture with 20 g L^{-1} polysaccharide added at the time of induction with 1.5 & 3.0 U L^{-1} reagent A;* ***(D)*** *TtPyNP expression in Enpresso B main cultures with different polysaccharide concentrations and 1.5 U L^{-1} reagent A after 12 hours of induction (soluble fractions of heated samples loaded on SDS-PAGE).*

In terms of the protein expression, different protein samples of different cultures supplied with three different concentrations of the polysaccharide were visualized on the SDS-PAGE (Figure 4-6(D)). The protein yields were almost the same over the different SBM concentrations. Samples from the cultures of two different reagent A concentrations were chosen for quantifying the enzyme activities (Figure 4-6 (A)). The protein activities were affected by the dosing of the Reagent A added simultaneously with the glucose polymer substrate. The combination of 3.0 U L^{-1} of the Reagent A and 20 g L^{-1} of the polysaccharide resulted in 23% higher volumetric protein activity compared to the volumetric activity obtained with the combination of 1.5 U L^{-1} of Reagent A and 20 g L^{-1} of the polysaccharide. However, compared to the protein yields of the previous experiment (addition of reagent A only), the protein activity was not significantly enhanced by the additional dose of the polysaccharide. Apparently supplying the cultures with 20 g L^{-1} of the glucose polymer simultaneously with the reagent A at the time of induction results in higher cell density but not necessarily better yields of an active protein.

❖ Effect of medium pH on the Enpresso B cultures

Reagent A activity is affected by temperature and pH. The cultivation is normally run under a constant temperature, usually at 30°C for the *E. coli* cells. However, pH is susceptible to change during the bacterial metabolism. Thus, the impact of the pH change during the main cultivation on the functionality of the reagent A in terms of the cell growth was investigated. Four different Enpresso B cultures were adjusted at different initial pH values (6.5 - 6.8 - 7.0 and 7.3) with the aid of HCL solution (20%) and NaOH (1M). This pH range represents the range that could possibly be reached through the metabolic reactions during Enpresso B cultivations. Additionally, different enzyme concentrations were added for each pH. A non-recombinant *E. coli* BL21 strain was also tested as control. All the cultures were inoculated with Enpresso B pre-cultures cultivated for six hours.

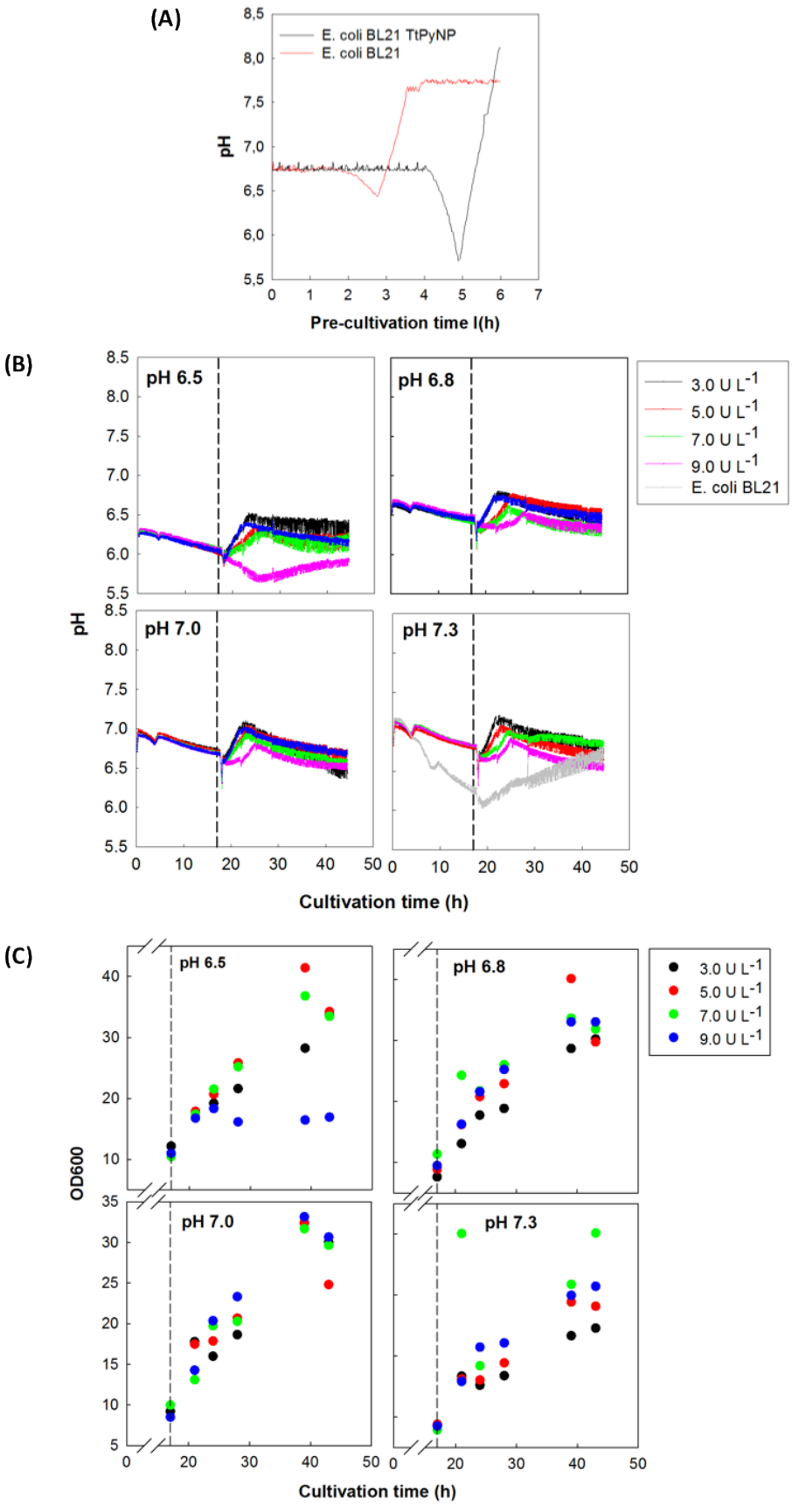

(A)
E. coli BL21 TtPyNP
E. coli BL21
pH
Pre-cultivation time I(h)
(B)
pH 6.5
pH 6.8
pH 7.0
pH 7.3
3.0 U L-1
5.0 U L-1
7.0 U L-1
9.0 U L-1
E. coli BL21
pH
Cultivation time (h)
(C)
pH 6.5
pH 6.8
pH 7.0
pH 7.3
3.0 U L-1
5.0 U L-1
7.0 U L-1
9.0 U L-1
OD600
Cultivation time (h)

Figure 4-7 Effect of the medium pH on the Enpresso B cultivations. (A) *pH profile of the pre-cultures of both recombinant and wild type E. coli BL21 in 3 mL fed-batch like Enpresso B;* ***(B)*** *pH profile of 3 mL fed-batch like Enpresso B main cultures of different pH_0 and different enzyme concentrations;* ***(C)*** *OD_{600} values of 3 mL fed-batch like Enpresso B main cultures of different pH_0.*

The pH of the recombinant *E. coli* BL21 pre-culture was rather stable during the pre-cultivation time until the 4 hours of the pre-cultivation time. At that time, the pH sharply decreased down to pH of 5.6. In contrast, the pH decrease was not sharp in the pre-culture of the wild *E. coli*. This could reflect the slower metabolism of the recombinant cells due to the strength of the T7 promoter and the metabolic load on the cells.
In the first part of the cultivation (before the induction time), the pH profiles were similar over the different cultures (Figure 4-7(B)). Regardless the pH_0 value, they decreased exponentially with the time down to pH 6.5; except for the culture of wild *E. coli* BL21 which recorded a pH decrease of down to pH 6.0. However, by addition of the second doses of reagent A at time of induction, the pH profiles were different in the cultures. The pH decrease was correlated to the concentration of the reagent A added to the culture. A high dose of reagent A resulted in low pH values. The decrease in the pH could be interpreted by the high metabolic rates of the culture producing acidic metabolic byproducts, and additionally by the consumption of ammonium ions from the cultivation medium and utilization for cell growth.
Addition of the high concentration of reagent A (9.0 U L^{-1}) to the cultures of low pH_0 value (pH 6.5) did not provide favorable pH conditions and resulted in the lowest final cell densities (≈ 15). With higher initial pH_0 values (6.8 – 7.0), the cultures with different reagent A concentrations reached the maximal OD_{600} values (≈ 30) at the end of the cultivation. However, the highest pH_0 value (pH 7.3), gave the lowest (OD_{600}≈ 20) final cell yield.
Clearly, (1) Reagent A was proven to have a broad range of the working pH values; (2) With a high reagent A concentration, highest OD_{600} values were obtained at the optimal pH range 6.8.-7.0.

4.2.2 Optimization of Enpresso B defined cultures

Enpresso B defined growth system was addressed in this part of the study as a defined medium, with a mixture of chemicals of exactly known composition. This type of growth systems is used particularly for the study of the growth and production kinetics. Like with Enpresso B cultivations, the optimal Reagent A concentration for Enpresso BD was investigated. The Enpresso BD cultures were inoculated with both LB and Enpresso B pre-cultures (Figure 4-8).

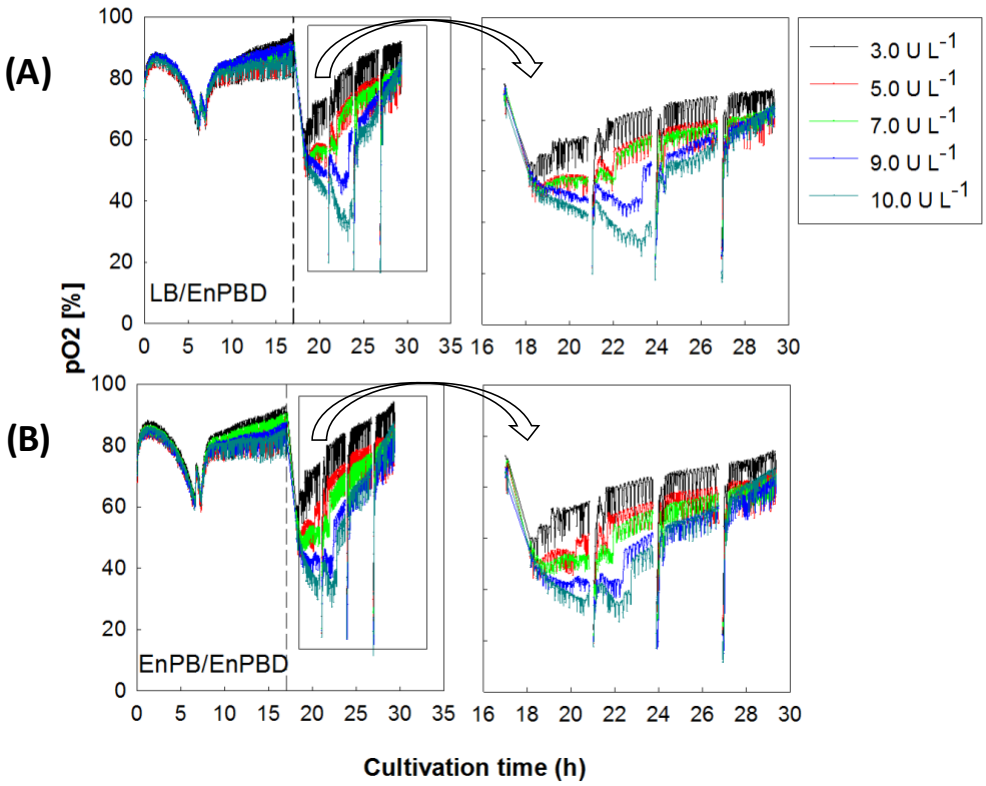


Figure 4-8 Effect of the pre-cultivation method on cell growth and respiration rate in defined medium Enpresso BD with different concentrations of Reagent A. *pO_2 [%] values of fed-batch like Enpresso BD main cultures originated from LB pre-cultures "LB/Enpresso BD"* ***(A)*** *and from Enpresso B pre-cultures "Enpresso B/Enpresso BD"* ***(B)*** *with different concentrations of reagent A added at the induction time which is indicated by a vertical dashed line. For clarification, the time after induction was indicated in the graphs (A & B) with a rectangle and enlarged as second graphs.*

Quite similar batch phases in the different cultures were observed. They ended after ≈ 5 hours and were followed by a long fed-batch phase. After overnight cultivation (induction and supplementation time), the oxygen consumption rates were different and increasing proportionally with the concentration of the reagent A. Enpresso BD has no nitrogenous organic compounds which in Enpresso B medium are offered through the boosting step and can serve as an additional carbon source. This is probably the reason of the high impact of the additional enzyme doses supplied to the cultures at the time of induction.

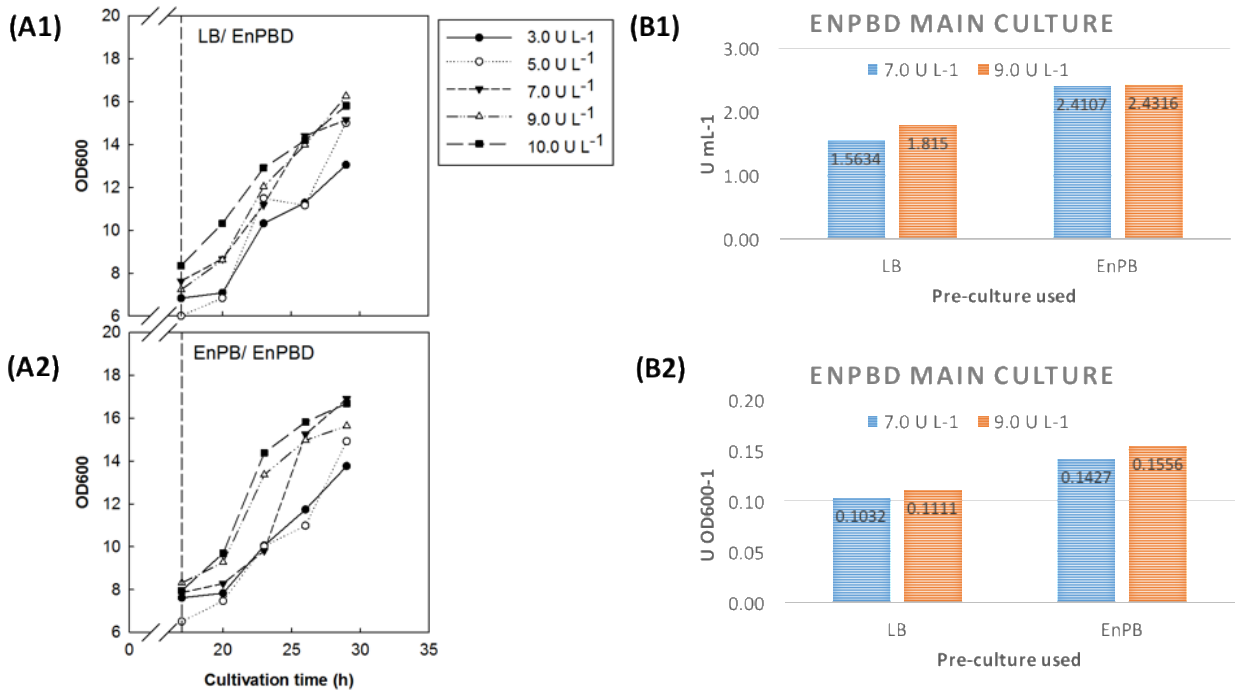


Figure 4-9 Effect of the pre-culture method (batch cultivation in LB or fed-batch in Enpresso B on growth and recombinant product formation in defined fed-batch medium Enpresso BD. (A) *OD_{600} values after induction of the Enpresso BD main cultures originated from different inocula (LB;* ***(A1)*** *& Enpresso B;* ***(A2)****); **(B)** final TtPyNP activity (per culture volume;* ***(B1)****) and specific activity (per cell;* ***(B2)****) of different cultures with different reagent A concentrations (7.0 & 9.0 U L^{-1}).*

OD_{600} profiles of the different cultivations are in a good agreement with the pO_2 profiles (Figure 4-9). With more reagent A added at the induction time, higher cell densities were obtained in both LB/Enpresso BD and Enpresso B/Enpresso BD main cultures. Yet, the reagent A dosing did not significantly affect the protein yields (Figure 4-9 B1 showing the volumetric yield and B2 showing the yield per biomass). The cultures supplied with 7.0 and 9.0 U L^{-1} Reagent A doses were chosen for activity measurement of the recombinant NP. These activities were higher in the cultures where Enpresso B was used for pre-cultures. Quite similar NP activities were obtained with different reagent A concentrations. This suggests that supplementation of the Enpresso BD cultures with a high reagent A concentration of (7-10 U L^{-1}) was effective only in terms of the biomass yield.

- **Supplementation of Enpresso B defined cultures with additional substrate along with the enzyme**

The Enpresso B defined growth system was optimized by supplying the cultures with an additional dose of the glucose-polysaccharide at the time of induction along with the reagent A. The Enpresso BD manufacturer's protocol does not include the boosting step of the cultures.

Therefore, at time of induction the cultures were supplied with polysaccharide solution in final concentrations of 10, 15 and 20 gL^{-1} along with additional Reagent A dose of 7.0 U L^{-1} (Table 4.2-2). Additionally, with the polysaccharide concentration 20 g L^{-1} two different doses of reagent A (7.0 and 9.0 U L^{-1}) were investigated. The cultures were inoculated with Enpresso B pre-culture and cultivated for six hours.

Table 4.2-2 *Change of supplementations at the time of induction of Enpresso BD cultivation*

#1	#2
Different polysaccharide concentrations	Different enzyme concentration (higher doses)
3. 10, 15 and 20 g L^{-1} 4. Fixed enzyme concentration of 7.0 U L^{-1}	3. 7.0 and 9.0 U L^{-1} 4. Fixed polysaccharide concentration of 20 g L^{-1}

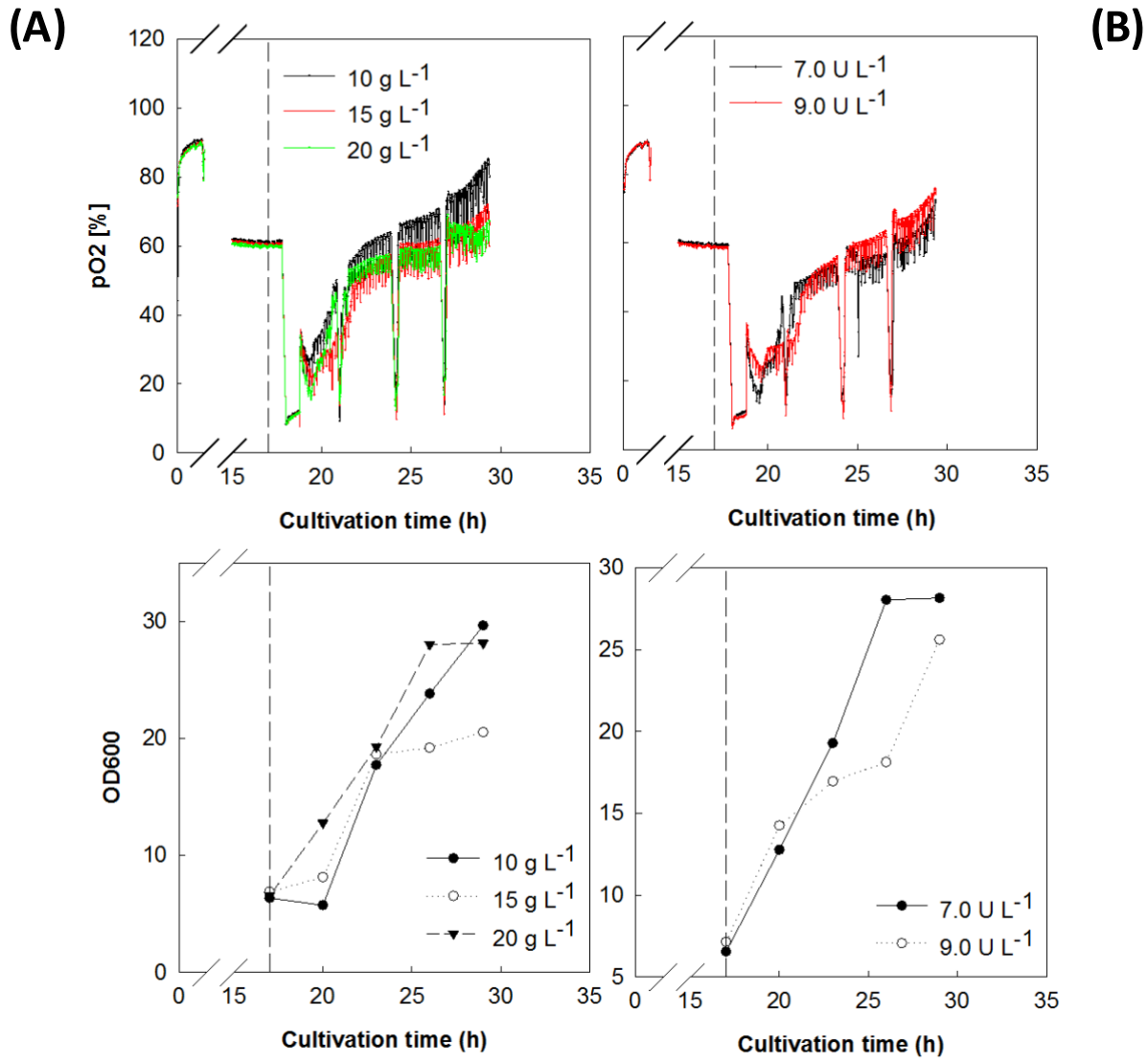


Figure 4-10 Effect of Reagent A and glucose polymer dosing on pO_2 and cell yield in Enpresso BD cultures. *pO_2 [%] and OD_{600} values of the with different glucose polymer concentrations* ***(A)*** *and with different enzyme concentrations* ***(B)****. All the additions were done at the time of induction (dashed line).*

In the cultivations oxygen consumption increased proportionally to the increase of the polysaccharide and the reagent A concentrations (Figure 4-10). However, the supplementation of a combination of substrate and reagent A resulted in longer batch phases after induction resulting in rather oxygen limited conditions. These limitations were not present in the cultures supplemented with reagent A only. Additionally, this influence was observed also on the cell biomass after the induction time in Figure 4-10. Compared to the bacterial growth in cultures having only reagent A supplementation (Figure 4-9), 1.7-fold higher biomass was obtained.

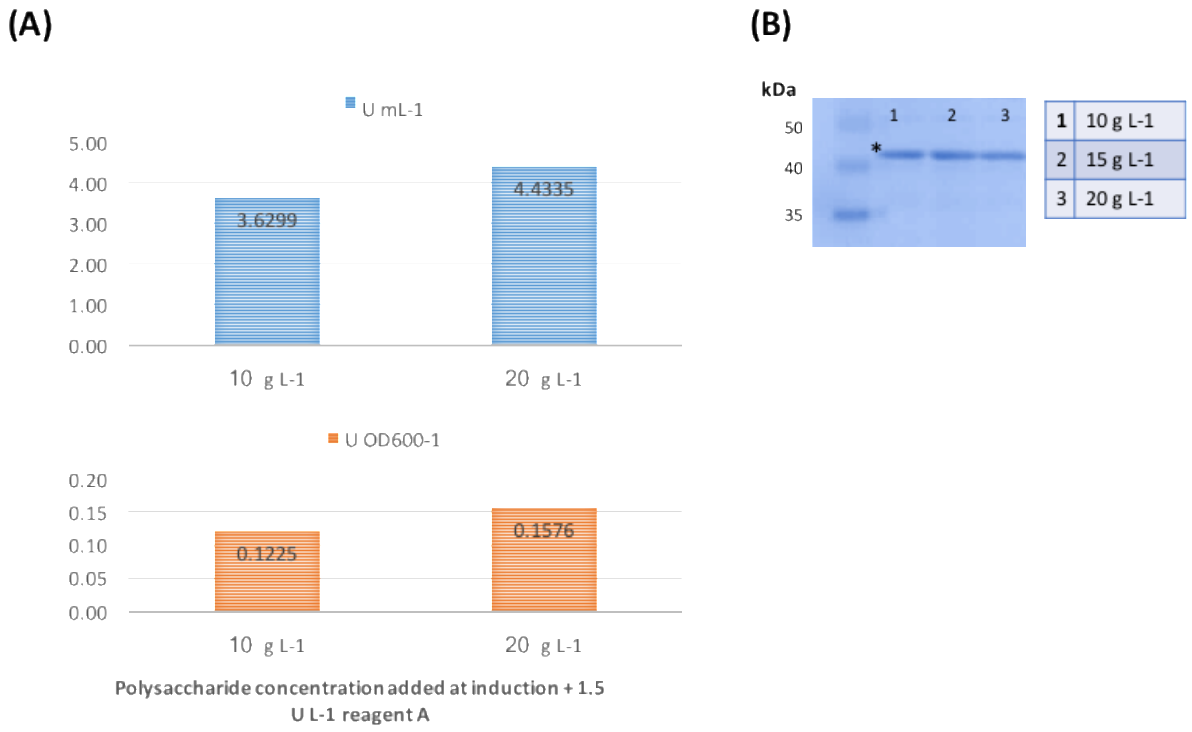


Figure 4-11 Effect of glucose polymer concentration and Reagent A dosing on recombinant protein yield in Enpresso BD cultures. (A) *Final TtPyNP activity (volumetric and specific activities) of fed-batch like Enpresso BD main culture with 7.0 U L^{-1} reagent A added at the time of induction with 10 & 20 g L^{-1} polysaccharide;* ***(B)*** *TtPyNP expression in Enpresso BD main cultures with different polysaccharide concentrations and 7.0 U L^{-1} reagent A after 12 h of induction (soluble fractions of heated samples loaded on SDS-PAGE).*

The protein expression levels per biomass were almost the same in different cultures of different polysaccharide concentrations. On the other hand, the protein volumetric activities were enhanced by the glucose polymer supplementation by approximately two-fold (Figure 4-11 (A)). This gives a conclusion that supplementation of the Enpresso BD cultures with the glucose polymer simultaneously with the reagent A was effective in terms of the biomass yield and protein activities.

4.3 Scale-up of the Enpresso B growth system

The process of scale-up is challenging due to the variations (e.g. geometrical differences) between the small-scale vessels and bioreactors, and difficulties to arrange sufficient aeration in large vessels. The performance of the recombinant *E. coli* BL21 in enzymatic glucose feeding system was investigated over three different scales; multiwell plate, shake flask and 2L benchtop bioreactor systems.

4.3.1 Multiwell plate cultivations

The optimization of the cultivation parameters for the nucleoside phosphorylase production was made in 3 mL scale using the optimized conditions for the Enpresso B cultivations in section **4.2.1.** The outcome of the optimization experiments was run for the Enpresso B cultivation with the first dose of reagent A (3.0 U L^{-1}). Overnight cultures were then fed at the time of induction with additional doses of the reagent A of 3.0 U L^{-1} simultaneously with the SBM solution containing additional glucose polymer (20 g L^{-1}). The cultures were run for 24 hours after induction/ medium supplementations.

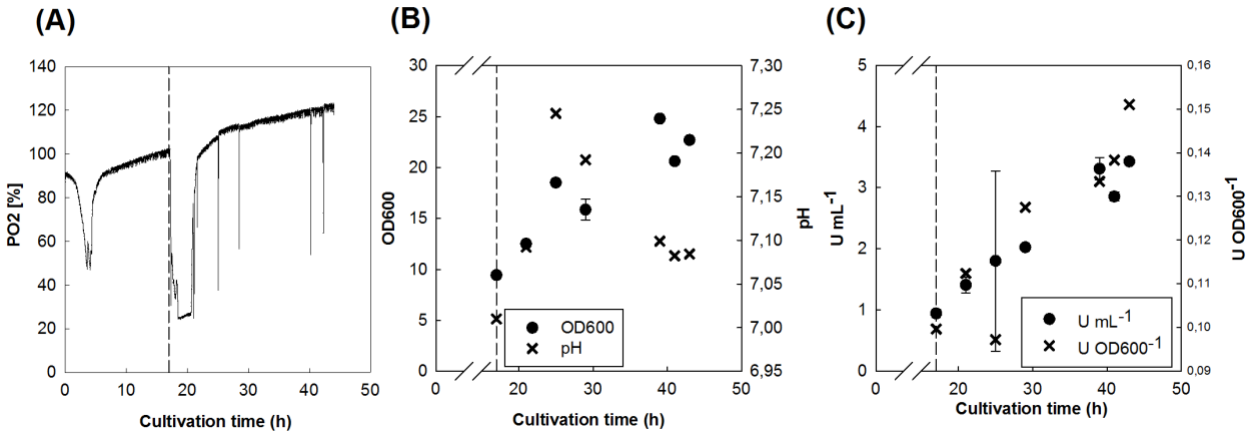
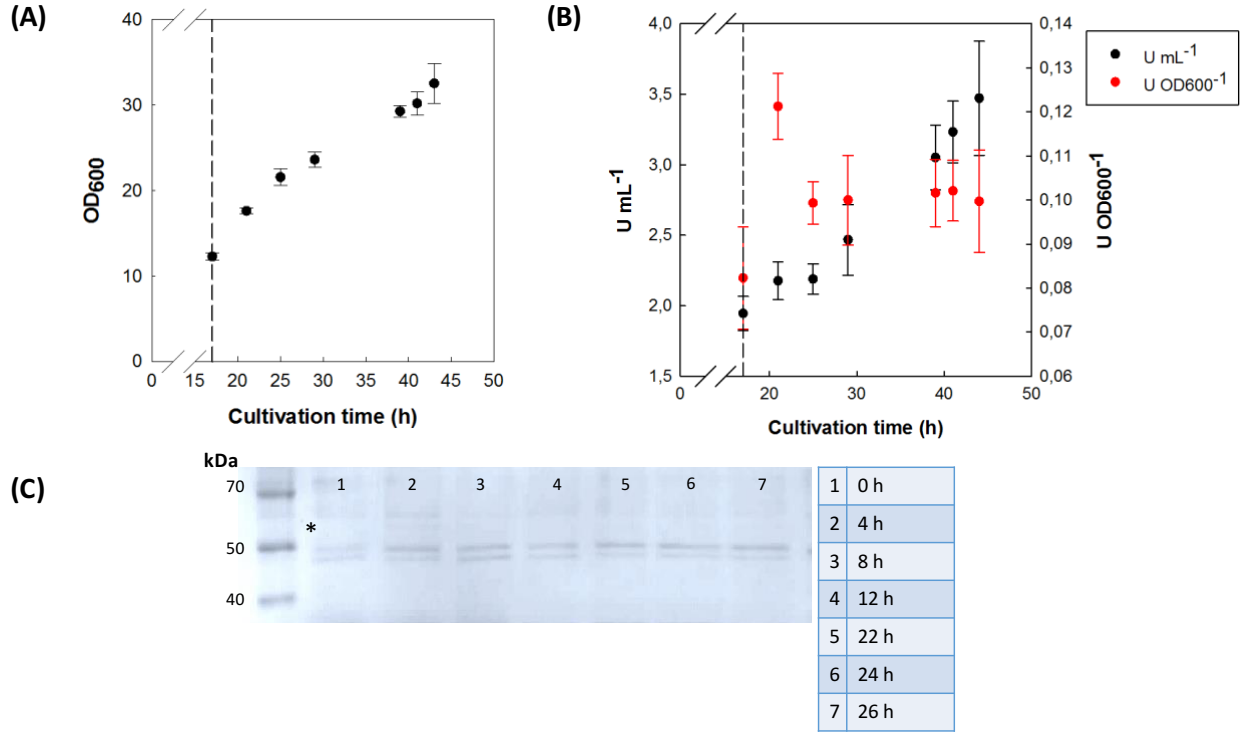


Figure 4-12 NP production in E. coli BL21 TtPyNP using Enpresso B growth system in a milliliter scale cultivation of. (A) *pO_2 [%] value;* ***(B)*** *OD_{600} & pH values;* ***(C)*** *TtPyNP activity (volumetric and specific activities). The vertical dashed line shows the time of induction. Error bars represent the Standard deviation of 3 parallel cultures.*

The growth of the cells in the first part of cultivation (before induction part) was reflected by the pO_2 graphs (Figure 4-12 (A)). Data shows that the cells grew exponentially until they finished the batch phase in 5 to 6 hours; then the fed-batch phase was started. After the induction and medium supplementation, the cells resumed in a few minutes the exponential growth for another 4 to 5 hours indicated by a higher oxygen consumption, and reached finally an OD_{600} of approximately 22 (Figure 4-12 (B)). The cultures started again in the fed-batch mode until the end of the cultivation. Interestingly, by cultivating the cells in the Enpresso B growth system, cultures did not go through a sharp oxygen limitation or culture acidification during the course of the cultivation (Figure 4-12 (A & B)). This could increase the success rate of the scale-up process. The profile of protein activity increased linearly with the time after induction reaching approximately 3.5 U L^{-1} and 0.15 U OD_{600}^{-1} enzyme yields at the end of the cultivation. Reproducibly to the results obtained before, the cell densities and the protein activities were rather similar to the values which have been obtained during the process optimization.

4.3.2 Shake flask cultivations

The cultivation in the shake flask scale is usually considered as a transition from the high throughput scale to the lab-scale bioreactor. The scale-up in shake flasks is demanding since in normal Erlenmeyer shake flasks the oxygen transfer capacity decreases significantly with the increase of the flask diameter. In order to ensure a sufficient oxygen availability, 50 mL and 500 mL cultivations were performed in special types of flasks; Ultra Yield Flasks (UYF). UYF's are known for enhanced bacterial growth due to a baffled system which creates a good aeration system in combination with air-permeable AirOTop membrane seals. UYF is usually filled with 20% of the cultivation vessel, therefore 50 mL cultivation was performed in 250 mL UYF and 500 mL was performed in 2.5 L UYF.



1	0 h
2	4 h
3	8 h
4	12 h
5	22 h
6	24 h
7	26 h

Figure 4-13 NP production using Enpresso B growth system in shake flask scale cultivation (50 mL) of E. coli BL21 TtPyNP. (A) *OD_{600} values of 50 mL fed-batch like Enpresso B main cultures in 250 mL Ultra yield shake flask;* ***(B)*** *TtPyNP activity (volumetric and specific activities. The induction time is marked by vertical dashed line. Error bars are representing the standard deviation of 3 parallel cultivations;* ***(C)*** *TtPyNP expression in Enpresso B main cultures at different time points after induction as mentioned in the attached table (soluble fractions of heated samples loaded on SDS-PAGE).*

The cells grew exponentially for 24 hours after induction and until the end of the cultivation due to the medium supplementations (Figure 4-13 (A)). By cultivating the cells in UY flasks a high biomass yield was obtained at the end of the cultivation (final OD_{600} of ≈32). However, the protein

volumetric activity obtained was quite similar to the values which had been obtained in the 24-wellplate, ≈ 3.5 U L^{-1} (Figure 4-13 (B)). The volumetric NP activity increased exponentially with the time which corresponds to the exponential increase of the biomass. Yet, the protein activity per cell was quite similar over the time after induction which was confirmed by the equal protein expression level which visualized on the SDS-PAGE (Figure 4-13 (C)).

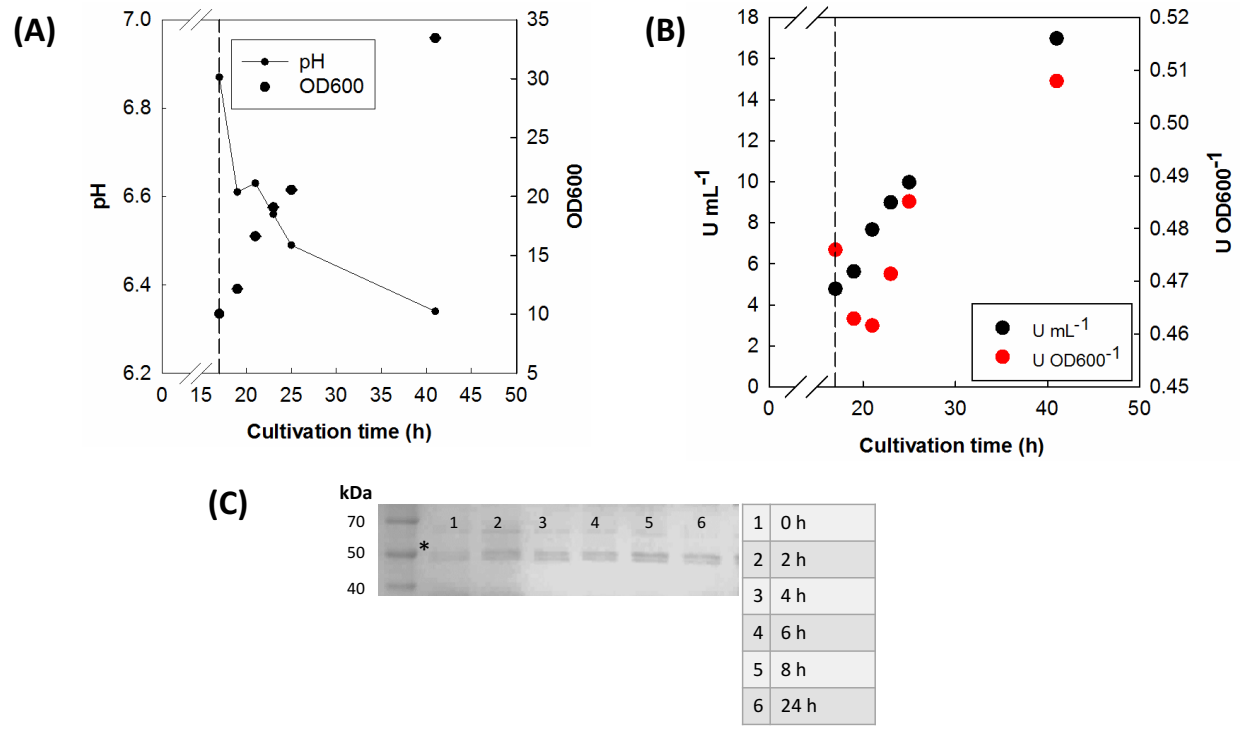


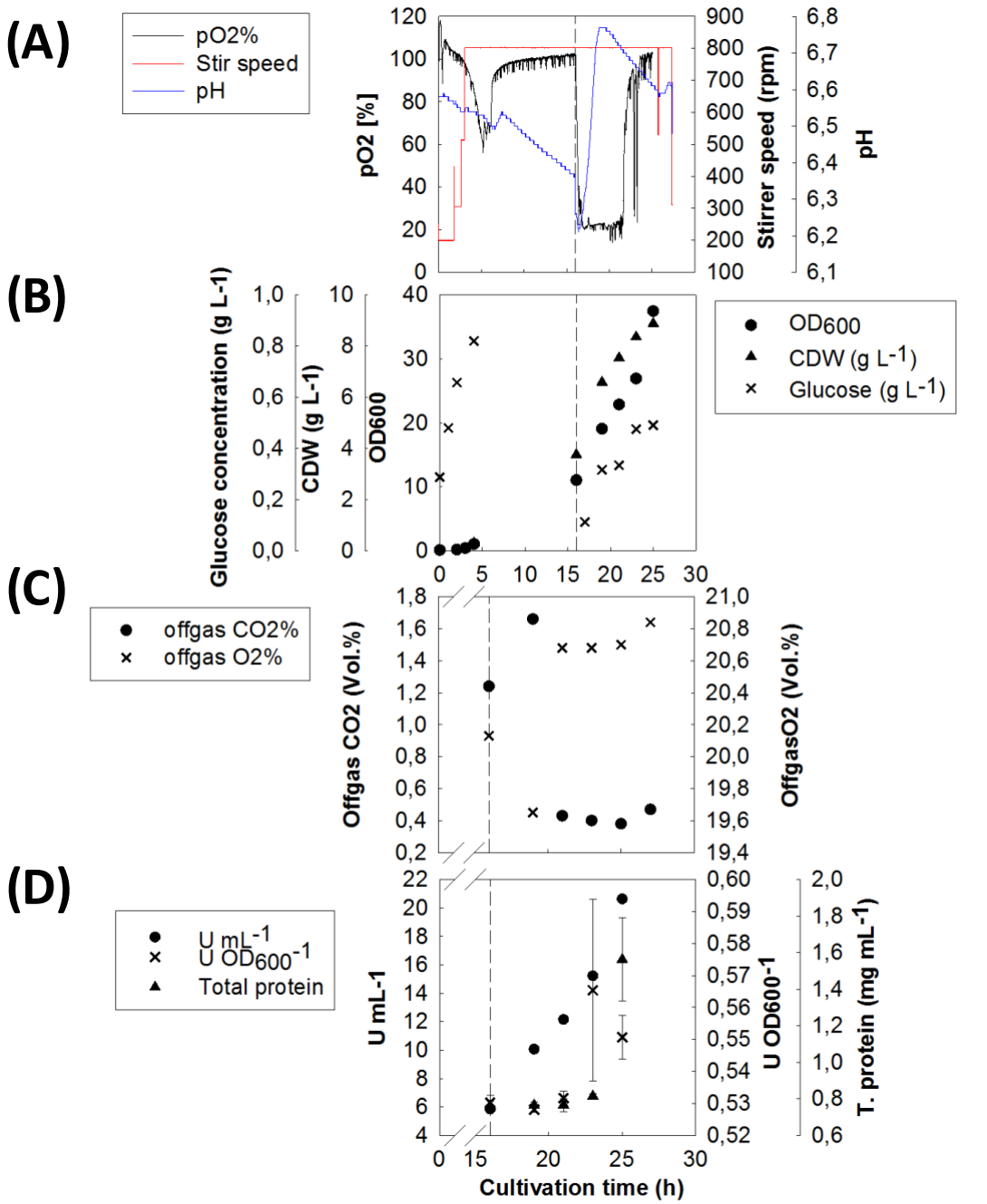
Figure 4-14 Recombinant NP production using Enpresso B growth system in a 0.5 L shake flask scale cultivation (0.5 L) of E. coli BL21 TtPyNP. (A) *OD_{600} & pH values;* ***(B)*** *TtPyNP activity (volumetric and specific activities) "marked by vertical dashed line";* ***(C)*** *TtPyNP expression in Enpresso B main cultures at different time points. Cultivations were performed in 2.5 UY shake flasks. The dashed line indicates the time of induction.*

The cells grew linearly after the induction for the first four hours (when biomass roughly doubled, after which the growth rate decreased reaching the final OD_{600} of ≈35 (Figure 4-14 (A)). The pH was 6.9 at the induction time and then decreased gradually down to pH of ≈ 6.3 at the end of the cultivation. Volumetric recombinant protein yields improved significantly during the growth, but the yield per biomass was quite similar over the culture time (Figure 4-14 (C)). In comparison to the 50 mL culture (Figure 4-13), the volumetric protein yields showed a four-fold increase with the higher scale (0.5 L). Possibly the 2.5 L UY flasks provide even better oxygen transfer than 0.5 L flasks. Thus, this improved aeration might result in higher specific yield of the recombinant protein. Differences in aeration may also affect the pH levels of the culture and thereby affect the final yields – however better aeration may not necessarily give the higher yield. It is also possible that there are batch to batch variation in cultivations (or starter glycerol stock) material.

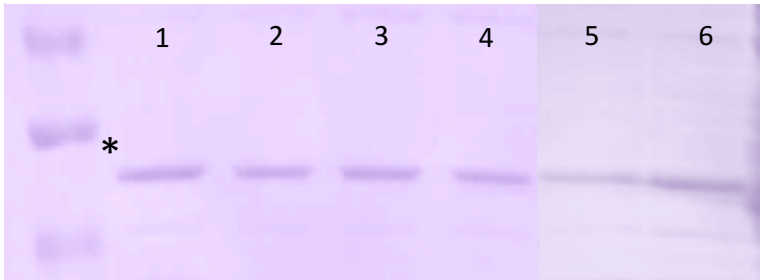
4.3.3 Fed-batch bioreactor cultivation using Enpresso B

A fed-batch bioreactor cultivation was performed in 3.7 L bench-top bioreactor to proof scalability of the Enpresso B growth system for recombinant protein production in high cell density cultures and to get detailed cultivation parameters. Pre-cultures and the reactor cultivation were done in the fed-batch like Enpresso B growth system. By this, change among different media, which probably lead to a long lag phase for cell adaptation to the new medium, was avoided. At the induction time (After 17 hours), bioreactor cultivation was supplied with 20 g L^{-1} of the substrate booster mixture (SBM). The culture run for 10 hours after induction and the samples were taken every 2 hours to monitor the level of the protein expression.

The stirrer speed, initially 200 rpm, was increased gradually in relation to the aeration level up to 800 rpm. This happened during the first 2 – 3 hours of the cultivation. After the inoculation the batch phase, characterized by a high oxygen consumption rate, took around 3 – 4 hours. This was followed by a long fed-batch phase. After the induction and medium supplementations, the pO_2 dropped immediately for a second batch phase which took approximately 6 – 7 hours before the start of the second fed-batch phase starts. In connection; it was seen that the emitted volume percentage of O_2 in the exhaust gases decreased sharply (19.6%) after the induction and medium supplementations (Figure 4-15 (B)). This reflects the immediate response of the cells towards the additional medium components in the terms of the cell respiration which accordingly resulted in corresponded increase in the volume percentage of CO_2 in the exhaust gases. In terms of the pH, a gradual decrease in the culture's pH was seen over the time course of the first batch phase (before induction). It has been increased again during the second batch phase (after induction, 17 hours) right after the addition of the induction and medium supplementation. This would reflect the impact of the buffering system which was introduced to the culture through the complex additives into the SBM. Expectedly, the pH decreased again (at ≈ 20 hours cultivation) which might be due to the glucose depletion and acetate accumulation instead, where the cells started to consume the accumulated acetate to generate energy for survival.



(E)



1	0 h
2	2 h
3	4 h
4	6 h
5	8 h
6	10 h

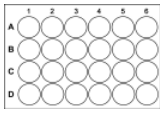
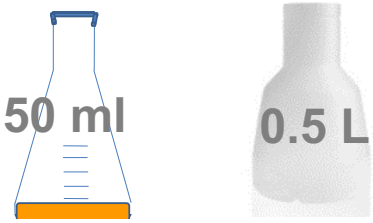
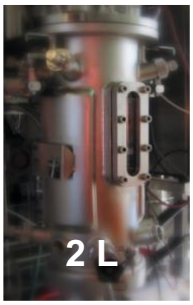
Figure 4-15 Bioreactor cultivation (2 L volume) with Enpresso B growth system for recombinant NP production. (A) *Profiles of pO_2 [%], pH and stirrer speed;* ***(B)*** *Cell growth (OD_{600}, CDW (g L^{-1})) and residual glucose concentration (g L^{-1});* ***(C)*** *Profiles of exhaust gases after the induction time "marked by vertical dashed line" (off-gases CO_2 and O_2 in Vol. %;* ***(D)*** *TtPyNP activities at different time points after induction (volumetric and specific activities);* ***(E)*** *TtPyNP expression at different time points after induction (soluble fractions of heated samples loaded on SDS-PAGE). Error bars represent the standard deviation of 3 parallel protein measurements.*

During the first part of cultivation (before induction), the cells grew exponentially with the continuous glucose release into the medium, up to OD_{600} of ≈ 10 which corresponds to ≈ 4 g L^{-1} cell dry weight. After induction and medium supplementation, cells grew up to OD_{600} of ≈ 37 which corresponded to ≈ 9 g L^{-1} cell dry weight. The glucose concentration increased in the medium linearly with the cultivation time up to 0.85 g L^{-1} by the action of the glucose releasing reagent A. At the time of induction, the glucose concentration was below 0.1 g L^{-1}. By further supplementation with additional dose of the reagent A, the glucose increased again gradually up to 0.5 g L^{-1} at the end of cultivation. During the same time the cells were exposed to a supply of the nitrogenous compounds which provide them with the amino acids. The drop of the oxygen saturation level suggests that stirring and aeration of the bioreactor did not provide enough oxygen for an efficient use of glucose (due to the enhanced glucose delivery) and the increased amount of nitrogenous compounds.

The protein expression level which visualized on the SDS-PAGE was similar over different time points after induction. Yet, the NP activities (volumetric and specific) increased exponentially with time after induction (Figure 4-15 (D)). A 21 U L^{-1} protein yield was obtained at the end of the cultivation. This corresponds to 23% increase of the protein yield compared to the yield from the 0.5 L shake flask culture (Figure 4-14(B)).

A summary for the cell and protein yields of the scalability work package is illustrated in *Table 4.3-1*. The scale-up from plate culture (3 mL cultures) maintained the productivity. A 357% increase in the protein yield was obtained with the 0.5 L shake flask culture, and further 23 % increase by the benchtop-scale bioreactor. It is possible that EnBase system works better in an improved aeration systems like baffled shake flasks and the bioreactors which are known with improved oxygen transfer rates. Also, presence of a dry oxygen permeable membrane affects the oxygen transfer into the culture particularly in the long-term cultivations. The filling volume is possibly affect the oxygen transfer too and thus the biomass and protein productivities. It is observed that the scale-up process of NP production from 24-wellplate to 2 L fermentation did not corrupt the protein production. This indicates the success of the scalability of the system.

Table 4.3-1 *Summary of the Enpresso B growth system scale-up*

	MWP	**Shake flask (50 mL & 0.5 L)**		**Benchtop reactor**
				
Cell density (OD_{600})	25	31	33	37
Volumetric protein activity	3.2 U mL^{-1}	3.5 U mL^{-1}	16 U mL^{-1}	20 U mL^{-1}
Specific protein activity	0.15 U OD_{600}^{-1}	0.1 U OD_{600}^{-1}	0.51 U OD_{600}^{-1}	0.57 U OD_{600}^{-1}
Cultivation time	40 h	40 h	40 h	25 h

4.4 Autoinduction of recombinant *E. coli* cultures

The conventional induction protocol of the recombinant proteins is the single shot induction which is usually done after reaching a certain cell density. The commonly known autoinduction system is the lactose-based autoinduction system which was introduced in 2005 by (Studier, 2005). Different concentrations of IPTG was investigated for autoinduction of fed-batch like Enpresso B cultivations (AI) in comparison to the single shot induction of overnight cultures (SSI) (Table 4.4-1).

Table 4.4-1 *Different concentrations of IPTG used for single shot induction and autoinduction of Enpresso B*

Induction source	Type of induction	Concentrations
IPTG	SSI	10, 20, 40, 100, 250 and 1000 µM
	AI	10, 20, 40, 70, 100 and 250 µM

4.4.1 Single shot IPTG-induction (IPTG-SSI) and IPTG-Autoinduction (IPTG-AI) of the Enpresso B growth system

Conventional induction protocol of Enpresso B cultures with IPTG (after 17 hours) along with IPTG-Autoinduction was performed in this part of work to investigate (1) the impact of using IPTG in autoinduction of Enpresso B growth cultures; (2) whether IPTG needs a certain cell density or growth phase for a maximal input; (3) the optimum concentration of IPTG for autoinduction of Enpresso B cultivations.

❖ IPTG single shot induction of Enpresso B cultures (IPTG-SSI)

In the single shot induction approach, the cultivations applied the standard Enpresso B protocol. The cultures were run for 15 - 18 hours, then the cells were induced with different concentrations of IPTG (10 – 1000 µM) and supplied with an additional dose of reagent A (3.0 U L^{-1}) and the substrate booster mixture (20 g L^{-1}).

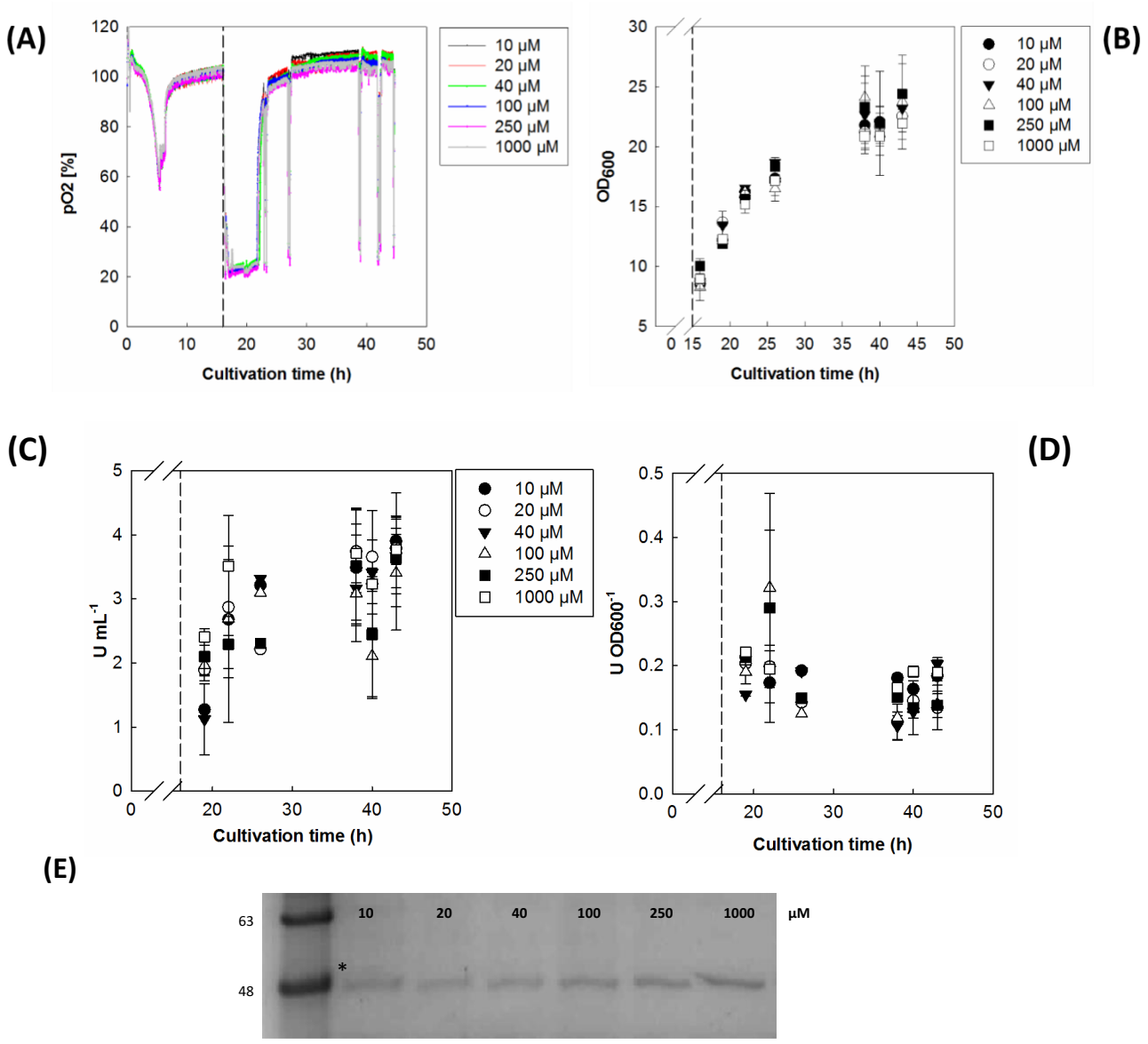


Figure 4-16 IPTG-Single shot induction of Enpresso B cultures. (A) *pO_2 [%] profile;* ***(B)*** *OD_{600} values;* ***(C & D)*** *TtPyNP activities (volumetric and specific activities). Values of OD_{600} and protein activities were measured at different time points after the induction "marked by vertical dashed line". Error bars represent the Standard deviation of 3 different measurements;* ***(E)*** *TtPyNP expression in different Enpresso B main cultures induced with different IPTG concentrations (soluble fractions of heated samples loaded on SDS-PAGE).*

Data presented in the Figure 4-16 (A) shows that the cultures grew with a batch phase of ≈ 5 hour and started then the fed-batch phase. After 16 hours of cultivation, the cultures were induced with different concentrations of IPTG at a cell density of ≈ OD_{600} = 9 (Figure 4-16 (B)). Unexpectedly, the cells grew after the induction with similar profiles over the different IPTG

concentrations. IPTG is known for the toxicity at concentrations higher than 1 mM, and indeed the cells were not highly affected by the dose of the IPTG up to 1000 µM (Figure 4-16 (B)).
The same was observed in terms of the recombinant protein yields (Figure 4-16 (C & D)). The profile of the volumetric protein activity was linear with time until the end of the cultivation over the different IPTG concentrations supplied to the cultivation (Figure 4-16 (C)). This would reflect that by cultivating the cells for longer cultivation time, higher volumetric protein activities could be obtained. Moreover, the activity of the protein per cells was rather stable over the time independent of the concentration of the IPTG added to the culture (Figure 4-16 (D)). On the other hand, the level of the protein expression was rather higher at high IPTG concentrations (100-1000 µM) (Figure 4-16 (E)). This indicates that the single shot induction of the Enpresso B cultures with IPTG concentration between 0.1 - 1 mM provides fairly similar yields of recombinant NP.

❖ IPTG-Autoinduction of Enpresso B cultures (IPTG-AI)

Different concentrations of IPTG were investigated for autoinduction of Enpresso B. Very low concentrations of IPTG are transported to the cell actively by the action of *lac* permease. while IPTG concentrations used for normal induction (0.2 – 1 mM) is transported by passive diffusion.
In the study of the IPTG-autoinduction of the Enpresso B cultures, the cultures were not interrupted by medium supplementations. The Enpresso B medium was prepared according the standard protocol of the Enpresso B growth systems. Reagent A was added at a concentration of 3.0 U L^{-1} at the beginning for glucose release, simultaneously with different concentrations of IPTG. The cultures were run for approximately 30 hours continuously with protein samplings at different points in time.

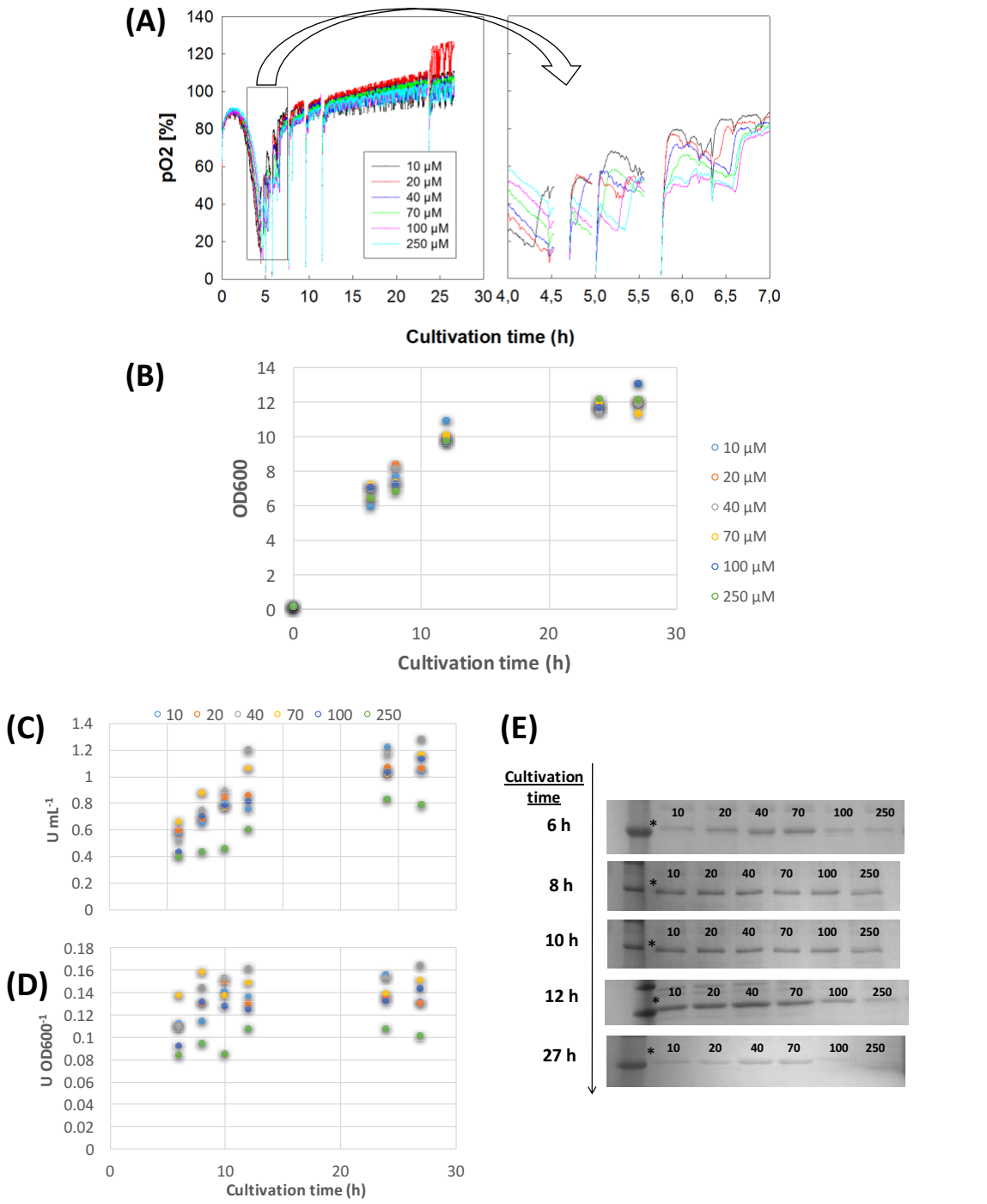
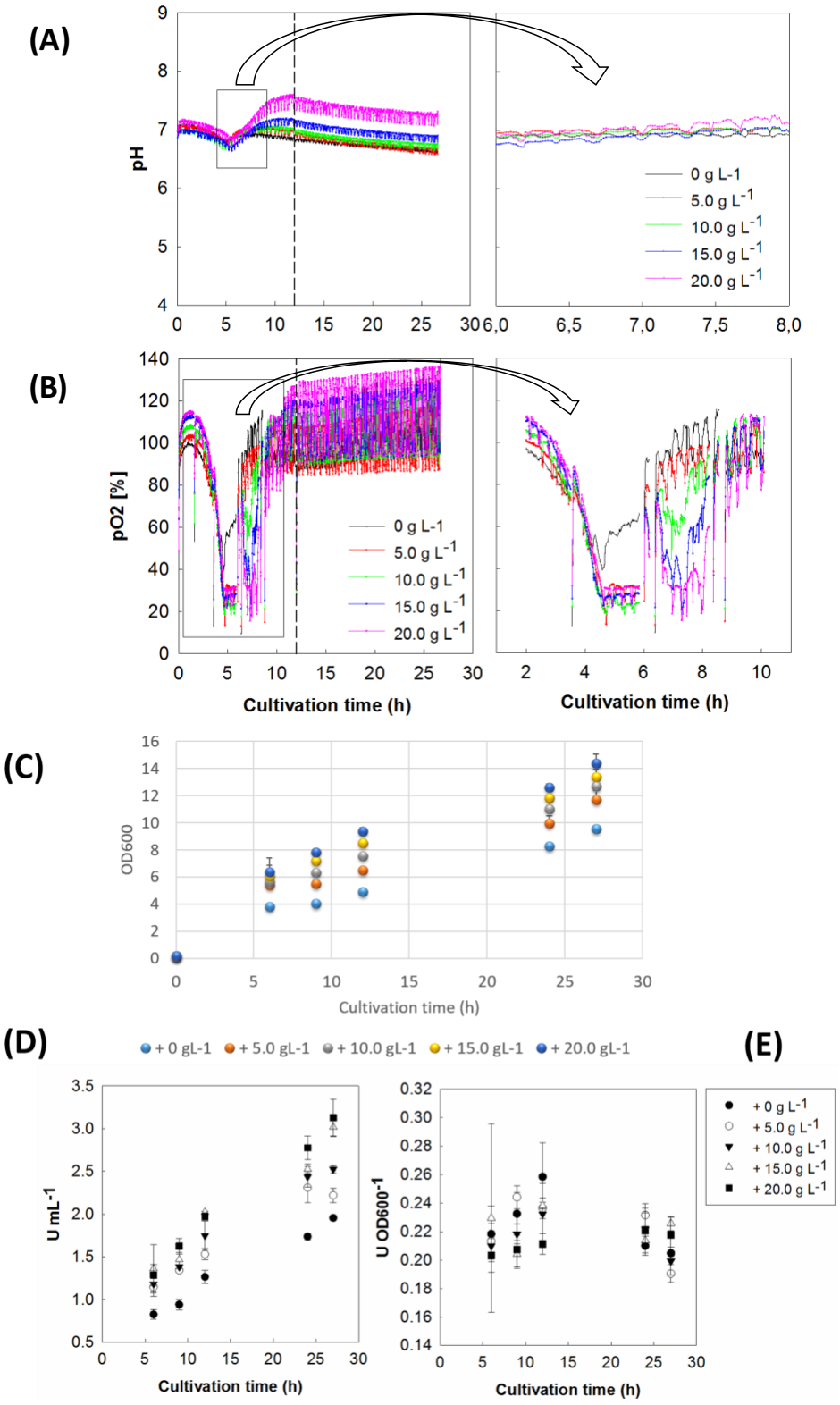


Figure 4-17 IPTG-Autoinduction of Enpresso B growth cultures. (A) *pO_2 [%] of different Enpresso B cultures with different IPTG concentrations. The time window (3 - 8 hours) showing pO_2 fluctuation has been enlarged;* ***(B)*** *OD_{600};* ***(C & D)*** *TtPyNP activities (volumetric and specific activities);* ***(E)*** *TtPyNP expression in Enpresso B main cultures at different time points of the cultivation (soluble fractions of heated samples loaded on SDS-PAGE).*

The pO_2 profiles presented in Figure 4-17 (A) show rather similar oxygen consumption profiles in cultivations with different IPTG concentrations. However, there are small differences in the pO_2 profile according to the IPTG dose. Obviously, with more IPTG concentration added to the culture a higher oxygen consumption was observed during the first 7 hours of the cultivation. Interestingly such correlation between the oxygen consumption and the IPTG concentration was not observed with the single shot induction. This suggests that IPTG has an impact on the growth and O_2 consumption if it is added at the beginning of the cultivation but not when the cells have already finished their batch phase and induced during the fed-batch phase. However, the cell densities showed no differences (Figure 4-17 (B)). Moreover, autoinduction provided much lower cell densities compared to in SSI cultures (maximal OD_{600} values of ≈ 12 vs 23), but this might be optimized by additional pulse of enzyme or polymer.
Different IPTG concentrations provided different yields of active recombinant (Figure 4-17 (C & D)). The highest IPTG concentration (250 µM) resulted in the lowest activity values (both volumetric and specific activities) during the whole cultivation. IPTG concentration between 40 and 100 µM was optimal for expression of an active protein (up to 1.2 U mL^{-1} & 0.16 U OD_{600}^{-1}). This was confirmed by SDS-PAGE (Figure 4-17 (E)). The maximum protein expression was obtained after 12 hours cultivation time in cultures induced with 20 - 70 µM of IPTG. With further overnight cultivation, the level of the protein expression per cell was lower. To sum it up, the impact of the IPTG dose was clearer in the Enpresso B-autoinduction system than in the conventionally induced overnight cultures. IPTG concentration between 40-100 µM seemed to be optimal for the autoninduction of the Enpresso B cultures.

The IPTG-autoinduction Enpresso B cultivation was not interrupted by any medium supplementation (e.g. booster or the biocatalyst). This might be a reason for obtaining low cell densities and the active protein yield. Therefore, an optimum concentration of the IPTG (70 µM) was chosen to test auto-induction combined with the culture boosting. The cultures were run in 24 deepwell plate with pH and pO_2 monitoring systems. 70 µM of the IPTG was added to the Enpresso B cultures at the beginning simultaneously with different concentrations of the booster solution (0, 5.0, 10.0, 15.0 and 20.0 g L^{-1}) and run ovenight. After 12 hours cultivation time, the cultures were supplied with another dose of the biocatalyst (3.0 U L^{-1}) of the biocatalyst and run further for another 17 hours. The OD_{600} and the protein samples were taken at the different time points during the cultivation.


(A)
pH
0 g L-1
5.0 g L-1
10.0 g L-1
15.0 g L-1
20.0 g L-1
(B)
pO2 [%]
Cultivation time (h)
Cultivation time (h)
(C)
OD600
Cultivation time (h)
(D)
+ 0 gL-1
+ 5.0 gL-1
+ 10.0 gL-1
+ 15.0 gL-1
+ 20.0 gL-1
U mL-1
Cultivation time (h)
(E)
U OD600-1
Cultivation time (h)

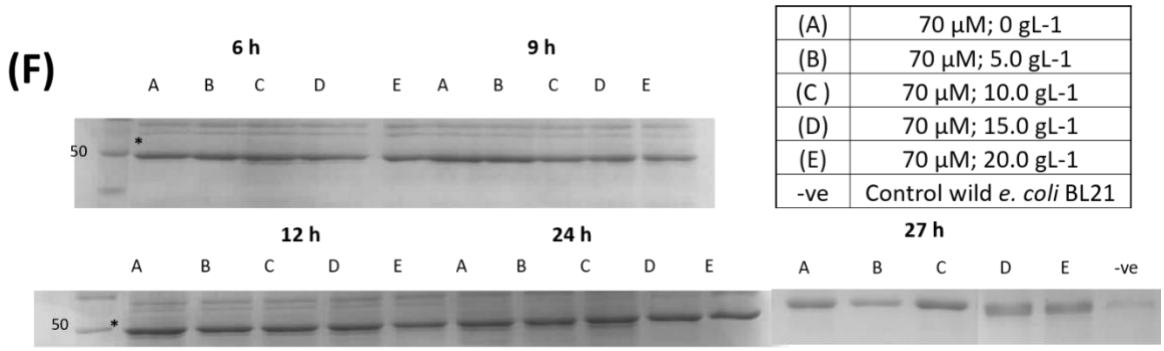


Figure 4-18 IPTG-Autoinduction of Enpresso B growth cultures with different concentrations of booster solution which was added at the beginning of the main cultivation. (A) *pH values of different IPTG autoinduced Enpresso B cultures. The time window (3 - 8 hours) showing the pH fluctuation has been enlarged;* ***(B)*** *pO_2 [%] values. The time window (2 - 10 hours) showing the pO_2 fluctuation has been enlarged;* ***(C)*** *OD_{600};* ***(D & E)*** *TtPyNP activities (volumetric and specific activities), error bars represent the standard deviation of 3 different measurements;* ***(F)*** *TtPyNP expression in Enpresso B main cultures at different time points of the cultivation (soluble fractions of heated samples loaded on SDS-PAGE). The time of the reagent A supplementation is indicated by a vertical dashed line.*

Despite the different concentrations of the booster in the cultures the pH profiles were nearly the same and provided the pH 7.0 which is optimal for the *E. coli* cells. Interestingly, the pH was well maintained during the whole cultivation; it decreased only from 7.0 to 6.5. After 12 hours cultivation, the differences in the pH values over the different concentrations of the booster were observed. The culture with the highest concentration of the booster (20 g L^{-1}) provided the highest pH (7.5), while the other cultures had pH close to 7.0. It seems that during the first six hours of the cultivation, the cells were growing apart from the effect of the booster. After 10 hours cultivation, the nitrogen content in the booster obviously started to be efficiently consumed.

Different booster concentrations provided different pO_2 profiles (Figure 4-18 (B)). The batch phase of the cells started right after the cultivation start and took approximately 5 to 7 hours. The cultures with no booster (0 g L^{-1}) started the fed-batch phase earliest (Figure 4-18 (B)). As expected, the lowest pO_2 values were obtained with high booster concentration.

The cultures with the high booster concentrations provided highest cell densities. This would verify the aim of supplying the autoinduced cultures with the booster. It was seen that the cell yield increased from OD_{600} of 10 to 14, when 20 g L^{-1} booster was used. Yet, the cell yields were lower than those obtained in the single shot induced cultures.

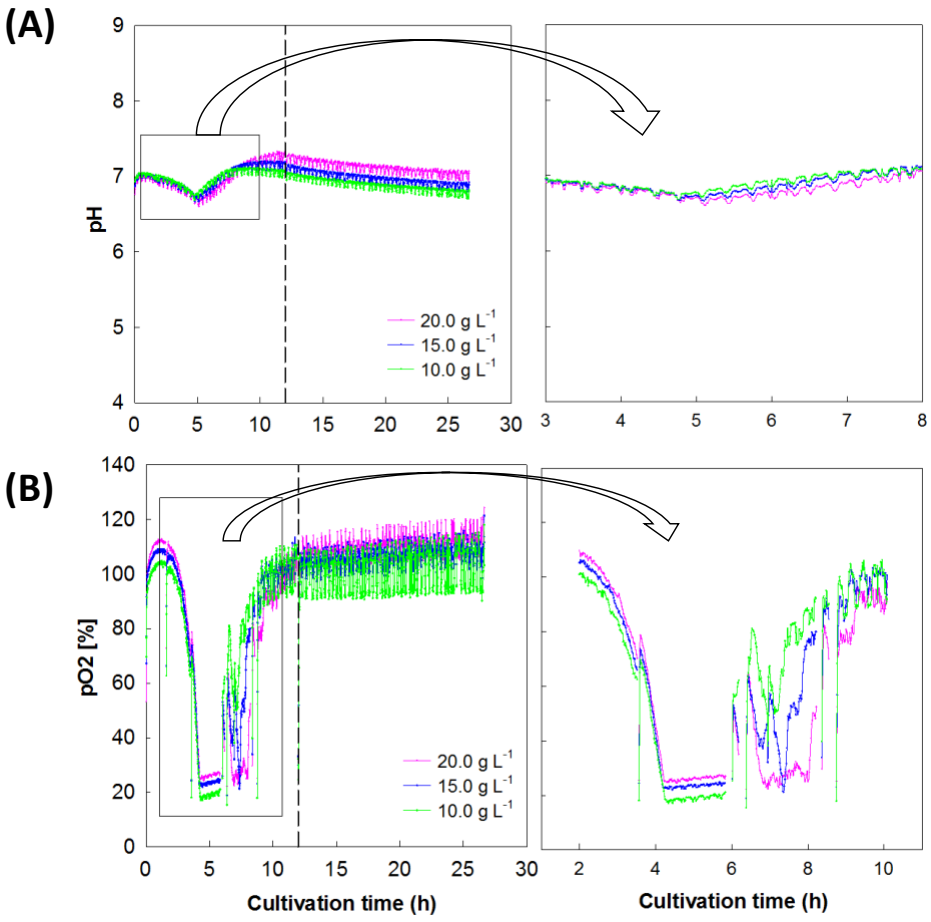
Similarly, the impact of boosting on the autoinduced cultures was observed in the profile of the protein activities (Figure 4-18 (D & E)). The expression levels visualized on the SDS-PAGE in the Figure 4-18 (F) shows that the protein expression levels per bacterial cell in the different cultures with and without boosting were the same over the whole cultivation time. However, the profile

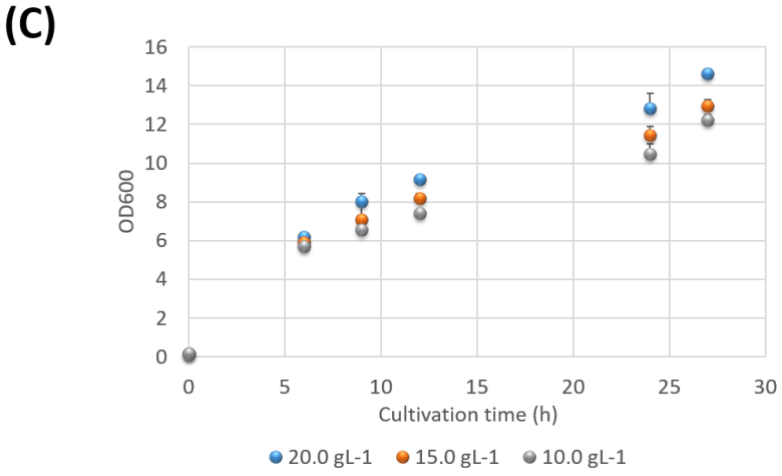
of the protein volumetric activity (Figure 4-18 (D)) shows higher protein activities when the booster was used. Boosted cultures gave increasing protein yields until to the end of the cultivation time, providing up to ≈ 3.3 U L^{-1} of active recombinant protein. With the use of 20 g L^{-1} booster, the protein yield improved from 1.5 to 3.3 U L^{-1} by providing a higher cell density while the productivity per cell was maintained. The maximum protein activity per cell was obtained after ≈ 12 hours is ≈ 0.25 U OD_{600}^{-1} compared to 0.16 U OD_{600}^{-1} obtained without culture boosting or by single shot culture induction after 17 hours of cultivation.

4.4.2 Autoinduction of the Enpresso B cultures with complex nitrogenous supplements

The impact of the booster on the IPTG-autoinduction cultures was shown above. Moreover, the impact of complex nitrogenous compounds on the autoinduction of *E. coli* BL21 cultures using T7 or tac promoters in a modified LB medium (mLB) has been reported before (Xu *et al.*, 2012). Therefore, the booster mixture was used here as the sole source of induction of Enpresso B cultures.

Accordingly, Enpresso B-Booster (mixture of yeast extract and peptone) was added to the Enpresso B growth system at the beginning of the cultivation in three different concentrations (20.0, 15.0 and 10.0 g L^{-1}) without any additional inducers. The cultures were run for 12 hours and then supplied with an additional dose of 3.0 U L^{-1} of the biocatalyst (reagent A). The cultures were thereafter run for another night. The pO_2 [%] and pH levels were monitored online during the whole cultivation while the cell growth and the protein analysis were investigated at different time points during the cultivation.


(A)
pH
20.0 g L-1
15.0 g L-1
10.0 g L-1
(B)
pO2 [%]
Cultivation time (h)
Cultivation time (h)


(C)
OD600
Cultivation time (h)
20.0 gL-1
15.0 gL-1
10.0 gL-1

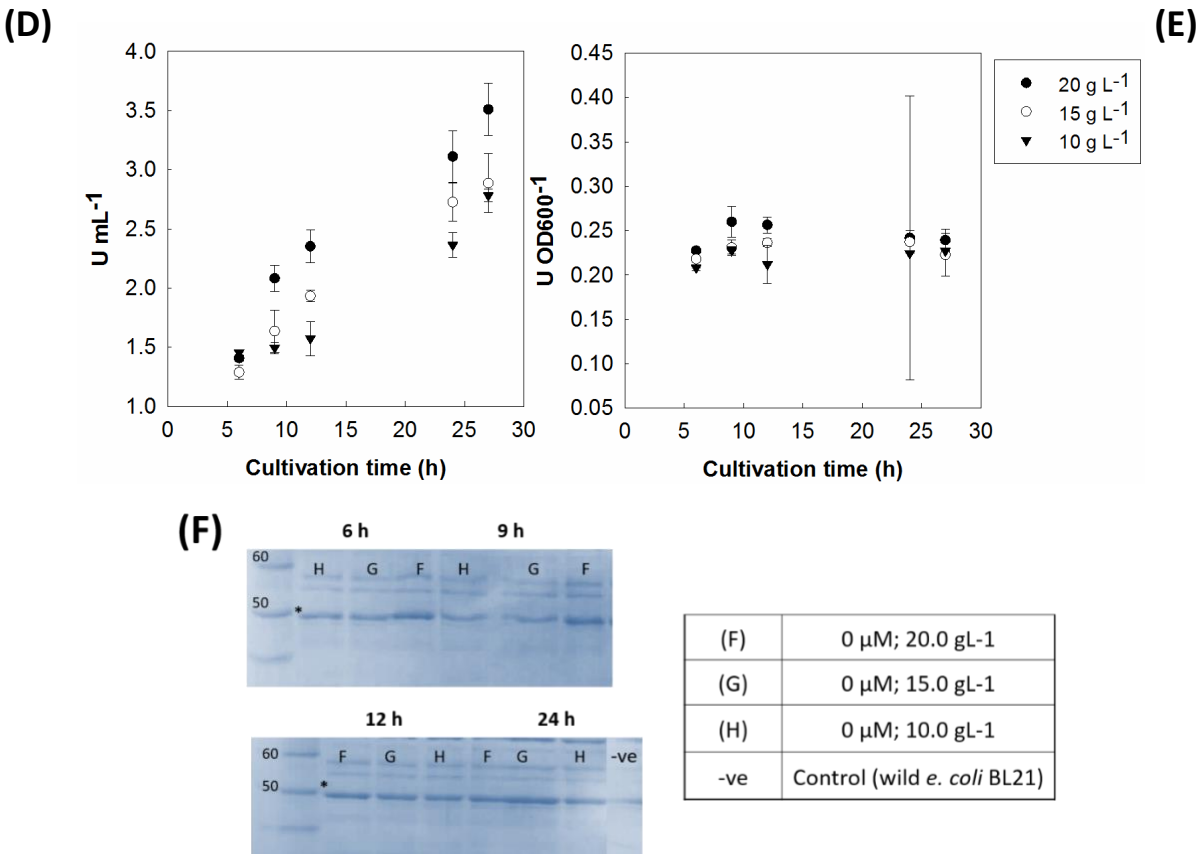


Figure 4-19 Booster-Autoinduction of Enpresso B growth cultures. (A) *pH values. The time window (3 - 8 hours) showing the pH fluctuation has been enlarged;* ***(B)*** *pO_2 [%] values. The time window (2 - 10 hours) showing the pO_2 fluctuation has been enlarged;* ***(C)*** *OD_{600} values;* ***(D & E)*** *TtPyNP activities (volumetric and specific activities), error bars represent the standard deviation of 3 different measurements;* ***(F)*** *TtPyNP yields demonstrated on SDS-PAGE. The same amount of bacterial cells was loaded to the each well. The time of the reagent A supplementation is indicated by a vertical dashed line.*

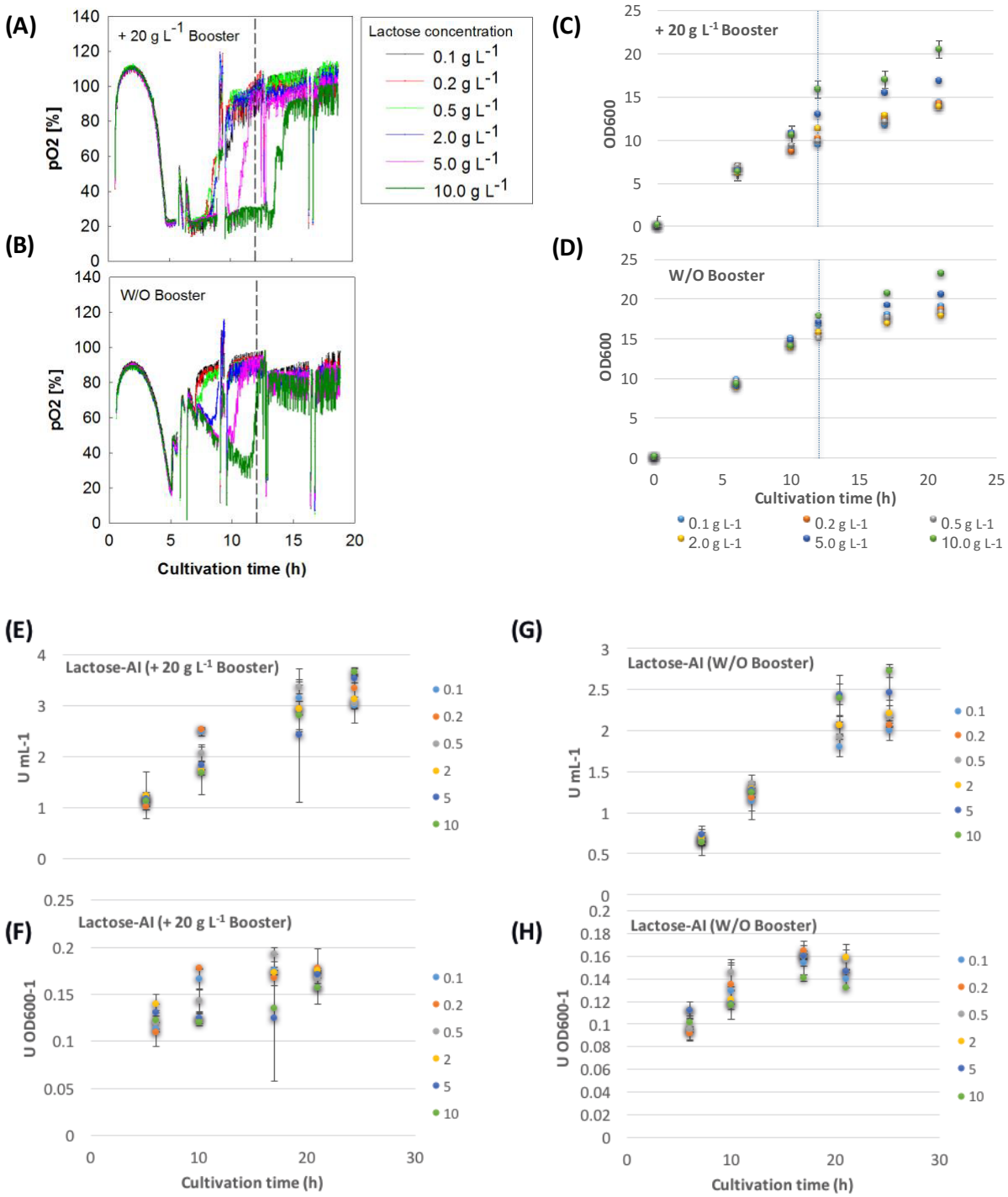
No significant differences between the pH profiles of the different samples was observed. Nevertheless, a slight variation was seen after the addition of the second dose of the biocatalyst after 12 hours (Figure 4-19 (A)). The pH was higher in the cultures of the high booster concentration with an overall range of pH of 7.0, the optimal pH of the *E. coli* cells.

The batch phase, indicated by a drop of the pO_2 value, started in a few hours after the start of the cultivation. During the batch phase, the differences in pO_2 profiles provided by the different concentrations of the booster were not very drastic. However clear and significant differences could be observed at the beginning of the fed-batch phase. The cultures supplied with low booster concentrations started their fed-batch phase earlier than the cultures with higher concentrations. With a higher amount of booster (higher nitrogen content) the batch phase of the culture was longer. Thus, the booster was important for the fine-tuning of the buffering system and as the nutritional factor on the cells. This was also confirmed by the cell densities

shown in Figure 4-19 (C). High concentrations of the booster resulted in higher cell densities until the end of the cultivation. The cultures with a booster concentration of 20 g L^{-1} had a cell density of approximately OD_{600}= 14.0 at the end of the cultivation. This was nearly as high as the cell density obtained from the IPTG-autoinduced culture supplemented with 20 g L^{-1} booster. This would confirm that the IPTG was able to promote the protein induction process but it was important to complement the cultures with additional nutritional additives.
In terms of the protein yield (Figure 4-19 (D & E)), nearly the same protein activities (≈ 3.5 U L^{-1}, 0.25 U OD_{600}^{-1}) were obtained by induction through boosting of the culture. It is possible that the low concentrations of lactose and galactose (or related compounds) in the booster mixtures (yeast extract and booster) were able to induce the recombinant *E. coli* BL21 cells for the NP expression. This could also be confirmed through the expression levels visualized on the SDS-PAGE (Figure 4-19 (F)). Cultures with 20 g L^{-1} booster expressed rather higher protein titers particularly in the first two-time point samplings (6 & 9 hours).

4.4.3 Lactose autoinduction

In this part, the lactose mediated autoinduction was performed using fed-batch Enpresso B growth system. Thus, six different concentrations of the lactose (ranging from 0.1 – 10.0 g L^{-1}) were added to the Enpresso B growth system at the beginning of the cultivation. Also 0.5 g L^{-1} glucose was supplied to the lactose-Enpresso B media to inhibit the lactose intake from the beginning. One group of the lactose-Enpresso B cultures were boosted with of 20 g L^{-1} booster mixture at the beginning while the second group of the cultures were grown without boosting. All cultures were supplied with a second dose of the biocatalyst (3.0 U L^{-1}) after 12 hours cultivation time; the cultures were thereafter run for additional 10 hours.


(A)
+ 20 g L-1 Booster
pO2 [%]
Lactose concentration
0.1 g L-1
0.2 g L-1
0.5 g L-1
2.0 g L-1
5.0 g L-1
10.0 g L-1
(B)
W/O Booster
pO2 [%]
Cultivation time (h)
(C)
+ 20 g L-1 Booster
OD600
(D)
W/O Booster
OD600
Cultivation time (h)
0.1 g L-1
0.2 g L-1
0.5 g L-1
2.0 g L-1
5.0 g L-1
10.0 g L-1
(E)
Lactose-Al (+ 20 g L-1 Booster)
U mL-1
(F)
Lactose-Al (+ 20 g L-1 Booster)
U OD600-1
Cultivation time (h)
(G)
Lactose-Al (W/O Booster)
U mL-1
(H)
Lactose-Al (W/O Booster)
U OD600-1
Cultivation time (h)

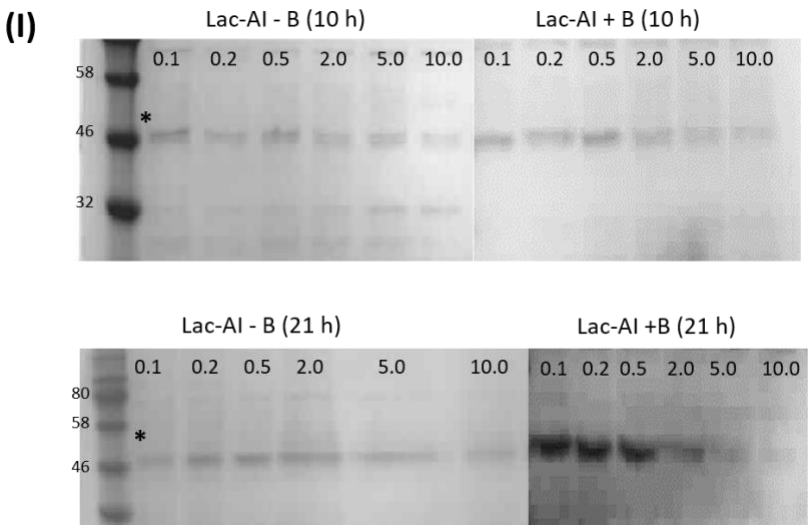


Figure 4-20 Lactose-Autoinduction of Enpresso B growth cultures with and without boosting. (A & B) *pO_2 [%] profiles of the lactose-AI Enpresso B cultures; the time of the reagent A supplementation is indicated by a vertical dashed line;* ***(C & D)*** *OD_{600} values;* ***(E & F)*** *TtPyNP activities (volumetric and specific activities) of the lactose-AI Enpresso B cultures with 20 g L^{-1} booster mixture;* ***(G & H)*** *TtPyNP activities (volumetric and specific activities) of the lactose-AI Enpresso B cultures without booster, error bars represent the standard deviation of 3 different measurements;* ***(I)*** *TtPyNP expression in different Enpresso B cultures (boosted & non-boosted) with different lactose concentrations after 10 & 21 hours of the cultivation (soluble fractions of heated samples loaded on SDS-PAGE).*

Figure 4-20 (A&B) shows similar batch phases of 5 hours in all cultures. During this time, the cells were growing and consuming the glucose released by Reagent A in the Enpresso B growth system. In the boosted culture, this was followed by a small peak in the pO_2 curve between ≈ 6 – 7 hours. This short-term period of reduced respiration can be considered as a lag phase (Figure 4-20 (A)) during which the cells were adapting to the use of lactose. Interestingly, this peak was shorter in the non-boosted cultures (Figure 4-20 (B)).

After the lag phase (≈ 1 – 2 hours) the cells resumed their growth on the lactose in the medium with a second batch phase which is indicated by a drop of the pO_2 value at 7 - 8 hours. The effect of the lactose addition on the oxygen consumption was different in boosted cultures than in the non-boosted cultures. *In boosted cultures* (Figure 4-20 (A)), the effect of different lactose concentrations on the growth was observed after 9 hours cultivation time (i.e., 2 hours after the start of the second batch phase). The cultures with high lactose concentration had a longer batch phase while the cultures with the low lactose concentration started their fed-batch phase earlier. *In the non-boosted cultures* (Figure 4-20 (B)), the effect of different lactose concentration on the growth was observed right after the start of the second batch phase (i.e. at 7 hours of the

cultivation). Cultures with high lactose concentration had longer batch phases (phases with high oxygen consumption).

A significant increase in the cell density was obtained by using the lactose-mediated autoinduction instead of using IPTG or the booster mixture as induction systems. The cells grew linearly with the time until the end of the cultivation achieving a final OD_{600} of 20 – 25, which is the highest cell density obtained with the tested autoinduction protocols. The cultures with high lactose concentrations gave the highest cell densities. For instance, OD_{600} of 20 was obtained in cultures with 10 g L^{-1} lactose, while OD_{600} of 15 was reached in cultures with 0.1 - 2.0 g L^{-1} lactose. Unexpectedly, the boosting of the lactose-mediated autoinduction cultures had no big influence on the cell growth/ density.

The protein activity values of different autoinduced cultures (boosted; non-boosted) with different lactose concentrations are showed in Figure 4-20 (E-H). Both volumetric activities and the specific protein activities increased with the time reaching up to 3.5 U mL^{-1} & 0.16 U OD_{600}^{-1} in boosted cultures and 2.5 U mL^{-1} & 0.16 U OD_{600}^{-1} in non-boosted cultures. Boosting of the lactose-AI cultures gave 40% higher titers of an active NP than the non-boosted cultures. This could address the role of the booster in the induction of the *lac* operon in the *E. coli* BL21 cells.
SDS-PAGE results confirmed the results of the activity measurements (Figure 4-20 (I)). The protein expression in the lactose-AI cultures without boosting was quite similar over the time and over the different concentrations of the lactose. In contrast, the protein yields in the lactose-AI- boosted cultures were high with the low lactose concentrations (0.1 – 2.0 g L^{-1}) and also higher than the non-boosted cultures. By this, it can be concluded that low concentrations (up to 2.0 g L^{-1}) of lactose should be used for autoinduction of Enpresso B cultures when booster supplementation is used.

4.5 Diauxic growth of *E. coli* strains

The lactose mediated autoinduction is principally based on the repression / de-repression of the *lac* promoter and influenced by the glucose concentration. Therefore, the aim of this part is to study the physiology of the *E. coli* cells that are growing under “diauxic conditions”.

4.5.1 Diauxie and diauxic lag phase in different *E. coli* wild strains

Four different wild *E. coli* genotypes were chosen for this part of the study; three *E. coli* K strains (BW25113, MG1655 and W3110) and one *E. coli* B strain (BL21). Mineral salts medium (MSM) was used to avoid the complexity and the interference with the continuous glucose release in the EnBase system. MSM medium was supplied with two different combinations of glucose and

lactose (3.0 and 5.0 g L^{-1} of glucose and 3.0 and 5.0 g L^{-1} of lactose). The study was performed in shake flasks equipped with a multi-sensor system for the online monitoring of the pO_2 and the pH over the cultivation time.

Figure 4-21 (A & B) show the pO_2, pH and cell density profiles of different wild *E. coli* strains grown under diauxic conditions. By this, the diauxic growth phases can be identified and conclusions about the substrates consumption can be drawn.

In the ***E. coli* BW25113** culture, the cells started their batch phase (characterized by an increasing respiration rate) right after the start of the cultivation. The cells grew on the available glucose during the batch phases. Once the glucose was depleted in the medium (after ≈ 6 hours with 3.0 g L^{-1} glucose and ≈ 8 hours with 5.0 g L^{-1} glucose), the cultures started their fed-batch phase with some fluctuations in the pO_2 values particularly in case of using 3.0 g L^{-1} lactose. Consumption of lactose started at the time where the pO_2 signal starts to decrease at ≈ 8 hours. Apparently, the pH was decreasing also during the diauxic lag phase which is most probably the time between 7-8 hours. The pH was decreasing also during the time of consumption of glucose coming from lactose hydrolysis at ≈ 9.5 hours. Afterwards, both the pO_2 signal and the pH started to increase. Very probably the time between 9.5 – 10 hours was the time of galactose consumption which coincided with the time of the pH increase. After 10.5 hours, the pH decreased again while the pO2 signal increased until the end of cultivation. Apparently using higher concentrations of the glucose / lactose did not affect the cell growth transition from one sugar to another. However, it prolonged the length of the batch phase from 6 to 8 hours.

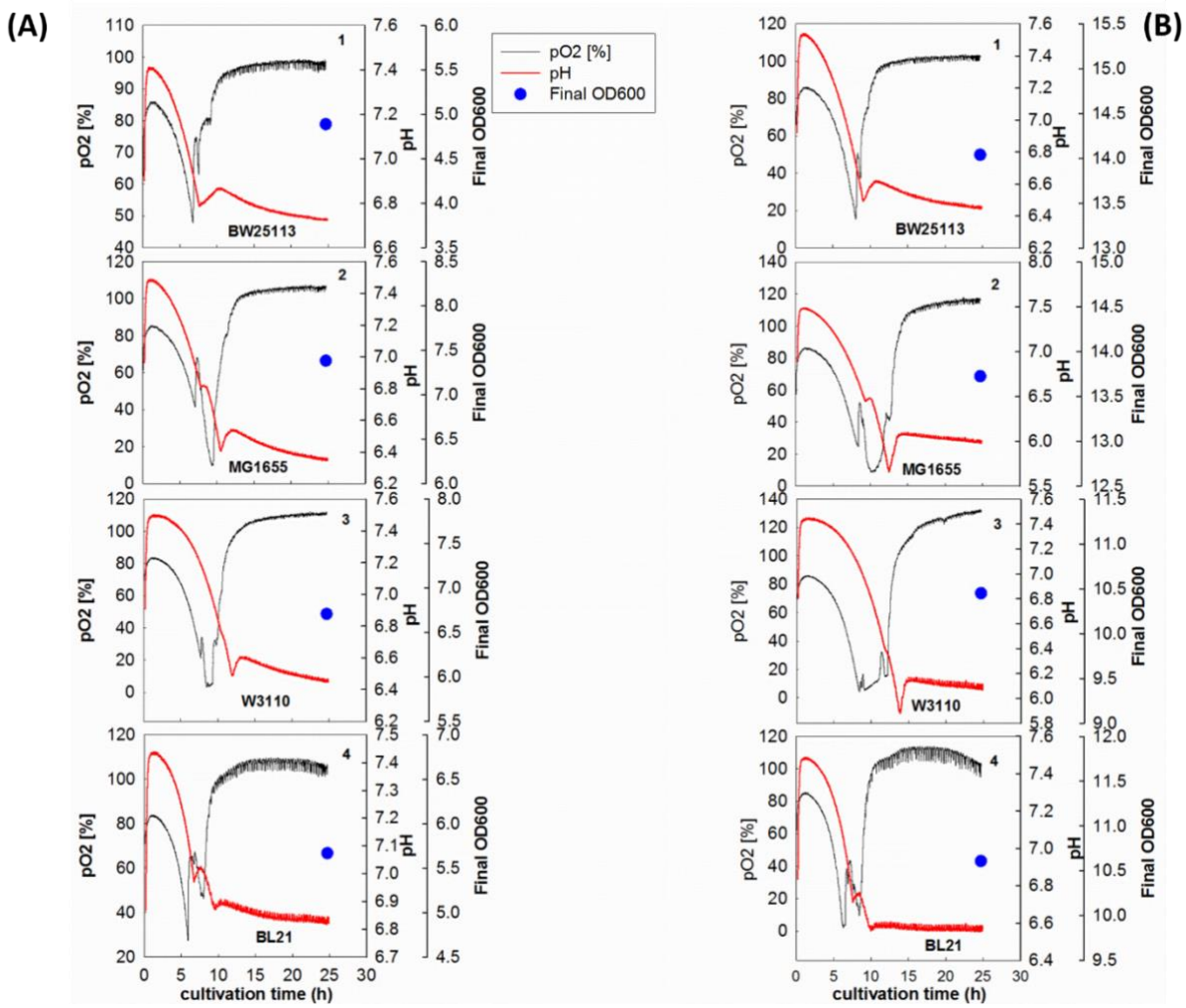


Figure 4-21 Diauxic growth of four different E. coli genotypes. (A) *pO_2 [%], pH and final OD_{600} values of different cultures grown on 3.0 g L^{-1} glucose and 3.0 g L^{-1} lactose;* ***(B)*** *pO_2 [%], pH and final OD_{600} values of different cultures growing on 5.0 g L^{-1} glucose and 5.0 g L^{-1} lactose. The name of each E. coli strain is written below on each belonging graph.*

Compared to the other strains, the ***E. coli*** **MG1655** cells grew differently in the diauxic conditions (Figure 4-21 (A2 & B2)). The cells grew on the glucose and started their batch phase which took the same time as the BW25113 cells (around 6 hours). A small peak then appeared in the pO_2 graph suggesting glucose was consumed at this point. After this point, the transition phase between the two different sugars was clear and took around 30 – 45 minutes (both for 3.0 and 5.0 g L^{-1} glucose/ lactose) Consumption of lactose started at the point where the pH starts to decrease! Very probably the diauxic lag phase is the time from the increase of the pO_2 signal until the start of the pH decrease. The lactose is consumed probably at the point where the O_2 increases drastically (and also the pH) – 9 or 12 hours respectively. Afterwards there is a small break in the pO_2 increase, possibly to either use of galactose or alternatively due to the finished

consumption of acetate. However, the length of the second batch phase was longer in the case of 5.0 g L^{-1} lactose. Moreover, a third batch phase was observed only in cultures with 5.0 g L^{-1} lactose. Probably this third batch phase represented the consumption of galactose which had resulted from the hydrolysis of the lactose. In this cultivation, the change in the pH was seen at the time of each pO_2-drop. The launch of a new carbon source seems to result in vigorous cell growth associated with the generation of metabolic byproducts which change the pH.

With the strain **W3110**, like MG1655 and BW25113 genotypes the batch phase finished after ≈ 6 hours (Figure 4-21 (A3 &B3)). The consumption of lactose started after ≈ 8 hours, at the time of the decrease of the pO_2 signal. Like strain BW25113, the pH was decreasing during the time of glucose consumption (both glucose in the diauxic medium and the glucose coming from the lactose hydrolysis). The time of lactose consumption here was longer than the former strains, took approximately 2 hours (between 8 – 10 hours) in case of medium with 3.0 g L^{-1} lactose and around 3.5 hours in medium with 5.9 g L^{-1} lactose. After 10 hours, the pO_2 signal increased for few minutes and then decreased slightly for around 15 minutes which could be the time of the galactose consumption or finished consumption of acetate. At 12- 13 hours, the pH increased gradually for around 1-2 hours. Compared to the other strains, the pH profile of the W3110 culture was quite different. The fluctuations which were observed in the pO_2 graph due to the sugar-consumption were not clearly visible correspondingly in the pH profile. Only the pH decreased during the cultivation due to the consumption of the different sugars, and the subsequent increase after ≈ 15 hours was seen.

For the ***E. coli*** **BL21**, after the batch phase of 6 hours the cells started their fed-batch phase which took approximately 20 to 30 minutes. The start of the lactose consumption was identified when the cells started the second batch phase with reduced pO_2% at 7 hours cultivation (Figure 4-21 (A4 & B4)). Apparently that the diauxic lag phase was the time between the end of the batch phase and before the pO_2 starts to decrease, between 6-7 hours. After ≈ 6 hours, the glucose from lactose was consumed where the pO_2 slightly increased with a beginning of the pH increase. At 6.5 hours, the pO_2 decreased gradually with a slight increase in the pH- probably this was the time of galactose consumption. After 9 hours, a gradual decrease in the pH was noticed which was broken at ≈ 10 hours which could be the time of the acetate consumption. The profiles of both pO_2 and pH were nearly the same in the medium with 5 gL^{-1} glucose and 5.0 gL^{-1} lactose with only shifting in the time hours of the sugar consumption due to longer phase.

As it was expected, with a high concentration of the glucose and lactose; the pH decrease was significant. A simple phosphate buffer was not sufficient. Due to uncontrolled growth, the cultures with high concentrations of the glucose and lactose were tending to grow under oxygen limitation. This happened in particular with the cultures of *E. coli* MG1655 and *E. coli* W3110. These two strains were able to consume all the available sugars into the medium. For this reason,

highest OD_{600} values were obtained with the W3110 and MG1655 cultures (≈ 10.4 and 13.7 respectively).

4.5.2 Diauxic growth in bioreactor cultivation of *E. coli* MG1655

The wild type *E. coli* strain MG1655 which is lactose and galactose utilizing was chosen for a detailed study of the cell physiology under the availability of multi-substrate conditions. A 2 L fermentation was performed in MSM medium supplemented with glucose and lactose, 4.0 gL^{-1} each. The cultivation run for 13 hours at 30°C with 1000 rpm mixing (Figure 4-22 (A)). The pH was controlled at pH 7.0 after ≈ 6 hours when the pH started to decrease below pH 7.0 (Figure 4-22 (C)).

Figure 4-22 (B) shows an exponential growth of the cells during the time of cultivation which was followed by a time of the diauxic shift after ≈ 8 hours. This profile was in a good agreement with the pO_2 profile shown in Figure 4-22 (C). The batch phase took 8 hours and was indicated by pO_2% decrease and exponential decrease in the glucose concentration until it was totally consumed (Figure 4-22 (F)). On the other hand, the lactose concentration was rather constant during the batch phase and the time of glucose consumption (Figure 4-22 (F)). Moreover, the concentration of O_2 (O_{2ex}%) in the off gases was decreasing during the batch phase while the level of the produced CO_2 (CO_{2ex}%) was increasing (Figure 4-22 (D)). This indicates that the C/E sources coming from the aerobic respiration of glucose are directed to the cell growth.

After 8 hours batch phase, a sudden increase in the pO_2 concentration was observed. This time is defined as the diauxic shift which is characterized by a diauxic lag phase. A lag phase of approximately 30-45 minutes was also observed in the bioreactor cultivation. During this lag phase, the profiles of O_{2ex} and CO_{2ex} were changed (Figure 4-22 (D)). A sudden increase in the level of $O2_{ex}$% & a sudden decrease in the level of CO_{2ex}% at the time of the lag phase were observed and it took approximately the same time of the diauxic lag phase (30 minutes).

A gradual decrease in the pO_2 [%] was then observed after this lag phase (at ≈ 9 hours) (Figure 4-22 (C)). This decrease in the pO_2% signals took two different slopes. The rate of the first pO_2-drop (between 9 – 10 hours) was higher than the rate of the second pO_2-drop (between 10 – 12 hours). Figure 4-22 (F) shows a slight co-consumption of lactose and glucose in the first phase of the culture (0-8 hours), but after 8 hours the lactose consumption rate increased very much. The initial co-consumption also is possibly the reason which is this case we do not see a longer diauxic lag phase, but it looks like there is, after exhaustion of glucose and the corresponding pO_2 shift a direct and fast metabolic shift to the consumption of lactose. It has earlier shown in different papers, that co-consumption of glucose and lactose depend from the specific concentrations of these both substrates. Figure 4-22 (F) shows also that galactose was detected only during the

time window between 9 -10 hours of the cultivation with a concentration of up to 2 g L^{-1}. This coincided with one hour after the time of the lactose metabolism.

Similarly, the O_{2ex}% decreased with the same pattern change of the pO_2 change after the lag phase. On the other hand, the CO_{2ex}% increased at the same time followed by a short time of CO_{2ex}%-decrease at ≈ 11 hours.

In connection, Figure 4-22 (E) shows that the O_2 uptake (QO_2) and CO_2 formation (QCO_2) rates decreased mainly two times over the cultivation time. First decrease in these rates took place at 8 hours of cultivation (during the diauxic shift). The duration of this change was the same time length of the diauxic lag phase (≈ 45 minutes). The second drop in the O_2 uptake and CO_2 formation rates happened after 11 hours of cultivation. Figure 4-22 (E) shows a third decrease in these rates a few minutes before the end of the cultivation (after ≈ 11.5 hours).

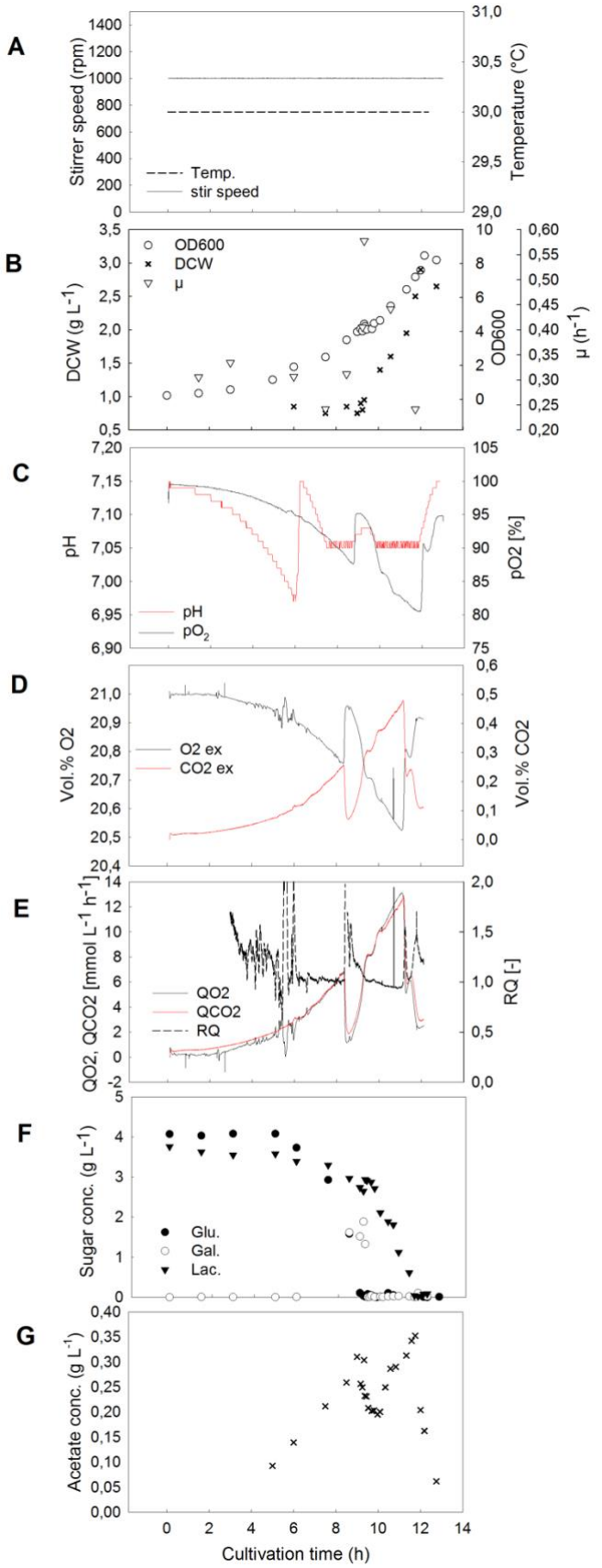

A
Stirrer speed (rpm)
Temperature (°C)
Temp.
stir speed
B
DCW (g L-1)
OD600
μ (h-1)
OD600
DCW
μ
C
pH
pO2 [%]
pH
pO2
D
Vol.% O2
Vol.% CO2
O2 ex
CO2 ex
E
QO2, QCO2 [mmol L-1 h-1]
RQ [-]
QO2
QCO2
RQ
F
Sugar conc. (g L-1)
Glu.
Gal.
Lac.
G
Acetate conc. (g L-1)
Cultivation time (h)

Figure 4-22 Bioreactor diauxic cultivation of E. coli MG1655. (A) *Stirrer speed and temperature;* ***(B)*** *Cell growth as dry cell weight & OD_{600} values and the specific growth rate;* ***(C)*** *pH and pO_2 [%] profiles;* ***(D)*** *The exhaust gas values;* ***(E)*** *volumetric O_2 uptake and CO_2 formation rates and respiration quotient (RQ);* ***(F)*** *Sugar concentrations in the cultivation medium (glucose "Glu.", galactose "Gal." and lactose "lac.") and* ***(G)*** *Acetate concentration in the cultivation medium.*

After 12 hours, the pO_2 concentration increased again up to 90% and then decreased slightly afterwards for short time (15 minutes, Figure 4-22 (C)). In connection, acetate was detected into the cultivation broth at two different time points (Figure 4-22 (G)). Frist acetate production is due to the growth on glucose (until 8 hours up to 0.3 g L^{-1}). Afterwards acetate is consumed in the diauxic lag phase and following the acetate concentration in the medium decreases (up to 0.2 g L^{-1}). Later after 10 hours, when the lactose is consumed and hydrolyzed by ß-galactosidase to glucose and galactose, glucose is consumed and the second increase of acetate is based on the consumption of glucose. Finally, after 12 hours the acetate concentration decreases (to 0.05 g L^{-1}) when all other carbon sources in the medium are exhausted.

Such changes in the pO_2% and corresponding O_{2ex}% & CO_{2ex}% together with the sugar analysis reflects the cell growth on different sugars at different times with the following order; glucose in the cultivation medium, glucose comes from lactose hydrolysis, galactose comes from lactose hydrolysis and finally some acetate consumption as a metabolic byproduct.

5. Discussion

5.1 Batch and Fed-batch pre-cultivation of recombinant *E. coli*

The *inoculum age* and the *pre-cultivation process* affected especially the lag phase and the length of the batch phase in the Enpresso main cultures during the first 5 hours of cultivation time. Enpresso B pre-cultures were continuously exposed to a glucose supply through the enzymatic glucose release system. By continuously exposing the culture to a glucose supply- in a lower concentration than in a batch cultures- through the enzymatic glucose release system, the culture will not go through a glucose starvation and the acetate synthesis rate is lower. The side effects of the acetate accumulation have long been extensively studied. Acetate accumulation is mainly affect the growth and carbon uptake rates when the level exceeds 1.0 – 1.5 g L^{-1}, depending also on the *E. coli* strain (Jensen and Carlsen, 1990; San *et al.*, 1994; Shiloach *et al.*, 1996; Van De Walle and Shiloach, 1998). It can change the central carbon metabolism that used for energy generation and biosynthetic pathways. It also affects the proton motive force which results in impairment of the cell growth and recombinant protein production (Luli and Strohl, 1990; Eiteman and Altman, 2006). Cultivations with high initial glucose concentrations or cultivations at high specific growth rates show higher rates for acetate accumulation and sometimes a reduced production rate of recombinant proteins (Turner, Gregory and Thornhill, 1994). The reason for acetate accumulation has been assumed to be a lower respiratory capacities or low activity of tricarboxylic acid cycle in relation to the glucose uptake (Andersen and Von Meyenburg, 1980).

Different strategies have been studied for feed control and prevention of the over-feeding of the glucose that lead to the overflow metabolism and acetate accumulation or the underfeeding which results in the cell starvation. Glucose limited fed-batch strategy is commonly used for limiting the growth and acetate formation with adjusted feed control in connection to the oxygen transfer capacity of the cultivation system.

The fermentation process is often characterized by the presence of the lag phase which has an impact on the initial phase (Pham, Larsson and Enfors, 1998; Xu, Jahic and Enfors, 1999). The lag phase is initiated when the inoculum is prepared as batch process due to part of the cells in the inoculum is dead or the time required by the cells to get adapted to the new environment. Inocula of fed-batch pre-cultivation systems were active when they have been transferred to a new medium in the main cultivation. This resulted in the shorter lag phase in the Enpresso B compared to the use of pre-cultures prepared in LB medium. Also in LB medium the cells are adapted to the uptake of amino acids but not to their synthesis. With an Enpresso pre-culture they are prepared for the synthesis of all amino acids and thus should have a shorter lag phase for adaptation of the metabolic network. Particularly the cultures inoculated with pre-cultures cultivated in Enpresso B for six hours performed well. The reason could be in these cultures the cell was

harvested at stage when the cells were growing in the exponential phase and not entered the fed-batch phase (starvation phase). From it should be also noted that the use of the same cultivation medium (Enpresso B) as in the main culture (Enpresso B) and same cultivation principle (fed-batch) have resulted in a shorter lag phase in the main cultures. This was in agreement with the observation of (Jõers and Tenson, 2016) that the cells which have ended their metabolism last are those cells that resume their growth fastest when transferred into a new fresh medium. This can be seen also in our experiments. The cultures inoculated with overnight pre-cultures cultivated overnight (and having a stationary phase or at least a period of a slow growth of at least 10 hours), had a much longer lag phase. As it was reported before that the *E. coli* cells can be exposed to an oxidative damage during the stationary phase. Therefore, they require additional time (than the cells harvested during their log phase) to repair their constituents before growth resumption in a new culture (Desnues *et al.*, 2003).

In connection to this, the study of Luidalepp et al. (Luidalepp *et al.*, 2011) demonstrated that long stationary phase results in heterogeneous pre-cultures providing heterogeneous growth resumption as well as release of more persisters, which are the cells characterized by a delayed growth resumption and antibiotics tolerance without undergoing genetic change (Wood, Knabel and Kwan, 2013). Persister's frequency is not only affected by the inoculum age but also by the type of the medium. For instance, growing the persisters on a simple carbon source like glucose can minimize the growth resumption time (Jõers and Tenson, 2016). The use of different media in the pre- and main cultures would probably have an impact on the length of the lag phase, since the persisters would need time to adapt to new substrates. In our study, the starter cultures prepared in LB instead of Enpresso B exhibited a bit longer lag phase when they were transferred to a fed-batch medium with slow glucose feeding strategy.

The *inoculum age* and *pre-cultivation mode* had no significant influence on the level of the produced protein yield or activity. Differences were present only during the batch phase (first 5 to 6 hours) of the main cultivations when the cells are adapted to the new environmental conditions. In all cultivations, by the time of induction the bacteria were growing in the stationary phase (slow growth) which was indicated by a low respiration activity suggesting similar physiological conditions. As concluded by (Jõers and Tenson, 2016) for the stationary phase cells it is difficult to estimate the timing of the growth resumption of each cell. They also showed that induction of GFP (green fluorescent protein of their study) at the stationary phase together with a new carbon source promotes the growth resumption and GFP expression. In the presented work, the main cultures inoculated with overnight inocula (in LB or Enpresso B) started the fed-batch phase, after induction, earlier than the cultures inoculated with 8 & 6 hours inocula. This is in line with the observations of (Jõers, Kaldalu and Tenson, 2010; Jõers and Tenson, 2016) that the cell population of the stationary phase may have heterogeneity in terms of the growth

resumption (start and end of their metabolism) when they are diluted to a new fresh medium (Balaban, 2004; Jõers, Kaldalu and Tenson, 2010).

The choice of the starter culture should be well-studied when designing the experiment. Some cultivations need a short-term pre-culture which is often the safest approach. However, some cultivations must be done with an overnight pre-culture which is more critical due to the effect of the stationary phase on the generation of the persisters and dormant cells. With the enzymatic glucose delivery system, overnight pre-cultures can be safely prepared for inoculation.

5.2 Optimization of the enzyme based substrate delivery system for recombinant protein production

Fed-batch process is commonly used in many biopharmaceutical bioprocesses in order to achieve high cell densities with high product yields. In contrast, the batch process is used for the high throughput and small scales. In this study, Enpresso B and Enpresso B defined cultivations were optimized for liter scale fed-batch fermentations.

One unit of reagent A releases approximately 0.048 g L^{-1} h^{-1} of glucose from the glucose polymer in the standard Enpresso B medium (See Appendix). Curiously the cultures inoculated with culture done in LB medium grew to higher cell densities than the cultures inoculated with bacteria grown in Enpreso B medium, although all the main cultures were started with an initial OD_{600} of 0.15. This suggests that the behavior of the cell growth is dependent not only on the dose of the added reagent A but also on the "metabolic memory" of the cells. Similar results have been shown by (Jõers and Tenson, 2016) who found a correlation between the cell condition at the end of the stationary phase and the first "starter culture". This "metabolic memory" reminds the cells about their former condition and thus controls the time length of the growth resumption as well as the metabolic rates in the next growth cycle (Labhsetwar *et al.*, 2013; Kiviet *et al.*, 2014). In terms of protein expression, around 2.4-fold higher protein activity was obtained in cultures inoculated with Enpresso B inoculant instead of LB inoculant, when the culture was supplemented with 0.3 U L^{-1} reagent A. The same observation was reported by Hortsch and Weuster-Botz (Hortsch and Weuster-Botz, 2011) who showed that the growth kinetics of *E. coli* BL21 (DE3) cells in EnBase cultivation were different according to the reagent A dosing. They obtained the fastest growth in cultures with a high reagent A concentration (up to 12.0 U L^{-1}) reaching a biomass yield of approximately 6 g L^{-1} in 6 hours and > 8 g L^{-1} at the harvest. They showed that fed-batch cultivation, by providing a slow growth and product formation rates, allows a proper protein folding into active form and also increases the extent of the protein glycosylation as it was mentioned in (Shelikoff, Sinskey and Stephanopoulos, 1994; Andersen *et al.*, 2000). Moreover, the growth rate control via fed-batch cultivation affects many factors related to the recombinant protein production like the level of the substrate allocated to cell

maintenance, RNAP activity, amount of ribosomes, plasmid copy number and plasmid stability (Sandén *et al.*, 2003).

One unit of reagent A releases approximately 0.09 g L^{-1} h^{-1} of glucose from a 20 g L^{-1} glucose polymer present in the substrate booster mixture (See Appendix). With the aid of this mixture and additional reagent A the cultures obtained approximately 1.5-fold higher cell density. The higher cell densities might have been obtained also due to consumption of the amino acids in the complex compounds of the booster.

However, the protein productivity per cell was not significantly improved. Possibly the expression of the recombinant nucleoside phosphorylase saturates the cell protein-synthesizing machinery (especially ribosomes). Whereas the increase in the yields of the protein was obtained largely due to the overall increase in the cell density. Similarly, Glazyrina et al. reported that addition of EnBase Booster had no influence on the biomass and protein yields (Glazyrina *et al.*, 2012). However, a study presented by (Peck *et al.*, 2014) showed about 8 times higher yields of capripoxyvirus protein per cell as well as the cell densities in the enzyme controlled fed-batch cultivations. Compared to the minimal medium or complex medium; (Krause *et al.*, 2010) reported a 10-fold increase of the volumetric recombinant protein yields by using the enzyme-controlled fed-batch cultivation.

Based on these findings, refreshing the Enpresso B system with new nutrients after overnight cultivation at the time of induction was expected to improve the bioprocess in terms of the cell density. Joly et al. (Joly, Leung and Swartz, 1998) had reported that the complex additives reduce the product proteolysis and extend the time of the efficient protein synthesis. Also Krause et al. (Krause *et al.*, 2010) showed that the supplementation of the fed-batch cultures with the nitrogenous compounds (booster) simultaneously with the inducer results in an enhanced protein synthesis. Neubauer and Winter (Neubauer and Winter, 2001) addressed the major influence of the expression of the recombinant protein on the cell growth and maintenance. This means that when the cell growth is slow, the metabolic energy is allocated to the cell maintenance instead of recombinant protein expression. Therefore, the addition of extra glucose polymer in addition with the boosting step was quite important for achieving higher growth rates and maintenance of the optimal pH. However, production of the periplasmic proteins may require different cultivation strategies. Ukkonen and Veijola (Ukkonen, Veijola, *et al.*, 2013) showed that by excluding the culture supplementation with complex nutrients at the induction time, a higher yield of the periplasmic proteins expressed in *E. coli* RV308 (Fab fragments) was obtained in well-aerated shake flask cultivations. They realized also that the exclusion of this step will reduce the Fab release to the cultivation medium and maintain higher overall Fab yields.

Culture supplementation with an additional glucose polymer in the substrate booster mixture at the time of induction improved the growth and protein yields significantly in Enpresso B defined

cultures. Supplementation of the culture with the glucose polymer simultaneously with reagent A addition after overnight cultivation enhanced the volumetric protein yield approximately two-fold while the specific protein activity per cell was not significantly enhanced. A similar observation was reported by Panula-Perälä et al. (Panula-Perälä *et al.*, 2008). They reported 8 to 10-fold increase in the volumetric yield of recombinant triosephosphate isomerase (TIM), compared to the standard minimal medium (M9) with the use of enzymatic glucose release system. This was done by achieving higher cell densities, but the productivity per cell was not enhanced.

It was found that the pH also affects the rate of the glucose release by the Reagent A (See Appendix). The release rate decreases rapidly at the pH values above 7.0. The results showed quite stable pH values, between 6.5 and 7.0 over the different Enpresso B cultivations during the whole time. Detailed, a lower pH recorded over the different Enpresso B cultivations was 6.3 in the 2 L bioreactor cultivation (Figure 4-15 (A)) while the maximum pH reached was 7.2. Therefore, a culture-pH ranges from 6.5 - 7.3 and was tested to investigate the activity of the reagent A in different concentrations.

Around 20% lower cell densities and a significant decrease in the culture pH (up to pH ≈5.5) were the results of a combination of using high enzyme concentration (9.0 U L^{-1}) at a low initial pH value (pH 6.5). Too high glucose release rate apparently negated the pH-elevating effect of booster. In the absence of the glucose, the cells start to consume the complex nitrogenous compounds for energy generation; while the excess nitrogen will be excreted to the medium in a form on NH_4^+ ions which also affect the medium pH (Krause *et al.*, 2010).

The impact of the pH change on the bioprocess by Ukkonen and Veijola (Ukkonen, Veijola, *et al.*, 2013). They reported that the extracellular pH might affect the leakage of the periplasmic protein by affecting the membrane fatty acids composition. This suggests that the high pH value may provoke a higher membrane permeability. Wang et al. (Wang *et al.*, 2014) showed that a culture pH of 7.5 can significantly reduce the acetate concentration and improve the specific growth rate. In the same study, they addressed the pH raise to promote the acetate tolerance of *E. coli* BL21 (DE3) cells. They reported that the acetate anion (Ac^-) accompanies the non-dissociated acetate form (HAc) whereas their proportion is affected by the medium pH. HAc can diffuse across the cell membrane and dissociates into Ac^-. It accumulates if the intracellular pH is higher than the medium pH leading to the collapse the transmembrane pH gradient (Wang *et al.*, 2014). Our results and the reviewed literature data suggests that the pH control mechanism of Enpresso cultivation system (Krause *et al.*, 2010) is one important benefit of this cultivation system.

5.3 Scale-up of Enpresso B growth system

The key point of any bioprocess for recombinant production is to obtain a high yield of the biomass together with a high yield of the good quality product.

Enpresso B cultivations were scaled from the µL scale to the mL scale and further to 0.5 L scale using ultra-yield flasks. These flasks enhance oxygen-liquid transfer even at low shaking frequencies (Tunac, 1989; Jiménez *et al.*, 2011). Ultra-Yield flasks have a KLa value of up to 350 h^{-1} while to maximum KLa values for normal round-bottom shake flasks is 100 h^{-1} (Schiefelbein *et al.*, 2013). Ukkonen et al. had addressed that the product yield in Enpresso B growth system is influenced by the volumetric oxygen transfer coefficient (KLa) (Ukkonen, Veijola, *et al.*, 2013). By using matched KLa values a successful scalability from small scale to fermenter scale can be obtained (Kensy *et al.*, 2009). The KLa values of the squared-multiwell plate is two-fold higher to the rounded-multiwell plate (Duetz and Witholt, 2004).

A good scalability of the system was observed over the shake flask scale (50 mL and 0.5 L) in terms of the biomass and the volumetric protein activities. Yet, the titer of the protein activities per cell was rather stable through the cultivation time after induction. The cell growth was enhanced by the change to larger cultivation vessels. Yet, by overexpression of highly active NP (up to 16 U mL^{-1} in 0.5 L shake flask); the cell growth did not increase further after reaching a plateau level at OD_{600} of ≈ 33. This could be due to the overexpression of a highly active recombinant protein which increase the metabolic burden on the cells which affect the cell growth and the final cell densities. The metabolic burden here is defined as the withdrawal of the raw materials and energy sources from the cell metabolism towards the expression of a foreign DNA. This might result in lowering the growth rate of the plasmid-carrying cells and overtake of the culture by the faster growing plasmid-free cells. The amount of the metabolic load is affected by plasmid copy number, size of the insert, level of the recombinant protein expression and toxicity of the expressed protein (Corchero and Villaverde, 1998; Summers, 1998; Hoffmann and Rinas, 2004). This reflects to the energy allocated to the synthesis of recombinant and stress proteins to the respiration rates. Some studies have addressed specific consequences of the overexpression of the recombinant proteins such as; 1. the housekeeping genes, including the protein synthesis genes, are downregulated (Weber, Hoffmann and Rinas, 2002); 2. most of the genes which are involved in the transcription and translation of amino acids biosynthesis are downregulated (Chang, Smalley and Conway, 2002).

The cultures did not pass through oxygen limitation over the multiwall plate and 2L bioreactor scales. The pO_2 values of the cultures did not go below 20% air saturation while it was reported that pO_2 values higher than 20% do not limit the growth of the bacteria unless a certain resistance is initiated in the culture like agglomeration of the cells (Palomares, Estrada-Mondaca and Ramírez, 2004); this was not observed in these cultivations.

As mentioned before, the pH level during the cultivation affects the success of the bioprocess. This is particularly true for the fast-growing *E. coli* cultivations which may be due to the overflow metabolism form undesired "acidic" metabolites like acetate. This is problematic in the all steps of the scale-up process. In bioreactor scales acetate accumulation tends to increase and adversely affect the level of the protein expression (Eiteman and Altman, 2006; De Mey *et al.*, 2007; Wang, Wang and Wei, 2009). The pH range of 6.0 – 7.0 seemed to be optimal working range for the cell growth and the reagent A activity. EnBase system seemed to provide a sufficient buffering capacity. Since *E. coli* BL21 cells do not form much acetate (Noronha *et al.*, 2000; Carneiro, Ferreira and Rocha, 2013), the small pH changes observed through cultivation are likely to be a result of the carbon and nitrogen (ammonium ions) consumption to metabolized peptides during the glucose limitation phase and release of ammonia from the complex compounds like amino acids, peptides and yeast extract (Krause *et al.*, 2010). Similarly, the study of (Hortsch and Weuster-Botz, 2011) reported almost no culture acidification during the fast growth of *E. coli* cultures. Some research groups use other different strategies to control in turn the concentration of the produced acetate and avoid the drastic culture pH change. For example, a genetically modified *E. coli* VH32 was constructed by (Lara *et al.*, 2008) with a modified glucose transport system lacking PTS. This strain can grow in glucose concentrations of up to 100 g L^{-1} producing a maximum of only 2.0 g L^{-1} acetate. Akesson et al. (Akesson, Hagander and Axelsson, 2001) created an automated glucose feeding system over three different laboratory-scale platforms and fed-batch cultivations with three recombinant *E. coli* strains; K-12 UL635, BL21(DE3) and K-12 UL634. The feeding strategy, using a feed-pump, was applied through the control of the dissolved oxygen (pO_2) in the culture by manipulating the stirrer speed. By this, they succeeded to reduce the acetate accumulation to less than 60 mg L^{-1}.

By the use of the enzymatic glucose delivery system, approximately 1.2-fold increase in the volumetric protein activity was obtained through the benchtop, fed-batch bioreactor fermentation compared to the shake flask and well-plate cultivations. Furthermore, the profile of the protein activity per cell increased linearly after induction reaching 0.57 U OD_{600}^{-1} after 8 hours of induction.

5.4 Autoinduction of recombinant *E. coli* cultures

Autoinduction is a simple method for induction of protein synthesis in *E. coli* strains with *lac*-derived expression system. It is based on the inducer exclusion phenomenon which is initiated in the presence of glucose. This prevents the intake of lactose to the cell and induction by lactose before glucose depletion. The metabolic burden from overexpression of the foreign genes has a great impact on the cell growth and protein formation (Neubauer *et al.*, 1992; Donovan, Robinson and Click, 1996). This can be controlled via the glucose concentration in the medium. High

concentration of the glucose can maintain a high proportion of inducible cells in the culture and thus a higher yield of recombinant product can be obtained (Kunze *et al.*, 2011). However, the unlimited availability of the nutrients with the exponential growth in a batch system results, as mentioned before, in overflow metabolism and production of side metabolites like acetate and consequent culture acidification. In Enpresso B cultivations, the pH is balanced by the NH_4^+ ions derived from the utilization of the booster (yeast extract and peptone). Therefore, 1. the pH auto-tuning system; 2. the constant glucose delivery until the end of the protein production phase (with the feed control via the amount of the biocatalyst added) makes Enpresso optimized for a strengthen recombinant protein production.

The physiological conditions of the cells at the time of induction is also important and affect the metabolic response to the recombinant protein expression. If the induction happens in the mid-log phase, the cells will have the sufficient level of energy for the recombinant protein expression in parallel with the growth. However, if the induction takes place at the late-log phase or during the stationary phase, the cells might not have enough energy for initiation a foreign protein expression (Carneiro, Ferreira and Rocha, 2013). In connection, a study by Miksch et al ((Miksch, Bettenworth, Friehs and Flaschel, 2005) aimed to broad the utilization of the stress promoters by construction of synthetic stationary phase/stress promoter for fine-tuning the gene expression for industrial applications. They obtained higher level of induction of these promoters at the time relative to the entrance into the stationary phase. About 91% of these synthetic promoters showed no or low background activity during the exponential phase.

IPTG is known for its rapid, drastic and constant induction potential for *lac* promoters in *E. coli* (Kilikian *et al.*, 2000). Some other researches addressed other inducible promoter systems like araB promoter which is easily induced by addition of arabinose (Guzman *et al.*, 1995), and modified *E. coli* glnAP2 promoter which is induced in nitrogen limited conditions (Schroeckh *et al.*, 1996). Yet, few studies addressed the applicability of the IPTG for autoinduction of fed-batch cultivations.

Enpresso growth system was used with IPTG-based induction of the recombinant gene encoding PyNP in *E. coli* BL21. IPTG was added to the Enpresso B growth system in two different ways. First the set of cultivations were single shot induced with IPTG after overnight cultivation (SSI) and the second set of cultivations were autoinduced by adding IPTG already at the beginning of the cultivation (AI).

The cell growth was not affected by IPTG that was added after overnight cultivation (SSI). Similar cell densities (up to $OD_{600} \approx 24$) were obtained with different IPTG concentrations and under oxygen sufficient conditions (≥ 20%). On the other hand, the cells grew differently according to the dose of the IPTG.

In samples containing IPTG in the beginning of cultivation, the cells grew under oxygen limitation during their batch phase reaching a cell density of approximately OD_{600} = 12 after 25 hours cultivation time and providing PyNP activity of ≈ 1.2 U mL^{-1}. The influence of the IPTG on the cell growth was thus clearer when it was added from the beginning of the cultivation than in the case of the single shot induction of overnight cultures though the same concentration of the complex additives added to the culture. However, the effect of the IPTG-AI on the volumetric activity was clearer in boosted induced cultures than the non-boosted cultures. This might reflect the relation between the IPTG dose and the cell density/number. In connection, a study for Fernández-Castané et al performed an experimental quantification of the inducer level into the medium and calculated its level inside the cell using the mass balance (Fernández-Castané *et al.*, 2012). They found that the production of recombinant RhuA (Rhamnulose-1-P- aldolase) depends on the initial inducer/biomass ratio (I/X0). The maximum protein yield was obtained at I/X0 =1

Remarkably, there is a drastic decrease in the final cell densities by the addition of the IPTG at the beginning of the cultivation. For easy comparison, the cell density was about OD_{600} ≈ 24 after 25 hours from the IPTG addition (in case of SSI); while it was ≈ OD_{600} of 12 after 25 hours from the start of cultivation (in case of AI). This would reflect an approximate decrease of 50% in the cell biomass by the change the induction time. Similarly, Faust et al. (Faust, Stand and Weuster-Botz, 2015) showed that the effect of the IPTG on the protein expression was higher in the autoinduction medium than in the normal fed-batch cultivations with single shot IPTG induction. This study also reported that the use of low concentrations of IPTG (< 100 µM) is mandatory for autoinduction of an efficient expression of recombinant eGFP in bacterial strains having an active lactose permease (*lac*Y). Also other studies (Sevastsyanovich *et al.*, 2009; Alfasi *et al.*, 2011) have reported that induction with a high IPTG concentration results in a strong induction of the T7 polymerase system but the cell growth rate and viability is adversely influenced. Thus, the cultures can become overgrown by the plasmid-free cells or non-producing cells.

In terms of the IPTG concentration, the IPTG concentration between 40 – 100 µM were found to be the optimal for induction of Enpresso B cultures and provided highest cell densities and protein yields. Due to the fact of the metabolic overload affects the growth rates and often results in poor protein yields. Many studies have addressed the adaptation of the induction mechanisms to reduce the metabolic load (Striedner *et al.*, 2003). For instance, the reduction of the inducer concentration to decrease the product formation rate (Sevastsyanovich *et al.*, 2009; Fernández-Castané, Caminal and López-Santín, 2012); fine-tuning the ratio of the inducer to the biomass (Durany, De Mas and López-Santín, 2005) or feeding the culture with the inducer gradually during the expression phase to adapt the cells to the recombinant protein expression (Zou, Duan and Wu, 2014; Su, Hong and Wu, 2015). For example, Hortsch and Weuster-Botz (Hortsch and Weuster-Botz, 2011) showed that the reduction in the IPTG concentration from 1000 µM to 5 µM resulted in higher specific product formation rate in a chemostat cultivation.

IPTG concentration above 25 µM resulted in a decrease of biomass by 10 to 30%. Fernández-Castané et al. (Fernández-Castané *et al.*, 2012) proposed that the cells are able to control the strength of the recombinant protein expression by controlling the uptake of the IPTG while the excess of the inducer remains in the medium. Additionally, they addressed the role of the *lac*-permease (*lac*Y) in promoting the active transport of the IPTG across the cell membrane. Thus, the cells could continue their growth normally without being affected by the product formation.

In autoinduction cultures, the profile of the protein activities increased linearly with time up to 12 hours, after which the cells reached the stationary phase and the protein produced reached a plateau level. Similarly, Diederichs et al. (Diederichs *et al.*, 2014) observed that the protein activity per biomass decreased after the target protein content reached its peak. This was due to the fact that the inducer lactose depletion in the medium may results in overgrowth of the culture by the non-productive cells. Tsao et al. (Tsao *et al.*, 2010) studied autoinduction using the native quorum sensing (QS) regulon; they hypothesized that the reduction of the target protein production is due to the diminished metabolic activity of the cells in the stationary phase. The study of Desnues et al. (Desnues *et al.*, 2003) suggests that the reduction in the product yield is due to the extensive protein degradation, growth of plasmid free cells or the oxidative damage that could happen to the *E. coli* cells living in the stationary phase.

Jõers and Tenson (Jõers and Tenson, 2016) showed that the addition of the inducer to the stationary phase cells affects the expression of the pBAD-Crimson protein. The addition of the inducer during the transition between the exponential and stationary phase resulted in a higher level of the protein expression. Moreover, they found that addition of the IPTG to the stationary phase of the cells without glucose supplementation did not provide protein expression at all. This could be due to the static conditions of the cells in the culture and absence of the actively multiplying cells. Therefore, it was necessary to add a new carbon source together with the inducer to initiate the growth resumption and GFP expression (Jõers and Tenson, 2016).

In the single shot induced cultures, the protein activity profile was linearly increasing with the time after induction until the end of this long cultivation (42 hours). Grossman et al. (Grossman *et al.*, 1998) have reported an increased level of the GST–human parathyroid hormone (hPTH) production by autoinduction of *E. coli* long-term cultivations, until the stationary phase, in a complex medium containing yeast extract. They also reported that the autoinduction in that case was not stationary phase-dependent and could be performed in 2 hours after the cultivation start.

The IPTG-AI cultivations were supplemented from the beginning with extra nutrients (SBM and reagent A). Quite stable culture pH profiles during the batch phase (first 5 – 6 hours of cultivation time) were observed in all cultures. Thereafter, the pH change was dependent on the concentration of the substrate booster mixture. Such fine-tuning of the pH level can be applied

for protein expression with pH-regulated promoters, as proposed formerly by (Tolentino, G.J., Meng, S.Y., George, N. Bennett, G.N., and San, 1992).

The medium supplementations clearly affected the oxygen consumption at the start of the batch phase. Expectedly, autoinduction cultures with high concentrations of the SBM had prolonged batch phases with corresponding higher cell densities. Furthermore, the protein activities were almost doubled by the culture supplementation with the complex nitrogenous compounds in SBM. The study of Li et al. (Li *et al.*, 2011) addressed the use of yeast extract as a source of induction for the polyvinyl alcohol-degrading enzymes (PVAases). They added the yeast extract two, three or four times between 18 and 24 hours with a total level of 2.0 g L^{-1}. They found that the PVAases activity increased with the number of the yeast extract additions. By this, it can be concluded that autoinduction of Enpresso B growth cultures should be done with medium supplementation and culture boosting at the beginning simultaneously with the inducer.

The focus in the last few years is directed towards the use of the mild induction conditions for recombinant protein expression. Therefore, the possibility to use the booster as source of induction is investigated. A study for (Xu *et al.*, 2012) has shown that complex nitrogen sources such as peptones and yeast extract can contain sugars that have an inducing effect. They show that the content (mg. g^{-1}) of galactose, lactose and glucose were 7.07, 0.01 and 74.5 in peptone and 0.01, 0.07 and 2.50 in yeast extract respectively.

Unlike IPTG, such mild inducers do not increase the metabolic burden on the cells (Studier, 2005; Faust, Stand and Weuster-Botz, 2015).

The protein activities obtained by booster-autoinduction (3.5 U mL^{-1} and 0.25 U OD_{600}^{-1}) were at the same level as obtained by IPTG autoinduced cultures supplied with booster together. This suggest that IPTG usage is not necessary, which is beneficial especially for the human biopharmaceutical products. Also Glazyrina et al. (Glazyrina *et al.*, 2012) has addressed the positive effect of the booster, particularly the role of the complex compounds to the production of recombinant proteins. Similar to our results, a study by (Jia *et al.*, 2011) addressed that the autoinduction in *E. coli* BL21 (DE3) started in 2 hours after the cultivation start and was highly dependent on the concentration of the yeast extract in the complex medium. However, they reported an unintended induction at the early stage of the cultivation with using a high concentration of yeast extract. This unintended induction results in a drop in the cell viability, plasmid stability, depending on the type of the recombinant protein.

Regarding to the strain *E. coli* BL21, Xu et al. (Xu *et al.*, 2012) reported the influence of the galactose in the complex media derived from the plant sources on induction of *E. coli* BL21 (DE3) cultures. BL21 strains lack the galactokinase genes and thus they cannot metabolize the galactose. For that reason, a minor amount of galactose can accumulate into the cell to the level

that causes a robust induction of the *lac* operon. They reported that galactose concentration of 0.4 mM is sufficient to induce a high level of protein expression by autoinduction.

Lactose has been earlier reported to be a substitute for the IPTG for induction of the *lac* promoter in fed-batch cultures (Neubauer *et al.*, 1991, 1992). The use of lactose was investigated for autoinduction of the Enpresso B growth cultures with and without culture boosting. The first batch phase (glucose consumption) lasted about five hours. Thereafter the cells started their second batch phase on the lactose. This is also referred back to the fact that *E. coli* BL21 is a competent strain for growth on lactose (Hoffman *et al.*, 1995). Therefore, the glucose concentration should be generally chosen so that its depletion occurs in the mid-to-late log growth phase to promote the autoinduction at this time as (Studier, 2014). A direct correlation between the growth of the cultures with the concentration of the lactose used in both boosted and non-boosted cultures was observed. Higher lactose gave higher the cell densities. The impact of the lactose concentrations on the growth was rather similar in the two groups of cultivations (with/without boosting). In autoinduction experiments, the highest cell densities (OD_{600} of ≈ 22) were obtained by lactose mediated autoinduction. Kilikian et al. have reported that the production rates of the heterologous proteins are slow in the lactose-autoinduction medium (Kilikian *et al.*, 2000). Lactose needs some time to bind to the *lac*-repressor protein. Additionally, lactose permease protein (product of the gene *lac*Y) must be synthesized to enable the efficient transport of lactose into cell. Finally, the cells need ß-galactosidase (product of the gene *lac*Z) to be synthesized. This enzyme converts lactose to the allolactose molecule which acts as the real binder to the *lac* repressor. It has been also reported earlier that the lactose uptake is regulated by the phosphorylated IIA^{Glc} while the unphosphorylated enzyme may prohibit the lactose uptake by binding to the *lac*Y (Osumi and Saier, 1982; Nelson, Lengeler and Postma, 1984). Differently, IPTG can enter the cell either by the active transport or by diffusion. It then binds directly to a few number of *lac*-repressor molecules (Miao and Kompala, 1992).

Results showed also that lactose was efficient in induction of the Enpresso B cultures to synthesize an active nucleoside phosphorylase. The profile of protein activities was increasing exponentially with the time until the end of the cultivation (up to 3.5 U L^{-1} in the boosted cultures & 2.8 U L^{-1} in the non-boosted cultures). However, the differences in the protein activities obtained from different cultures with different lactose concentrations were not significant. This is probably due to the interference of the small lactose content in the booster mixture and the pure lactose added to the cultures which probably made the values fluctuating over the time. Nevertheless, SDS-PAGE showed higher expression levels at low concentrations of the lactose (0.1 – 2.0 g L^{-1}) during the log-phase (≈10 hours) and stationary growth phases (21 hours); in the boosted cultures.

The use of high lactose concentrations or glucose/lactose combination could result in fast growth cultivation with consequent oxygen limitation, which impair the recombinant protein synthesis. Slow growth, by the use of fed-batch strategy, provide slow protein synthesis as it is recommended by many research groups (Graslund, 2008; Panula-Perälä *et al.*, 2008; Krause *et al.*, 2010). This could suggest that the low lactose concentrations would be preferable for autoinduction systems.

Contrarily, some other research groups have addressed that high concentration of lactose is better for the autoinduction systems. For instance, (Studier, 2014) reported that the small amount of lactose is not able to promote a good induction of the cultures at the log-phase growth. Similarly, Faust et al. found that the high lactose concentration (10 g L^{-1}) was more efficient in the classical lactose-autoinduction for expression of eGFP more than the use of low lactose concentration (2.0 g L^{-1}) (Faust, Stand and Weuster-Botz, 2015).
A similar study for (Kunze *et al.*, 2011) for lactose autoinduction using OnEX system (Novagen) reported two to four times higher protein formation rate with a two to four-fold increase in the concentration of lactose compared to the original 2 g L^{-1} lactose. Additional 2 g L^{-1} lactose lead to the extension of the protein production phase from four to eight hours while an additional 6 g L^{-1} lactose extended it to 16 hours. However, Mayer et al. reported that lactose concentration of 0.5 g L^{-1} was sufficient for induction recombinant protein biosynthesis in the enzymatic glucose feeding system (Mayer *et al.*, 2014).

E. coli BW25113 and BL21 had very similar growth profiles in diauxie cultivations. Both grew on glucose with the first batch phase for about six hours. This phase was followed by diauxic lag phase of about 30 – 45 minutes. Then cells resumed growth again with a second short batch phase. *E. coli* BW25113 is known to be arabinose and lactose non-utilizing. This gives a conclusion that the second batch phase in BW25113 was not due to lactose consumption. Most likely the accumulated acetate is consumed during this phase. The *E. coli* strain BL21 is known to be galactose non-utilizing. Therefore; the second batch phase in BL21 cultures was either due to consumption of glucose originated from lactose hydrolysis, or due to the consumption of the accumulated acetate.

The strains *E. coli* MG1655 and W3110 grew differently. They showed three different batch phases with two different lag phases. These were particularly well seen in cultivations using high concentrations of the glucose/ lactose. This was in agreement with a former study by (Solopova *et al.*, 2014) which proposed that the small doses of glucose can facilitate the diauxie shift between sugars in the diauxic growth. Yet, the absence of the diauxic lag phase can reflect the presence of an elevated level of cAMP which stimulates the *lac* transcription rate but might fail to relieve the repression caused by the presence of glucose (Narang and Pilyugin, 2007). First batch phase was due to glucose consumption. Since both strains are lactose utilizing; the

followed batch phases are due to consumption of lactose and galactose and maybe at some extent of acetate, if produced. Therefore, it can be claimed that MG1655 and W3110 were growing under triauxic (or even tetraaxic) conditions.

Since *E. coli* MG1655 and W3110 were able to utilize all the provided sugars, MG1655 was chosen for the diauxie studies at benchtop scale for better understanding of the physiology of the cells growing under multi-substrate conditions. The results showed a typical diauxic growth profile which is in a good agreement with the profiles of diauxic cultivations which were obtained at the shake flask scale. Results reflected a multi-substrate availability in the cultivation medium suggesting the involvement of also substrates other than the glucose and lactose which were supplied to the medium at the beginning of the cultivation. Cell growth on each substrate started with a short lag phase. The time of these lag phases was different with different substrates.

The cultivation started with 4.0 g L^{-1} glucose and 4.0 g L^{-1} lactose which was chosen based on the data have been generated from the shake flask experiments. During the first 8 hours (1st batch phase), the culture was growing exponentially with a growth rate of 0.3 h^{-1} consuming the glucose. During this time, the glucose is transported into the cells via PTS system while EIIA, one of the PTS components, is dephosphorylated and inhibit the transport of any sugars other than glucose which is known by the inducer exclusion mechanism.

After the glucose consumption after 8 hours, cells started adaptation to uptake the second sugar (lactose). This cellular adaptation mechanism takes time which is defined as the diauxic lag phase which was detected here by the sudden change in the pO_2 [%], O_{2ex}, CO_{2ex}, QO_2 and QCO_2 profiles after 8 hours and took about 30 minutes. This diauxic lag phase is the main characteristic of the diauxic growth. The non-diauxic growth can exhibit the same carbon source preference but lacks the diauxic lag time (Narang and Pilyugin, 2007).

Being *E. coli* MG1655 a lactose & galactose utilizing strain; lactose is transported into the *E. coli* cell by the lactose permease (*lac*Y), component of the *lac* operon of *E. coli*, and hydrolyzed then into glucose and galactose by the aid of β-galactosidase (*lac*Z) when the galactose started to be detected in the cultivation broth. The second batch phase then appeared. It was divided into two successive parts with different rates (from 9 – 10 hours & from 10 – 12 hours). Since that glucose coming from lactose hydrolysis is the preferential carbon source for *E. coli*, it was consumed firstly during 9 – 10 hours of the cultivation with corresponding accelerated growth rate of approximately 0.57 h^{-1} followed by the consumption of the galactose which took around 2 hours with corresponding growth rate of approximately 0.44 h^{-1}.

A third decrease in the pO_2 values which was detected after 12 hours of the cultivation seems to be due to acetate consumption which was confirmed by the significant decrease in the acetate level (from 0.35 to 0.05 gL^{-1}) observed in the cultivation broth during this time. However, the acetate consumption time did not exceed a few minutes and followed by fed-batch phase until

the end of the cultivation. By this, four different batch phases were detected along the time course of the cultivation. It was due to different substrates consumption with a distinct order. Glucose in the cultivation medium was consumed firstly followed by the glucose comes from lactose hydrolysis. This was followed by the galactose comes from lactose hydrolysis and finally the acetate formed as a metabolic byproduct. By this, the theories behind the bacterial growth/ sugars uptake order under diauxic conditions have been confirmed.

6. Conclusion

The enzyme controlled substrate auto-delivery cultivation system (EnBase) has been extensively studied as a fed-batch like growth system for a well-controlled microbial growth with high yields of recombinant proteins. In this study, 1. A new application for the fed-batch like EnPresso growth system as a pre-cultivation system is introduced; 2. Application of the EnPresso with different induction protocols was addressed to open different possibilities for the EnBase application according to the type of the expression system used; 3. Scalability of the EnBase system, up to the benchtop-bioreactor scale, has been demonstrated.

The use of the slow enzymatic glucose feeding mechanism (EnBase) provides potent pre-cultures for recombinant protein production in *E. coli*. When the time used for pre-culture making was limited to 6-8 hours, both batch cultures (in LB medium) and fed-batch cultures (Enpresso B medium) provided good results. When overnight pre-culture was used, Enpresso B cultures were superior. The use of fed-batch like pre-cultivation system avoids the initiation of a long stationary phase, which has an adverse impact on the cell performance in the main cultures.

Enpresso Growth Systems have been optimized for shake flask cultivations. However, we found that for our cultivation purposes it could be further improved by the use of additional glucose polymer together with the booster. The scalability study of the enzymatic glucose feeding system showed a good performance over the different cultivation scales. Around 32% increase in the cell density was obtained by shake flask cultivations (UYF) when compared to the cell densities obtained from the multiwell plate scale. About 25% improvement in the yield of the recombinant nucleoside phosphorylase activity was obtained in the benchtop bioreactor scale cultivation when compared to the shake flask level in 15 hours less cultivation time. In this work, nucleoside phosphorylase was successfully scaled-up from well-plate to 2 L scale with the consistent fed-batch cultivations. Based on this fed-batch like Enpresso growth system, a process development from small to larger scale as well as simple-to-perform parallel cultivations were well performed.

The influence of the IPTG on the expression level of the nucleoside phosphorylases was significant in the autoinduced cultures. Moreover, autoinduction of Enpresso B cultures resulted in similar protein activity titers as the normal single shot induction though provided 64% lower cell density but with 15 hours shorter induction time.

A low concentration of IPTG (< 0 .25 mM) or lactose (≤ 2.0 g L^{-1}) were sufficient for induction of Enpresso B cultures. The final volumetric protein activity yields were 3.0 -3.5 U mL^{-1}. The inducing components present in the booster mixture could cause expression in cultures where no IPTG or lactose was used, with a high yield (3.5 U mL^{-1}). By this, different efficient protocols were developed for autoinduction using Enpresso growth system.

Diauxic cultivations confirmed that during the exposure of *E. coli* cells to a mixture of two sugars, glucose and lactose, the cells prefer to consume glucose first. This was followed by a series of metabolic changes in a few minutes according to the strain (around 30 minutes in this study). By these changes the cell takes the second sugar (lactose) which is then internally hydrolyzed into glucose and galactose which will be consumed afterwards. The cells start to consume also the accumulated acetate after all sugars are consumed.

By application of Enpresso growth system as a starter culture for fed-batch cultivation, high biomass yield and protein yield could be achieved. This has a considerable importance in industry to avoid such problems of overfeeding and acetate accumulation from batch cultures. EnBase provides a scalable and simple operation protocol for recombinant protein production. Application of Enpresso growth system with different induction protocols could be demonstrated with high protein yields.

7. References

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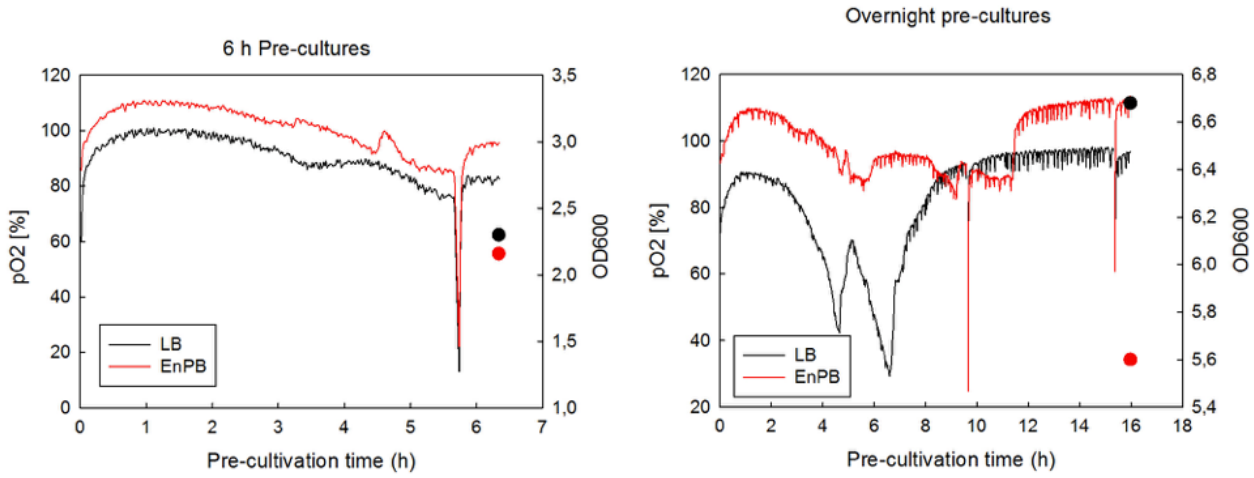
8. Appendix

Composition of the booster powder mix and preparation of the different solutions of different concentrations of the substrate booster mixture (SBM)

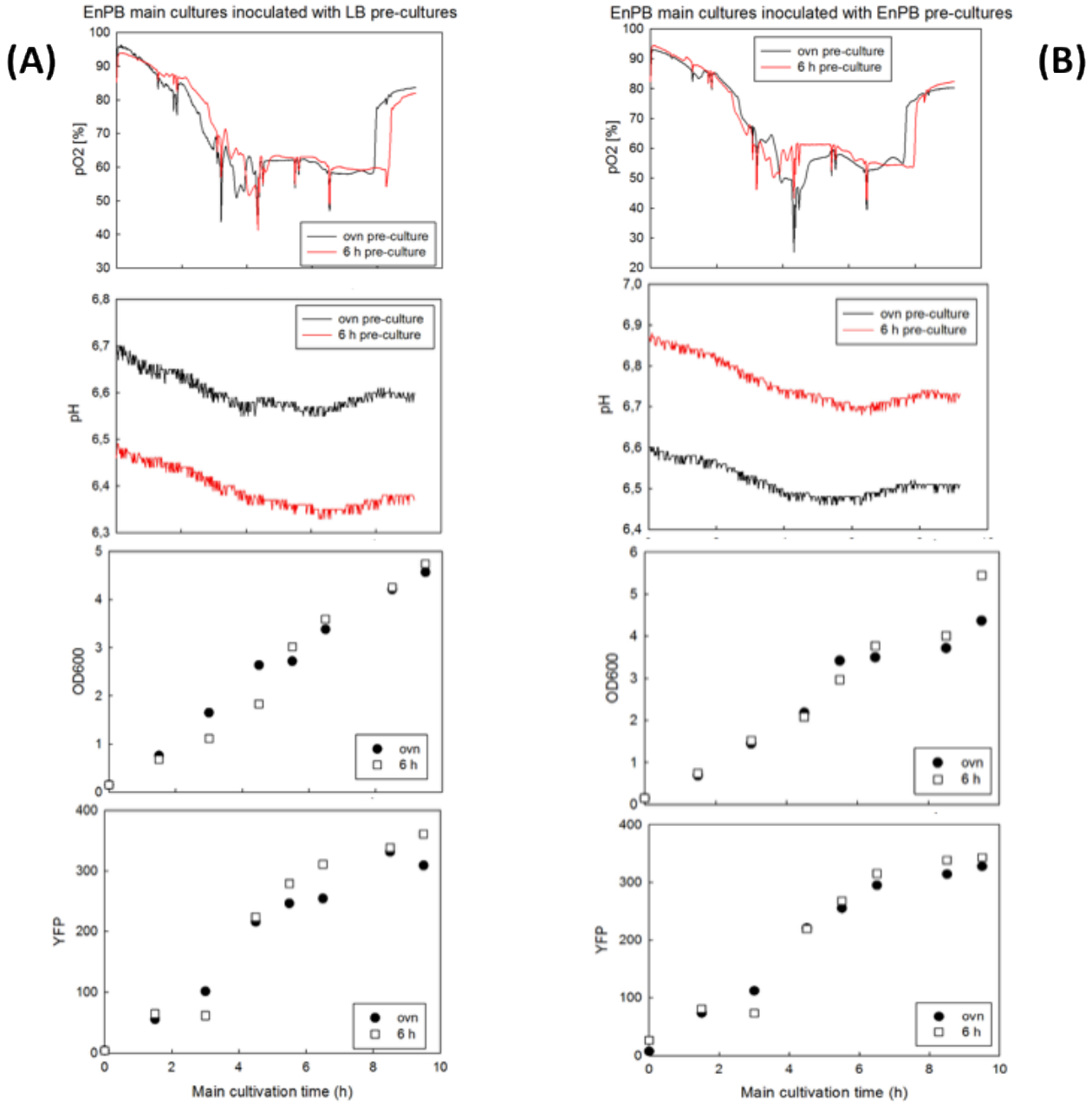
230 g/L Booster powder contains 120 g/L yeast extract; 60 g/L tryptone and 50 g/L glucose polymer		
Concentration of stock substrate booster mix solution [g/L]	Booster mix powder [g/40 mL]	Glucose polymer (EnPump) [g/40 mL]
200	9.2	6.0
150	9.2	4.0
100	9.2	2.0
SBM was prepared as concentrated solutions for further dilution into the cultures (300 µL/3 mL culture volume; 10 X dilution). The final concentrations of the yeast extract and peptone are the same in all solutions, only different final glucose polymer concentrations were obtained (20, 15 and 10 g/L respectively).		

Different pre-cultures of recombinant *E. coli* BW25113 expressing yellow fluorescent protein (YFP)

(A) **(B)**

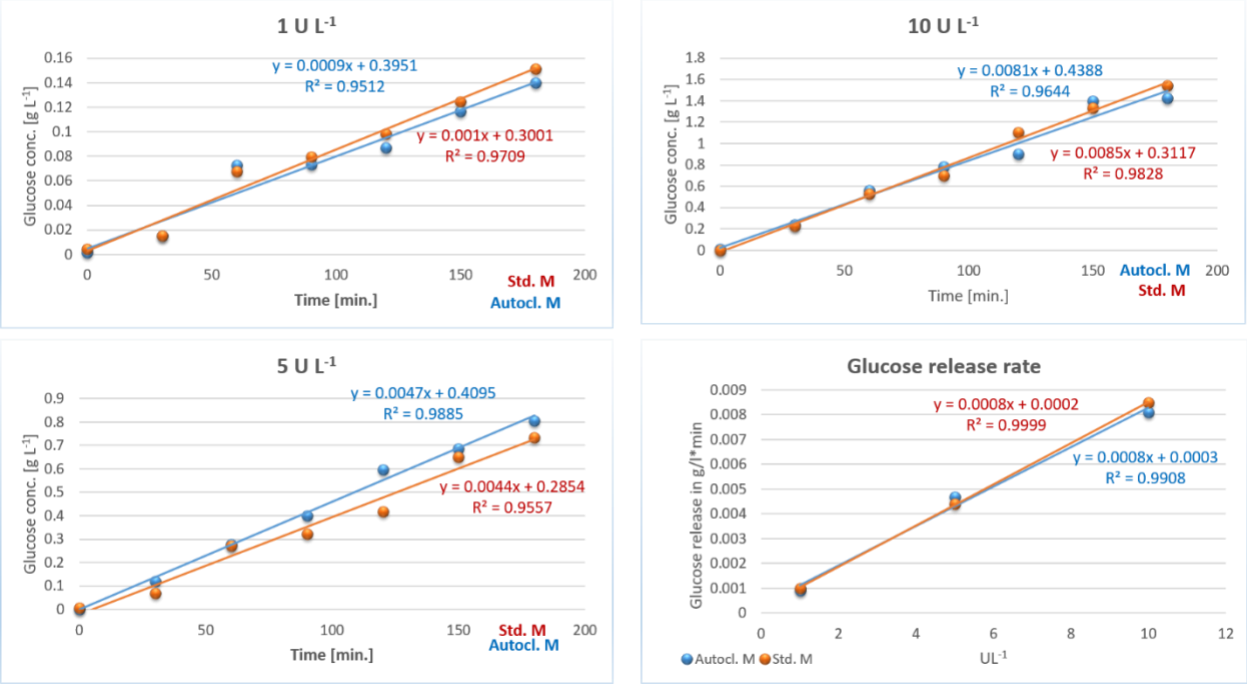


Batch (in LB) & fed-batch pre-cultures (in Enpresso B) of E. coli BW25113 YFP. (A) 6 hours pre-cultures & (B) overnight pre-cultures. pO_2 [%] (represented by solid line) and final OD_{600} values (represented by rounded symbols) of 3 mL pre-cultures in 24 deepwell OxoDish.

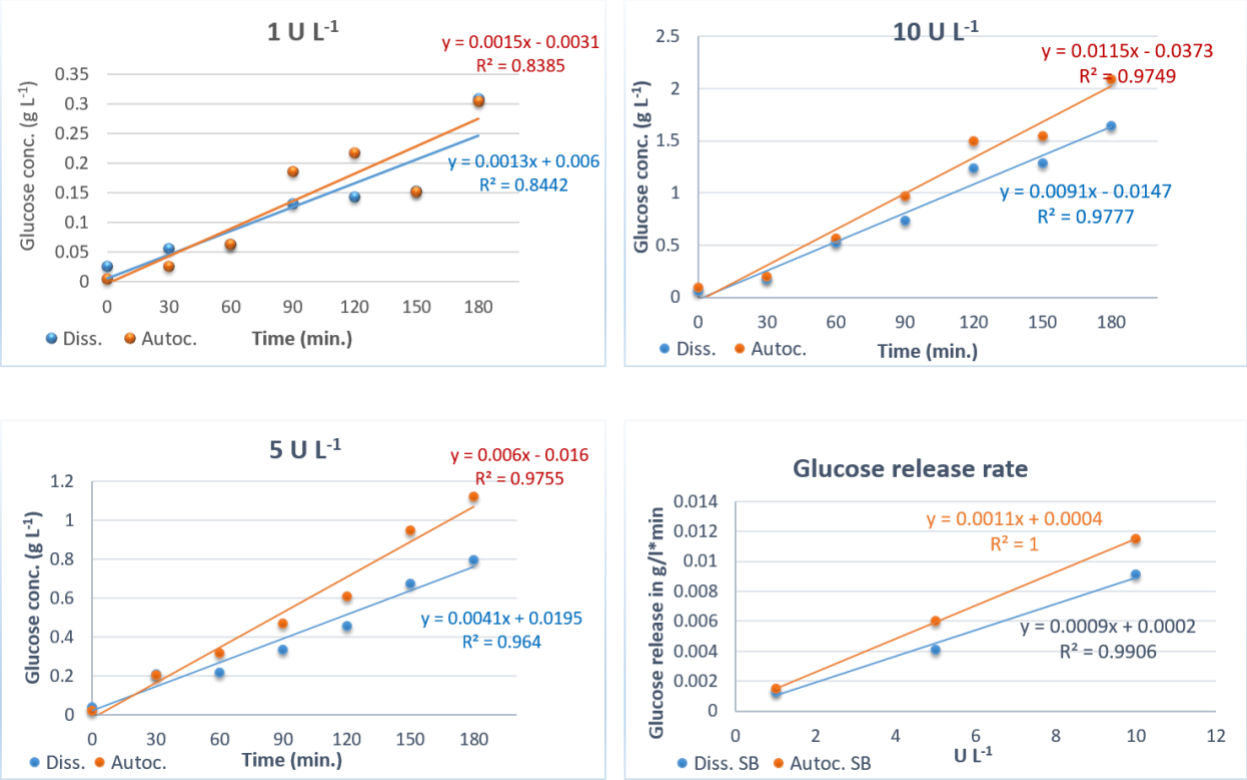


Enpresso B main cultures inoculated with different pre-cultures of E. coli BW25113 YFP (overnight & 6 hours pre-cultures in LB (A) and Enpresso B (B)). pO_2 [%], pH, OD_{600} and YFP expression (fluorescence intensity) respectively.

Glucose release rates of the Enpresso B medium and the substrate booster mixture (SBM)

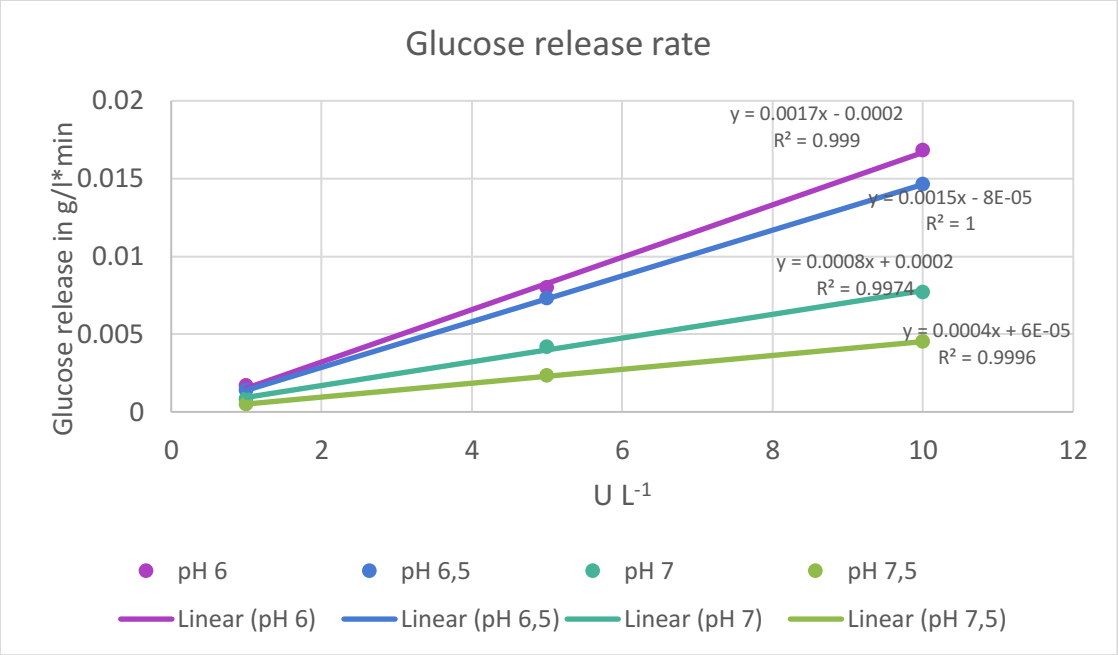


*Concentrations of the glucose released from Enpresso B medium (dissolved & dissolved/ autoclaved tablet) by different concentrations of reagent A (1, 5 and 10 U L^{-1}) in g L^{-1} and the glucose release rate in both cases in g L^{-1}*min.*



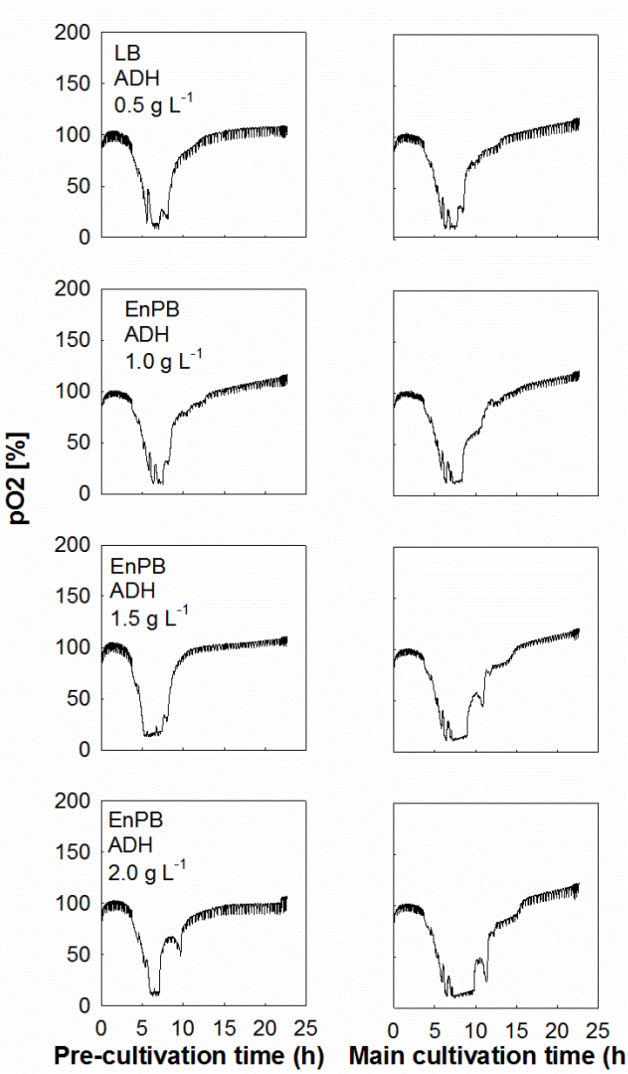
*Concentrations of the glucose released from substrate booster mixture (dissolved & dissolved/ autoclaved powder) by different concentrations of reagent A (1, 5 and 10 U L^{-1}) in g L^{-1} and the glucose release rate in both dissolved and dissolved/autoclaved substrate booster mixture in g L^{-1}*min.*

APPENDIX



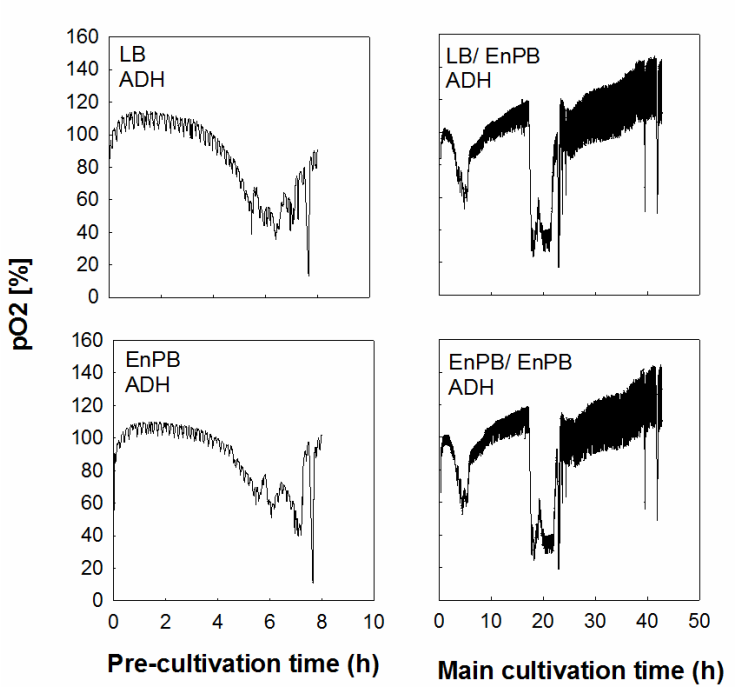
Glucose release rate of Enpresso B medium at different pH values with different concentrations of reagent A.

The effect of different concentrations of glycerol in glycerol stocks of *E. coli* RB791 ADH on the length of the batch phase in the Enpresso B main cultures



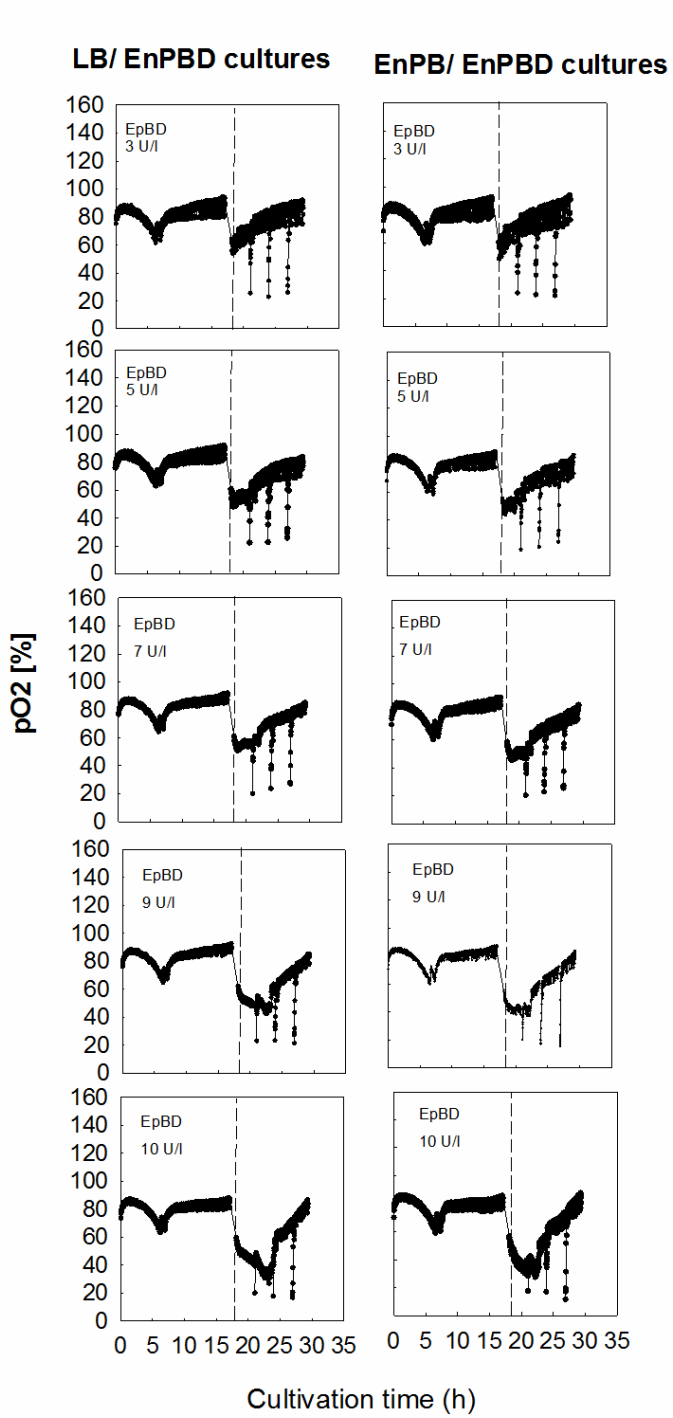
Effect of different concentrations of glycerol (0.5, 1.0, 1.5 and 2.0 g L^{-1}) in the glycerol stocks of E. coli RB791 [pQE30: ADH] on the pre-culture and the batch phase in the main cultures.

The effect of different pre-cultivation system (batch: LB and fed-batch: Enpresso B) on the length of the batch phase in the Enpresso B main cultures.



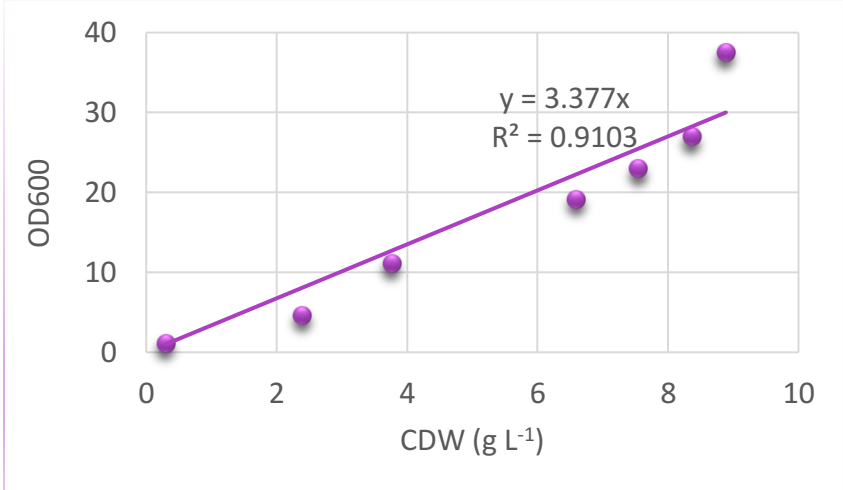
The impact of different pre-cultivation system (batch: LB and fed-batch: Enpresso B) of E. coli RB791 [pQE30: ADH] on the length of the batch phase in the Enpresso B main cultures. The Enpresso B main cultures were induced with 100 µM IPTG after 17 hours of the cultivation.

The effect of different concentrations of the EnBase-biocatalyst on the behavior of *E. coli* BL21 TtPyNP in Enpresso B defined cultivation medium



pO_2 (%) profile of different E. coli BL21 TtPyNP cultures in Enpresso B defined medium with different concentrations of glucoamylase (reagent A)

Correlation between the optical density (OD_{600}) of *E. coli* BL21 and the dry cell weight.



Linear correlation between the OD_{600} and CDW of E. coli BL21 TtPyNP