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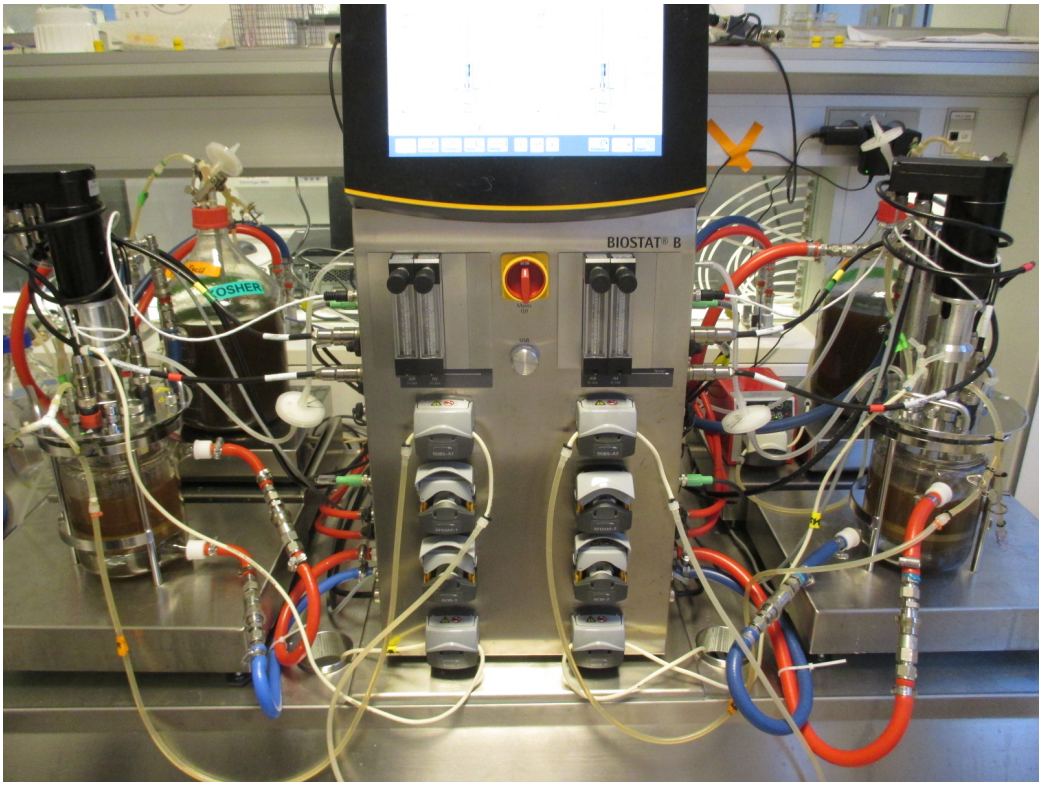

Process physiology study of *Staphylococcus carnosus*

Development for increased nitrate reductase activity

Master's thesis in Biotechnology

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Department of Biology and Biological Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2017

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Cover: Experimental Set-up of 1L fermentors in Chr. Hansen Research and Development laboratory in Hoersholm, Denmark.

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Abstract

Staphylococcus carnosus is a bacterium used as a starter culture in the food application industry, particularly used for fermented sausage production. In this application its specific property is to reduce nitrate to nitrite which subsequently reacts with myoglobin to form a characteristic red color. The strain has been found efficient in this by a mechanism suppressing nitrite from further reduction to ammonia, this is mainly under the control of the nitrogen assimilatory pathway and in particular the enzyme nitrate reductase. This process have also been found to be strongly influenced by oxygen. Chr. Hansen A/S is a food applications company that produces *S. carnosus* at an industrial scale. Recently, a patent has been been filed by the company for a production procedure in which aerobic fermentation of the cells is switched to anaerobic conditions. This has been found to increase the sought after properties of the cells. The mechanisms for increase of the nitrate reducing process in *S. carnosus* when grown on different carbon sources under different aerated conditions are still unknown. In this master's thesis project the effects of different oxygenated states on two different carbon sources, glucose and glycerol, have been studied. KNO_3 was found to severely limit the growth, and a 2.5-fold increase of KNO_3 concentration was therefor implemented. Using a continuously stirred tank reactor set-up as a chemostat cells were grown at steady states with the fixed dilution rate 0.1 [h^{-1}] using two levels of air flow and one level of nitrogen flow. This yielded cells at different physiological states in regards to oxygen availability. Residual metabolites and media components were quantified by HPLC to gain insight to metabolic differences between the physiological states. The nitrate reductase activity for each of these physiological states was determined through an enzymatic assay which resulted in lower activity of the enzyme of interest under anaerobic conditions compared to aerobic conditions when grown on glucose a result contradicting theory. The results of enzymatic activity for growth on glycerol was no able to be determined for the two oxic conditions.Transcriptional levels of genes coding for the enzymes nitrate reductase and nitrite reductase were determined with qPCR. Results showed an increase in expression of reductase genes for cells grown on glycerol anaerobically in comparison to cells grown in the aerobic environment. For glucose, there were no significant differences between cells grown under aerobic and anaerobic conditions.

Keywords: Staphylococcus carnosus, nitrate reductase, nitrite reductase, chemostat, qPCR, HPLC.

Acknowledgements

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Martin Wånge, Gothenburg, February 2017

Contents

1
Introduction

Starter cultures in the food application industry are used for many purposes. The organisms used have a very broad range depending on the application purpose. These application purposes range from use of fungal starter cultures that can contribute to texture and flavour compounds to bacterial starter cultures for acidification in for example the dairy or wine industry. Starter cultures often contain a mixture of microorganisms that have a desired processing effect on the starting material and the applications can be highly diverse [13][21].
A specific field of where starter cultures are used to process foods is the meat industry. These starter cultures can for example be used in the aim of meat preservation by the acidification of the raw material producing for example lactic acid. Fermented meat products can be defined as meats that have been deliberately inoculated with a microorganism to sufficiently cause a lowering of pH controlled by microbial activity[5]. This may hinder issues of rancidity and food spoilage posed by other microorganisms that my even be pathogenic. However other beneficial outcomes of the microorganism may also be present.

A specific application of starter cultures are the case of fermented sausages. Fermented sau sages are prepared from raw meat stuffed in casings where the fermentation process takes place. The starter culture used is often a mixture of lactic acid bacteria (LAB) and catalase-positive cocci such as non-pathogenic staphylococci to influence antioxidant activity and limit off-flavour compounds [18][20].

Nitrite is an important component in the meat industry where it works as a preservative acting with its antioxidant properties to prevent rancidity. It also acts as a suppressor of growth for other bacteria that may cause food poisoning [6]. Nitrite's most desired feature in the meat industry however is its ability to form red colour. Nitrite is used as an additive for colouring to achieve the required aesthetics for the market [20]. By optimizing the existing production organism to convert nitrate to nitrite there is a possibility of lowering the added amount of nitrite and relying on the microorganism to produce the red coloring from its sources of nitrogen.

Staphylococcus carnosus (*S. carnosus*) is a bacterium with an important use in the processing of dry fermented sausages. Species of *Staphylococci* can be grouped into pathogenic and non-pathogenic staphylococci where *S. carnosus* can be categorized into the latter [29]. When used in the mixture of the starting culture it expresses several positive and desirable characteristics; an expression of characteristic flavour development through conversion of amino acids and free fatty acids [20], moderately lowering the pH, and reduction of non-desirable compounds from other organisms

in the starter culture [16]. The most sought after characteristic is however its ability to gradually reduce nitrate to nitrite with the favourable effect that nitrate concentration is lowered to produce nitrite which combined with myoglobin produces the aforementioned red color wanted for the sausages [16]. There have been several studies into the metabolic workings and protein expressions of *S. carnosus* [31][29][24][17]. Neubauer *et.al.* investigated the physiological effects of nitrate reduction in *S. carnosus* [23]. They found that nitrite accumulation was 8 to 10-fold higher under anaerobic conditions than under aerobic conditions. However, knowledge of the underlying mechanisms for how the nitrate reductase, the enzyme found responsible for the conversion of nitrate to nitrite, functions in detail at different physiological states is limited.

1.1 Chr. Hansen A/S

Chr. Hansen A/S is a global bioscience company that develop solutions for the food, nutritional, agricultural and pharmaceutical industries. The company develop and produce a wide range of cultures, enzymes, colours and probiotics for these industries. As they communicate and work with such a versatile collection of industries they have many different sets of processes to accommodate these products. The company is conducting a large portion of their research & development in-house as their R&D headquarters holds a broad knowledge and utility base [1]. This project was coducted with the *Fermentation*-group in *Fermentation R&D* who focuses on development, optimization and up/down-scaling of processes.
The company have issued a patent for the production process of *S. carnosus* where an increase of enzyme activity have been shown when conditions affecting physiology changes from aerobic to anaerobic fermentation [25]. The process physiology of the strain have not been verified and an underlying mechanism hypothesis is not yet determined.

1.2 Aims

As Chr. Hansen has filed a patent for their process of producing *S. carnosus* to increase desired parameters such as enzymatic activity of nitrate reductase there is a need for further evaluation of the process physiology of the strain. This project aims to study the enzymatic activity of nitrate reductase under conditions with different oxygen availability to the bacterial strain.
The specific aims of this project are to:

- Determine the enzymatic activity as well as genomic expression determination for nitrate reductase at a range of delivered oxygen.
- Conclude if media changes are needed as no prior optimization of the medium has been performed .

2

Theoretical Background

This section will describe the background needed to understand the aim of this project. It will in addition contain some theoretical background in regards to bioreactors and the application of them.

2.1 Bacterial physiology of *S. carnosus*

S. carnosus is a gram positive bacterium belonging to the family of Staphylococcaceae. Another well studied staphylococcaceae is the pathogenic bacterium *S. aureus* which produces toxins and causes infections [37][12]. *S. carnosus* has shown low levels of DNA homology to *S. aureus*. It does not produce toxins or clumping factors and has a low level of extracellular proteolytic activity [31], indicators of pathogenic activity.

2.1.1 Nitrogen in *S. carnosus*

For the synthesis of nitrogen-containing molecules in the bacterial cell amino-groups are required. Sources of nitrogen can include nitrogen (gas), amino acids and nitrate as depicted in figure 2.1 corners. These nitrogen-containing compounds are formed by three processes; the assimilatory process by reducing inorganic nitrogen to ammonia, the catabolic use of amino acids through urea to ammonia or nitrogen fixation from the surrounding atmosphere [11]. The assimilatory process of nitrogen that may occur aerobically or anaerobically and nitrate can eventually be reduced to ammonia (NH_3) which is subsequently incorporated in the bio-synthesis process through for example amino acid production. An alternative element for nitrate is its use in the respiratory pathway where it is used as an alternative electron acceptor under low oxygen conditions [4] this to act as a redox balance for NAD/NADH recycling. This is called anaerobic respiration as an alternative electron acceptor that is inorganic.

As depicted in figure 2.1 there is only a solid arrow from NO_3^- to NO_2^- indicating the only path for reducing nitrate to nitrite is a respiratory one. Others have included this to be in the pathways of the assimilatory pathway as well [28][23]. The reduction of nitrate to nitrite by Nar and nitrite to amonia by Nir are both facilitated by NAD(P)H as the reducing agent.

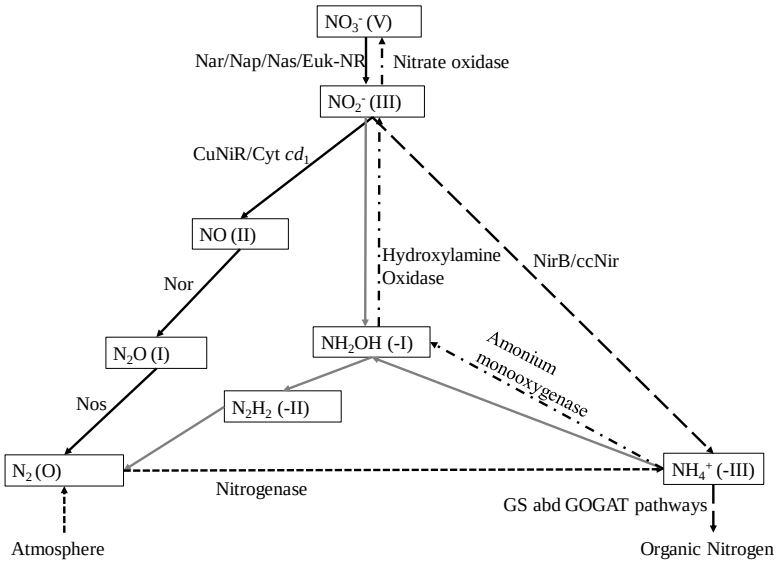

Figure 2.1: Schematic overview of the inorganic nitrogen pathways in microorganisms with responsible enzymes for each step. The oxidation state of each compound is indicated between parentheses. The pathways are donoted as: Solid black line, denitrification ; dashed line, dissimilatory and assimilatory ; dotted line, nitrogen fixation ; dash-dot line, nitrification ; gray solid line, the ANAMOX pathway. The figure is replicated from an article by Gonzáles *et. al.* [15]. The enzymes of interest in this project are nitrate reductase (Nar) and nitrite reductase (Nir) both of which are NADH dependent in its reduction, here described in the respiratory and assmilatory pathways.

For the assimilatory process where nitrate is reduced to nitrite which is subsequently reduced to ammonia, the bacterial strain of *S. carnosus* has shown a feedback inhibition of nitrite, the compound of interest. This acting so that nitrite does not further get reduced before nitrate is depleted [23]. Possessing this feature makes the strain a good candidate for its food application where nitrite concentrations can be kept high to yield red coloring [16].

2.1.1.1 Effect of oxygen

The two enzymes in the assimilatory pathway of nitrogen of interest are; nitrate reductase and nitrite reductase as shown in figure 2.1 for Nar and Nir enzymes. In a study by Neubauer *et.al.* [23] the synthesis of these enzymes were studied under different oxygenated states of *S. carnosus*. The nitrate reductase was found to be inhibited by oxygen when studying cells grown aerobically without addition of nitrate and a slightly higher activity was found with nitrate added. The highest observed nitrate reductase activity was found when cells were grown anaerobically with nitrate. When supplementing with nitrite the activity of nitrate reductase was higher than without any addition of nitrate or nitrite. Nitrite reductase showed no activity for aerobically grown cells. Further results showed that nitrite was only taken up by the cells after nitrate was depleted suggesting an inhibition of nitrite reduction by nitrate.

A metabolic shift occurs for organisms able to grow both aerobically and anaerobically when the oxygen levels available change. The energetically favourable TCA cycle in the lack of oxygen as electron acceptor may be shut down if no other acceptor is present. This often due to not being able to recycle coenzymes such as NADH. This leads to a need for recycling of such intracellular molecules [3]. *S.aureus* copes with this by initiating fermentation and production of Lactic acid. In a study on *S. aureus* performed by Ferreira *et.al.* [12] the effect of oxygen and its deprivation on glucose metabolism in aerobically grown *S. aureus* was studied. They found that lactic acid production is reduced and acetic acid was increased as an effect of oxygen deprivation. No such study have been performed on *S. carnosus* and the mechanism for coping with anoxia there is a possibility of using a similar fermentatitative recycling as well as a possible anaerobic respiration option.

A factor influencing the shift in metabolism over the availability of oxygen is the carbon source. Glycerol catabolism occurs in *E. coli* through gluconeogenesis to be able to shuttle the carbon source into the glycolysis via pyrovate. It has also been shown to be able to do so under anaerobic conditions [7]. Similarly *S. aureus* is believed to be able to utilize glycerol catabolism to be the electron donor [36]. It is not yet determined if it can do so under anaerobic conditions. *S. carnosus* can therefore be assumed to be able to utilize glucose as the carbon source under both aerobic and anaerobic conditions and growth on glycerol under aerobic conditions as well. Under anaerobic conditions on glycerol as the carbon source there is uncertainty on whether it can be functioning as a sole carbon source.

2.2 Reactor Theory

A bioreactor is an apparatus that is used to carry out any kind of biological process [22]. The process can be carried out in several settings. In this section there will be a brief explanation of some theories on the topic that is relevant to this project.

2.2.1 Batch Fermentation

A batch fermentation is constructed of a closed system in which all liquid or solid components are placed in the reactor at the start of fermentation. In this system the concentrations of the constituents change over time as cells multiply [33]. The batch set up is commonly used for a range of fermentations. It is considered an easy option to scale up processes as to mimic what is commonly used in production due to its low utility cost and high flexibility [19].
Using Monod kinetics $\mu_{\max}$, maximum specific growth rate, for a bacteria is determined as the maximum relative growth rate that is possible under specified conditions for the medium where S»K_S (as $\mu \to \mu_{\max}$ when $S \to \infty$). Determination of $\mu_{\max}$ can be performed using a batch fermentation set-up by measuring biomass dynamics during the exponential growth phase which fit a linear regression equal to $\mu = d(lnX)/dt$, where μ is the specific growth rate [26].

2.2.2 Continuous fermentation

In a continuous set-up of a bioreactor new media is continuously being pumped into the reactor and fermentation broth is simultaneously being pumped out. This can be represented by a chemostat, a continuous stirred tank (CSTR) reactor that continuously feeds fresh media to maintain the nutrient concentration in the tank. Usually all substrates except one are added in excess which renders one to be the limiting substrate of the reactor. The excess volume is discarded by pumping out the effluent. This set-up results in the volume being constant and it further helps to keep the cells grown in the system at a selected state for their physiology [19]. The system does however become more prone to contamination as it runs over longer time and the conditions of having a limiting substrate also puts a selective pressure onto the organisms which can cause mutations to accumulate in the population [33].

2.2.2.1 Steady state of Chemostat

The residence time of a chemostat performed in a CSTR is defined as $\tau_{\mathrm{CSTR}} = V/F$, where V is the working volume of the tank and F is the flow through the reactor. As conditions change a delayed response on the cell population occurs. Direct effects can be seen after two to three residence times but outcomes of changing the conditions can have long-term responses that are dependent on the specific organism grown [32]. There is therefore a "rule of thumb" of minimum of five volume changes or residence times that is used as the guideline to ensure a steady state can be achieved [35][27][34][14]. To ensure that the physiology of the cells are not in a dynamic state output variables from the fermentor can be used to assume steady state or the case of a pseudo-steady state. The most common parameter that is used is optical density [35] but other parameters such as the composition in the off-gas or acid production by the cell population can also be used.

2.2.2.2 Mass balance theory for chemostat

The general dynamic mass balance equation for a bioreactor can be conceptualized for a compound j in volume V as ; mass flow j in - mass flow j out + mass j generated - mass j consumed = mass j accumulated inside a defined system boundary. The system boundary may be real, such as the walls of a cell or a reactor or imaginary resulting in that the mass balance is a general thermodynamic term and not an occurrence solely for bioractors. The system boundaries can be defined as a closed or an open system where the process causes changes in the system only or to its surroundings respectively. The differential equation for the change of mass (M) of a compound (j) can be described as

$$dM_{\mathrm{j}}/dt = M_{\mathrm{ji}} - M_{\mathrm{jo}} + R_{\mathrm{jG}} - R_{\mathrm{jC}} \tag{2.1}$$

where $\mathrm{M_{ji}}$ is the flow of mass of compound j into the system, $\mathrm{M_{jo}}$ is the mass flow of compound j out of the system, $\mathrm{R_{jG}}$ is the rate of generation of mass of compound j and $\mathrm{R_{jC}}$ is the rate of consumption of mass of compound j. For biomass this can be further explained as $\mathrm{M_i} = \mathrm{Fx_i}$, $\mathrm{M_o} = \mathrm{Fx_o}$ and $\mathrm{R_G} = \mu\mathrm{xV}$ where F represents

the volumetric flow rate, x is the biomass concentration and μ is the specific growth rate. At steady state where

$$\frac{dM}{dt} = 0 \tag{2.2}$$

and the consumption of biomass (cell death) is assumed to be negligible it ends with the equation

$$Fx_{\mathrm{i}} - Fx_{\mathrm{o}} + \mu xV = 0 \tag{2.3}$$

and therefore as no biomass in a chemostat is added to the inlet flow ($\mathrm{x_i} = 0$)

$$Fx_{\mathrm{o}} = \mu xV \tag{2.4}$$

if the dilution rate, D, is defined as D=F/V the relation between dilution rate and growth rate can be described as

$$D = \mu \tag{2.5}$$

By this it is possible to select the growth rate of the cells in the reactor by controlling the dilution rate and letting the culture reach steady state. It is worth noting that the specific growth rate is limited by $\mu_{\max}$ which means there exists a maximum dilution rate described as

$$D_{\max} = \frac{\mu_{\max} s_i}{K_S + s_i} \tag{2.6}$$

where $\mathrm{s_i}$ is the steady state substrate concentration and $\mathrm{K_S}$ is the monod constant. As $\mathrm{K_S}$ is usually much smaller than $\mathrm{s_i}$ $\mathrm{D_{crit}} \approx \mu_{\max}$. This determines the boundary for the chemostat to be subject to washout, the process where the biomass in the reactor is washed out and replaced by fresh media. It can further be stated that it is desirable to not be too close to the maximum dilution rate as smaller changes make the system sensitive to washout [9][10][8]. Furthermore, as described in section 2.2.1, the determination of $\mu_{\max}$, could also be performed using a chemostat at steady state. This by using different sets of dilution rates until you reach washout or spiking of the limiting substrate at different concentrations [26]. This can also be estimated through a set of steady states below the maximum.

3
Materials & Methods

This chapter describes methods that were used in a chronological order as the project was performed. The details are limited due to confidentiality of exact figures. The explanation is therefore described in broader terms so that a clear picture of what was done can be told.
The temperature for all experiment was controlled at 37°C and an pH of 6.5 was used as a starting value of the medium used. Further, all experiments were monitored for contamination using microscopy where pictures were taken and examined. Further it should be stated that all experiments not conducted in smaller scale than 1 L fermentors were subject to limited oxygen supply as no aeration or agitation was available to the closed vessels that were used. These experiments are therefore considered to be anaerobic.

3.1 Strain and inoculum process

The strain used for all experiments was a strain of *S. carnosus* owned my Chr. Hansen A/S. For all experiments of cultivating *S. carnosus* a Direct Inoculum Material (DIM) was used as the basis of starting the growth. DIM was thawed and diluted in a pepsine substrate solution prior to inoculation with selected V/V %. Experiments utilizing 1 L fermentors used the DIM and pepsine mixture as dictated by stanard procedure whereas the experiments in smaller scale (described in section 3.2) used thawed DIM re-frozen in 15 mL tubes that was subsequently thawed prior to inoculation. These will be referred to as miniDIM.

3.2 Media determination

This section gives insights to the proceedings prior to the continuous fermentation set-up being used. All media used was based on the production scale medium composition for *S. carnosus* at Chr. Hansen with an extension of the carbon source to include both glycerol to glucose. Due to no prior media optimization for *S. carnosus* it was desirable to investigate possible options to alter concentration in the recipe and/or adding of compounds to the medium.

3.2.1 Nitrogen assimilation

To study nitrogen assimilation of *S. carnosus* two different media compositions were used to determine if the need for nitrogen could be sufficiently provided solely by

KNO_3. The experiment used one medium composition where the complex medium component of yeast extract was removed and the other composition was remained unchanged. In both compositions KNO_3 was present in equal concentration as originally set. The experiments were performed using a 200mL reactor system monitoring pH and controlling temperature. miniDIM inoculation rate of the double of the previously used V/V % was adopted and a negative control for each media composition was used. pH and final optical density (OD) measured at 600nm was used as indication of growth of *S. carnosus*. Subsequently a similar set-up using two 1L fermentors was performed using identical conditions for the medium composition and inoculation concentration. To assess this further experiments using 1L fermentors with a batch set up was used to evaluate growth with the two different media compositions.

3.2.2 Vitamin supplement addition

As the results from some previous experiments (data not shown) and from experiments described in 3.2.1 had in some cases shown to be low growth on the selected medium an investigation to whether vitamin supplementation could increase growth for *S. carnosus* was performed. This was a further investigation to ensure carbon source limitation in upcoming chemostat experiments. This was performed by sterile-filtering a Chr. Hansen standard vitamin mix to the production medium to the final concentration of 5g/100L (as a higher vitamin concentration would be considered toxic to the bacteria). The experiments were performed in 50mL plastic tubes in a conditioned water bath with quadruplicate tubes for each of the two media compositions (with and without vitamin addition to standard medium recipe). Medium pH had been adjusted prior to inoculation to 6.5 and miniDIM inoculation rate of the double of the previously used V/V % was used. Final OD at 600nm was measured to indicate growth for the bacteria.

3.2.3 KNO_3 concentrations

Investigation into whether potassium nitrate was limiting growth of *S. carnosus* was done using a range of concentration of KNO_3 in the medium. This was performed to investigate limiting substrate source as well as to increase the possible growth conditions for *S. carnosus*. Duplicate 50ml tubes in conditioned water bath were used for each concentration of KNO_3 that was added in a five point range of increasing concentration from the original recipe concentration. The experiment used two negative controls with the lowest KNO_3 concentration. Final OD at 600nm was measured to determine growth. miniDIM inoculation rate eight times of the previously used V/V % was used.

3.2.4 Carbon concentration determination

To determine the concentration of the carbon source used for the continuous reactor media feed in upcoming trials experiments were set up using different carbon concentrations for both carbon sources of interest; glucose and glycerol. Final OD at 600nm and final pH was measured to study the growth of *S. carnosus* at a lower

concentration than the one being used in the original recipe. The original recipe's KNO_3 concentration had been altered after results from the experiments described in section 3.2.3. The experiment used 50mL tubes in single points of eight different concentrations for each carbon source (1, 2.5, 5, 7.5, 10, 15, 20, 35 g/L). miniDIM inoculation rate of the double of the previously used V/V %.

3.3 1L bioreactor experiments

After investigation to medium composition described in section 3.2 the altered medium composition was used for experiments in the following 1L bioreactor experiments. The set-up consisted of two 1L bioreactors with possibility to control parameters such as; temperature, gaseous flow, agitation etc. The reactors was monitored by a Sartorius© control station (tower). An additional external pumping system as well as scales were connected and the tower and the additional components were run by a software called Lucullus-PIMS©. All experiments at 1L used a working volume of 0.75L with fitted DO-probes and pH probes that were autoclaved dry inside the fermentor to limit contamination risks. To control pH at the selected optimal for *S. carnosus* 18% NaOH was fitted to the pump on the tower and was PID controlled by the pH value output through the software. The DO probe was calibrated after addition of initial medium by flushing with air and nitrogen to perform a two-point calibration. pH probes were calibrated using a standard solution prior to autoclavation and the pH functionality after autoclavation was verified using an at-line pH meter.

3.3.1 Batch fermentation - μ determination

Previous results performed by George Nabin Baroi, supervisor of this project, had yielded maximum growth rates for *S. carnosus* under aerobic conditions (data not shown). Experiments to determine maximum growth rate under anaerobic conditions were therefore performed. The set-up was used as previously described in section 3.3 with one fermentor for each carbon source (glucose & glycerol). Gaseous flow sparging with nitrogen gas, N_2, at a rate of 0.2 L/min was used. The previously established lag-phase of *S. carnosus* (data not shown) was run with no or limited sampling and subsequently sampling during the exponential phase was performed every 30 min. Samples taken were measured for OD at 600nm externally during growth phase of *S. carnosus*.

3.3.2 Chemostat

Maximum growth rate, μ_{max}, for *S. carnosus* under both fully aerobic and anaerobic conditions was previously determined. To gain comparable results, using a strategy to 'uncouple' growth rate effects on nitrate reductase activity, a dilution rate below the highest determined μ_{max} was chosen. The set-up consisted of the previously described equipment where the external pump of inflow of medium was PID controlled by a scale the feed-reservoir was situated on. Further the effluent was similarly controlled by a scale that the reactor was placed on. This set-up yielded the possibility

to run the reactor as a chemostat. The outlet gaseous flow was connected to an off-gas analyzer (Mass spectrometry) to quantify the composition of gases. Steady state was assumed after four to five volume changes after which samples were taken for analysis. The possibility of controlling the dissolved oxygen level in the reactor through parameters of agitation and airflow was present. However the risk of break-down and cost of reparation was deemed to great since the experiments took place outside office hours. The selected method to gain varied oxygen levels was to have a fixed agitation and a fixed airflow and after a steady state was achieved alter solely the airflow.
To study cell physiology at different oxygen flow rates, agitation was fixed at 300 rpm and alternating gaseous flow in the working volume of 0.75L; 0.5 lpm (1/3 VVM), 0.2 lpm (apprx. 1/4 VVM), N_2 0.2 lpm (apprx. 1/4 VVM) for each steady state was used. Samples were taken with a syringe though the effluent flow tubing where a forked tube fitting was placed with a small check-valve. The first 5 mL were discarded to obtain a representative sample of the reactor.
DIM inoculation rate for these experiments was done using eight times of the previously used V/V %. As inoculation concentration do not matter when running a chemostat, since growth is controlled by the dilution rate, the increase DIM concentration was used to shorten the batch phase. This was done to achieve sufficient biomass to start medium feed and effluent pumping. The chemostat was run with the described set-up twice; first on glucose and then on glycerol. This yielded results for each steady state on each of the carbon sources in duplicates.

3.4 Set-up of fermentations

For experiments described in section 3.2 and section 3.3.1 the equipment used is deemed to not need further explanation. The media preparation for these experiments was performed by mixing medium components and autoclaving at 121°C for 45 min with the carbon source autoclaved separately and added prior to inoculation.

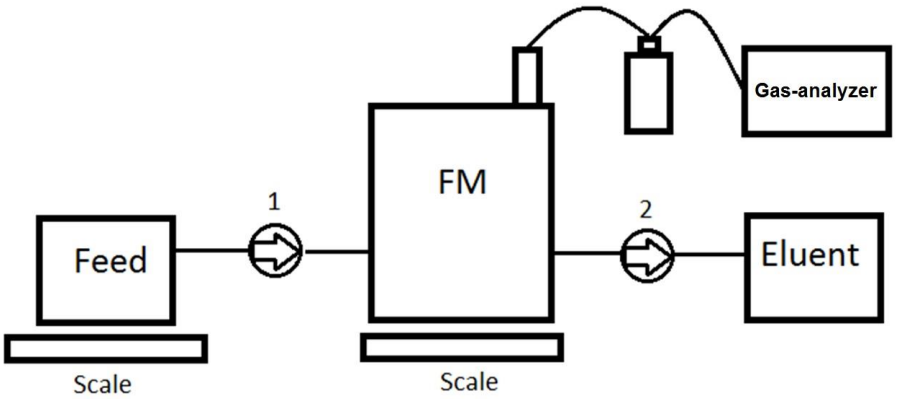

Figure 3.1: Schematic overview of the chemostat set-up, 1 & 2 are showing pumps and there is further a safety bottle between fermentor (FM) and off-gas analysis.

Experiments described in 3.3.2 for chemostat experiments was determined through an evolution of solutions. This to facilitate the requirements regarding for example;

feed volume available to autoclave, size of media bottle and available sterile filters. The overall set-up of the chemostat is portrayed in figure 3.1

In the figure two pumps are shown. Pump 1 used the output values of the scale of which the feed was situated on to provide the desired volumetric (assuming $\rho = 1$ g/cm^3) flow of medium to the fermentor (FM). Pump 2 used the output of of the scale for which the fermentor was stationed on to keep it at a fixed value which was set as the scale was tared prior to fermentation. In the software programing a safety mechanism was Incorporated stating that if the fermentor scale output value was $\pm$ 200 g the fermentation would shut down. This was implemented to secure the fermentor from overflowing, especially into the gas analyzer.

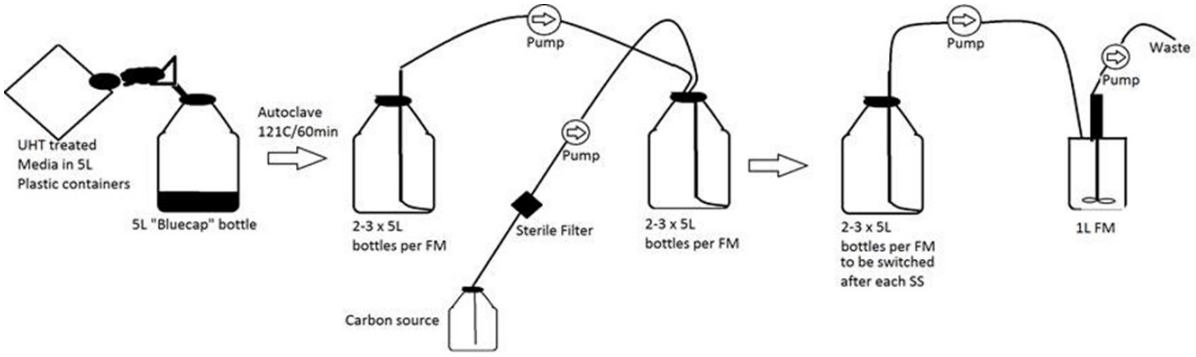

Figure 3.2: Schematic overview of the medium preparation set-up.

Figure 3.2 is a schematic overview of the preparation of medium. Ultra high temperature (UHT) treatment was performed on a batch of 100 L output volume of medium. This was used as a primary step of medium preparation as it was deemed to be a high risk option for usage in a chemostat setting. Hence a secondary step of medium treatment was implemented where 5 L bottles were filled and autoclaved at 121°C for 60 min. The carbon source was prepared and autoclaved at 121°C for 45 min separately. The autoclaved bottles were in a laminar flow bench filled with the desired concentration of carbon source pumped though a sterile filter. The feed bottle was then placed on the scale depicted in figure 3.1 to function as the medium reservoir. Notable here is that since the scale was controlling the flow of medium any alteration to the scale would cause a fluctuation in the flow of medium hence disrupting the steady state. Because of this the feed reservoir was filled to facilitate the desired amount of residence times before changing the bottle thus disrupting the assumed steady state.

3.5 Analyses

In this section the different analyses performed will be briefly described. Sampling from experiments conducted in section 3.2 were all end value samples and the need for precautionary meusseres considering contamination were not needed. Sampling from experiments in section 3.3 were performed using a syringe connected to a small check valve to reduce risk of contamination. The sample port used was placed in the

middle of the reactor to give a representative sample. The same port was connected to the effluent flow described in 3.3.2. The tubing on either side was shut with clamps to inhibit possible back-flow and inlet of gas from outside.

3.5.1 Optical Density - *OD*

OD was analyzed at-line, diluted in cuvettes with 0.1% NaCl water solution to be measured at 600nm in a spectrophotometer to show absorbency values between 0.100 and 0.600. Each sample was measured in quadruplicates with the 3 most similar being used to obtain an average value. A maximum of 0.010 difference between two values was used. On-line sampling of OD for experiments in section 3.3 were not possible due to restrictions of available ports as well as not being included for the new software being used for these two bioreactors in particular.

3.5.2 Microscopy

All samples acquired from both section 3.2 and section 3.3 were analyzed using optical microscopy. Pictures were taken of the sample to evaluate the state of the fermentation and assure that no contamination had occurred. Further, all media was evaluated using microscopy as well were a background for all media containing yeast extract was found, these contained rod-shaped bacteria which were mostly non-viable but still present.

3.5.3 Packed cell volume, *PCV*

Samples at each steady state from section 3.3.2 were in duplicates added in 10ml to graduated glass tubes and spun down at 2000G for 15 min. This was done to study the ability for cells to be packed, an important property for downstream processing. This analysis was also used to validate cell mass results from OD and Flow Cytometry.

3.5.4 *HPLC*

Samples taken at steady state in duplicates section 3.3.2 were diluted at a 1:2 ratio with ethanol and frozen and stored at -50°C. These were analyzed quantitatively for NO_3 and NO_3 content. Samples were also in duplicates diluted with 1M H_2SO_4 at a 1:5 ratio and frozen and stored at -50°C. These samples were quantitatively analyzed for; glucose, glycerol, acetic acid and lactic acid content. Analysis of HPLC were performed by the in-house *Biochemical Analysis* group and yielded results back in g/L content in the fermentate for each steady state.

3.5.5 Flow Cytometry

Samples taken at steady state from section 3.3.2 were in duplicates frozen and stored at -50°C. These samples were sent to the *Microbial Service* group for in-house analysis and were analyzed for total cell count and active cell count per gram of fermentate sample.

3.5.6 *NRA*

This method was developed by the *Enzyme* group at Chr. Hansen. Therefore the detailed explanation will be limited. Samples taken at each steady state from section 3.3.2 were disposed in 50mL tubes and subsequently frozen and stored at -50°C. Samples were thawed and concentrated to obtain the desirable cell concentration. This was done using results from section 3.5.5 of [active cells/g]. Concentration of samples were performed by spinning down samples for 20min at 2000G, supernatant discarded and re-suspended with buffer twice to obtain biomass concentration within the range given by the assay description. Cell concentrates were grown in quadruplicates on substrate containing a known concentration of NO_3 and samples taken over time to examine the conversion of NO_3 to NO_2. Each sampling point were measured using a Griess reagent protocol to measure absorbance at 540nm. By using an external standard, concentrations of NO_2 could be converted from absorbance values and resulting in values for conversion rates in units of [nM/h/cell] or [mM/h/mL fermentate]. For reagents used, further explanation and methodology please see appendix A.1 (confidential)

3.5.7 *RT-qPCR*

Gene expression was evaluated for two selected genes found responsible for reductase genes; nitrate reductase gene narG and nitrite reductase gene nirB. Samples taken at each steady state from section 3.3.2 were diluted 1:2 with RNAprotect Bacteria Reagent and frozen in liquid nitrogen (LN_2). RNA was extracted using a standard method procedure. To ensure that a too high cell concentrations were not used per extraction (which would cause clogging of extraction procedure) results from section 3.5.5 in [total cells/g] was used to calculate the concentrations of each sample to be used for extraction according to the method. From each sample triplicate RNA extractions were performed. To each RNA extraction gDNA wipeout was used to remove DNA from each sample. Reverse transcription of extracted RNA was performed to generate cDNA and samples were stored at -50°C. RT-qPCR was performed in triplicates for each extracted RNA sample. Primers for the, in total of, 6 different genes were used on separate runs on the PCR machine. The qPCR was run using an external standard to convert cycle threshold (CT) values to quantities. This was done using equation 3.1

$$Q = E^{\text{minCT-sampleCT}} \tag{3.1}$$

where Q is sample quantity relative to the sample with the highest expression, E is the amplification efficiency calculated statistically using the standard curve and minCT is the CT value of sample with the highest expression. The expression level for the two genes of interest, narG & nirB, was normalized to the expression of the four household genes to obtain a relative expression level of the samples. Using the software coupled with the qPCR hardware, *7500*, results flaged as outside the range were omitted (with resulted in a minimum of 2 sample points to generate the CT mean value for that sample). An average of each of the normalized expression levels was then calculated. This yielded a relative expression of the genes of interest for all

the samples. For equipment, reagents, further explanation and methodology please see appendix A.2 (confidential)

4

Results & Discussion

This section will illustrate the results yielded by the experiments performed for this project and give insight to the decisions that were taken and an interpretation of these results.

4.1 Media Composition

This section describes the outcomes from the experiments described in section 3.2. As previously mentioned the media had not been optimized for the growth of *S. carnosus* but instead a somewhat modified medium from previous Staphylococci production fermentations by Chr. Hansen had been used. To start the media analysis, removal of the complex media component yeast extract was studied. As shown in figure 4.1 A) there seems to be a small difference between removal of yeast extract and addition of it, this to the effect of yeast extract seems to be needed for *S. carnosus* to yield a higher optical density. The aim for this particular experiment (described in section 3.2.1) was to investigate if *S. carnosus* could grow with KNO_3 as the sole nitrogen source. However, previous studies have shown that *S. carnosus* is expressing auxotrophy to several amino acids and would in theory need the addition of either yeast extract or amino acids in a defined medium [29]. The results of experiments depicted in 4.1 A) are in a very low range of optical density where it could be questionable whether or not there is any growth. Unfortunately though, pH was sub-optimized as pH dropped drastically after inoculation. However, the samples had a reduced pH at the end of growth indicating metabolic activity and growth. The microscopy examination showed low numbers of Staphylococci, corresponding to the low OD_{600} values. There was, as shown in figure A) an increase in OD_{600} indicating that *S. carnosus* can indeed grow on KNO_3 as the only nitrogen source. There was also a significant drop in pH for the flasks without yeast extract which lack the buffering effect of yeast extract. Extended results can be found in appendix A.3.

Figure 4.1 B) shows the results of vitamin supplementation. There was no significant increase of growth with vitamin supplementation compared to without vitamin supplementation. It should be noted that these experiments too place in a temperature conditioned water bath in 50mL plastic tubes meaning that there was no possibility of aeration or agitation which led to sedimentation of cells and medium in the tubes. The lack of mixing may have led to limited transfer of nutrients to the cells. The lack of optimal growth conditions is a probable reason for the low OD_{600} values for the experiment. As shown later when the bacteria were grown under optimal

conditions the OD_{600} could reach values 30-fold higher. Nevertheless, vitamins were not supplemented in the following experiments.

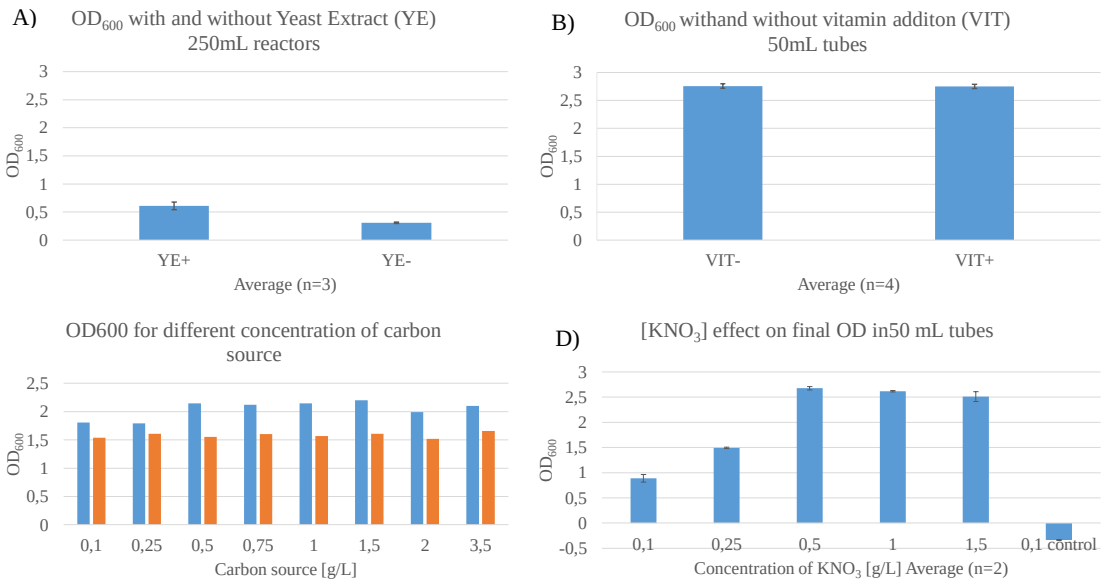

Figure 4.1: Results of the media determination experiments performed; A) shows the average OD_{600} for 250mL stirred flasks with and without yeast extract, B) shows the average OD_{600} for 50mL tubes in water bath with and without vitamin supplementation, C) shows the single point OD_{600} for glucose and glycerol of a range of concentrations grown in 50mL tubes in water bath, D) shows the average OD_{600} for a range of KNO_3 concentrations for growth in 50 mL tubes in a water bath (error bars in this figure represent SEM)

To further prepare for chemostat experiments an investigation as to whether the carbon source is the limiting substrate for the medium was conducted. The ideal outcome of such an experiment would return a linear increase of cells from lower carbon concentration to a higher as if it is the limiting substrate it would control the final biomass concentration after the carbon is consumed. With increasing concentration of the carbon source to a point where it is no longer limiting the curve would plateau as another component then would be limiting the biomass accumulation.In figure 4.1 C) the OD_{600} are shown for a range of concentrations of the two carbon sources of interest, glucose and glycerol. The chosen concentrations were in a range lower than the medium recipe concentration of 3.5 g/L. The results do not indicate an increase in OD_{600} over the range of concentrations which suggests that the carbon sources are not limiting the growth at any of the chosen concentrations. The set-up for this experiment was as previously described 50 mL stationary tubes which in itself constricted the cellular growth due to sedimentation. The results then seem more probable since important complex medium components such as amino acids may not be properly available for the cells. However the possibility of carbon not being the limiting factor also had to be accounted for. The further investigation of limiting substrate are shown in figure 4.1 D) where OD_{600} values for a range of con-

centrations of KNO_3 are depicted. Previous results (data not shown) at Chr. Hansen indicated that the concentration of KNO_3 could be increased as it had resulted in a biomass increase. For this reason concentrations above the original concentration were chosen. An increase in KNO_3 concentration, from 0.1 to 0.5 g/L, resulted in a 5-fold increase in OD_{600}. The experiments were set up in an identical fashion but only with glucose at the maximum previously tested concentration. These results could be viewed as a confirmation to the previous ones described in figure 4.1 C), since both indicate that the carbon did not seem to be the limiting substrate. Given these results the concentration of KNO_3 in the medium was increased 2.5-fold to keep it as a limiting substrate but to also increase the biomass. The reason not to increase the concentration further was based on the desire to stay within feasible concentrations for production and to avoid possible toxicity of too high a concentration of KNO_3.

In conclusion the medium was altered so to increase the KNO_3 concentration 2.5-fold to have it the possible limiting substrate for the rest of the project. It should be noted that these experiments overall yielded very low OD_{600} results and it could therefore be argued that they are not representable for *S. carnosus* growth. Further it should be stated that the low OD_{600} results prior to increasing the KNO_3 could be due to the concentration of nitrogen in the medium and that it could be starved for nitrate under semi-anaerobic conditions. Further, even though the OD_{600} are low the conditions of the 50 mL tubes are all equal resulting in that the results relative to one another should still be considered significant. The decision was hence taken to move forward with the results given and to interpret them as indications of possible changes that were made to the medium.

4.2 Cell physiology

This section will describe results from the fermentations performed to generate the different physiological states of *S. carnosus* cells studied in this project.

4.2.1 Batch fermentations

The aim was to study the effect of oxygen and carbon source choice independent of growth rate, which was the reason that a chemostat set-up was chosen. To ensue that washout would not occur as described in section 2.2 the $\mu_{\max}$ at each of the conditions was required to select the dilution rate. This meant that the growth rate of which the bacterium should be growing should be equal across all experiments. Hence a dilution rate was chosen that was below the lowest of the experimental values as theory described by equation 2.5.
In table 4.1 the experimentally determined growth rate under anaerobic conditions together with the previous results under aerobic conditions are displayed. There was no growth showing under anaerobic conditions on glycerol after 7 hours. This means that *S. carnosus* might have trouble growing under anaerobic conditions and have no metabolic mechanisms to cope with anoxic conditions with glycerol being the sole carbon source. However, it could also be possible that 7 hours was insufficient for *S. carnosus* under anaerobic conditions that might yield a prolonged lag-phase.

Table 4.1: For each sampling point; Residence times (RT), base usage over the last RT prior to sampling, the coefficient of determination (R^2) for regression of base usage and the dissolved oxygen averaged over the last RT.

	Glucose		Glycerol	
	Anaerobic	Aerobic	Anaerobic	Aerobic
μ_{max} [h^{-1}]	0,222	0,671	N.A	0,333

The experiments were performed in single fermentations anaerobically and the previous data given on growth rates for aerobic growth represented a duplicate run on glucose and a single run on glycerol. This could be considered a low amount of data to move forward with a continuous set-up. It was considered to perform further validation. The concern was not alarming though as growth under anoxic conditions was thought to yield lower growth and the dilution rate had to be picked below the lowest established growth rate. Further data description can be found in appendix A.4

4.2.2 Chemostat fermentations

This section will give insights to the results regarding the physiology of the cells for the steady state chemostat samples.

The chemostat experiments were set up using a dilution rate of 0.1 [h^{-1}] so as to be below the lowest experimentally determined $\mu_{\max}$ described in table 4.1 to avoid washout. Three different gaseous flows for sparging the reactor (0.5 L/min air, 0.2 L/min air, 0.2 L/min N_2) were used to generate the different physiiological states.

Depicted in table 4.2 are the reached residence times for each of the paramter combinations tested. As mentioned in section 2.2.2.1 the established consensus is five (minimum) residence times to assume steady state. The achieved residence times for this project many times fell short of that due to logistic requirements ranging from laboratory restrictions for available sampling times, to media feed exchange possibilities. Stating this it is still assumed that the residence times are sufficient for acquiring a steady state.

Table 4.2: Residence times (RT) for each of the set up experiments and their sampling points, Base (NaOH) used to maintain set-point pH averaged over the last RT, coefficient of determination (R^2) for regression of base used and DO for each of the sampling points. The fermentor ID is shown to indicate duplicates and the steady state settings as follows: 1SS; 0.5 L/min Air, 2SS; 0.2 L/min Air, 3SS 0.2 L/min N_2.

	Residence times	Base use over last RT		DO last RT [%]	Fermentor ID (Duplicate description with steady states)
		Regression [mg/min]	Regression R^2		
Glucose	4,08	40,0	1,00	9,32	FM19 1SS
	4,09	45,3	1,00	7,79	FM20 1SS
	4,64	35,9	1,00	10,2	FM19 2SS
	4,64	45,4	1,00	8,03	FM20 2SS
	6,73	1,63	0,60	0,98	FM19 3SS
	6,73	48,5	1,00	0,54	FM20 3SS
Glycerol	4,79	8,55	0,99	0,12	FM19 1SS
	4,81	15,7	1,00	17,1	FM20 1SS
	6,59	13,6	1,00	0,16	FM19 2SS
	6,59	10,6	1,00	4,00	FM20 2SS
	5,19	-6,04	0,65	0,17	FM19 3SS
	5,19	0,18	0,53	0,12	FM20 3SS

The aim of choosing different gasous flow levels, as explained in section 3.3.2, was to study the physiological state that the population is expressing at these different stages. A parameter to study then could be using dissolved oxygen levels for each of the steady states to study the effect of oxygen availability to the cells. This was not fully expressed in the data as DO levels, as previously mentioned, fluctuated due to experimental equipment that is highly sensitive at lower ranges of oxygen. When studying table 4.2 for DO values one can observe that the values for some of the sample points are not what they intuitively would show. For example looking at the DO levels for FM19 grown on glucose one can observe that the value when sparging 0.5 L/min is lower then that when sparging with 0.2 L/min. This could be explained either by the total cell count later depicted in table 4.3 but the more probable reason is the fluctuation of the equipment. As shown in appendix A.5 over the last residence time for FM20 on glycerol the DO is shown in table 4.2 at 33.9% but the standard deviation over the last residence time was determined to be $\approx$ 10%.

This project did not have availability to monitor the optical density on-line of the fermentors which meant that other parameter outputs had to be used to evaluate if a steady state could be assumed. The values were chosen over the last residence time (RT) to be able ensure that fluctuations in the on-line monitoring did not affect the results and presentation of the data significantly. In table 4.2 the data of the average base used (NaOH) is shown. This was one of the parameters used as the acid production of Staphylococcus can be correlated to the metabolic state of the cells [12]. Other values were also considered to evaluate the steady state, oxygen and carbon dioxide in the off-gas was for all assumed steady states seemingly stable

over the last residence time as is shown in appendix A.5.

At the different steady states samples were taken and flow cytometry analysis and at-line OD_{600} were performed to yield results on biomass in the reactor and can be seen in table 4.3. What can be noted in table 4.3 and as well for the base usage in table 4.2 is that there is a quite severe discrepancy between what should be duplicate run on each of the carbon sources (*e.g.* Glucose FM19 1SS and FM20 1SS have a 9-fold difference in active cells and 3-fold difference in OD_{600}) This is why the results are presented as separate results as they are not considered as comparable duplicates.

Table 4.3: Biomass determination at the different sampling points. The (*) next to the results indicate the anaerobic states where the assumption of a steady state could not be fully determined. The fermentor ID is shown to indicate duplicates and the steady state settings as follows: 1SS; 0.5 L/min Air, 2SS; 0.2 L/min Air, 3SS 0.2 L/min N_2.

	Flow Cytometry		OD_{600}	Fermentor ID (Duplicate description with steady states)
	Total/g	Active/g		
Glucose	7,08 10^8	6,52 10^8	28,6	FM19 1SS
	5,09 10^9	5,04 10^9	64,5	FM20 1SS
	6,22 10^8	6,07 10^8	25,5	FM19 2SS
	5,30 10^9	5,27 10^9	65,1	FM20 2SS
	4,42 10^8	2,58 10^8	5,17	FM19 3SS*
	4,36 10^9	4,19 10^9	38,1	FM20 3SS*
Glycerol	1,25 10^10	1,25 10^10	84,3	FM19 1SS
	4,92 10^8	4,82 10^8	33,9	FM20 1SS
	1,51 10^10	1,48 10^10	83,7	FM19 2SS
	5,73 10^9	5,70 10^9	45,7	FM20 2SS
	1,80 10^9	1,76 10^9	12,7	FM19 3SS*
	1,88 10^9	1,86 10^9	13,1	FM20 3SS*

Looking at the data in table 4.3 several interesting interpretations that can be made, especially when focusing on the anaerobic sample points. These have a lower OD_{600} value for all sample points when comparing to their previous oxygenated sample points. The data shown here should be viewed as a single moment in what otherwise can be described as a dynamic process. Because there is a lower OD_{600} does not mean that cells are washing out. This would require a constant decrease of the biomass in the fermentor and such data is not available. FM19 on glucose under anaerobic conditions have a lowered amount of viable cells. This could be an indication that the population is not coping well with the parameter change (from aerobic to anaerobic fermentation) but no assumptions on washout can be made. The fact that the cells here have a lower viability could be caused by inconsistent media feed. The further interpreting of the results should however be based on that there is a lowering of the biomass under anaerobic conditions in comparison to aerobic fermentation. According to table 4.2 the base use as a parameter for steady

state assumption does not look particularly fitting but the graphs in appendix A.5 show that the base use is halted and kept stable without change in pH. There is howere a questioning of the assumption of steady state for these and the results on both glycerol and glucose under anaerobic conditions will hence be marked with an asterisk (*) in the following results indicating issues with assuming steady state.

The difference in biomass for the different steady states may have been caused by several parameters, one of which likely being the medium preparation of the feed. The fact that the medium easily sediment, as mentioned in section 4.1, could have caused a difference in between different feeding bottles with suspect to the sediment and its constituents such as amino acids. There was for example a noticeable difference in the sediment size between two media bottles during the last chemostat fermentation.

Table 4.4: Quantitative HPLC analysis for each steady state, result are presented in g/L fermentate. The (*) next to the results indicate the anaerobic states where the assumption of a steady state could not be fully determined. The fermentor ID is shown to indicate duplicates and the steady state settings as follows: 1SS; 0.5 L/min Air, 2SS; 0.2 L/min Air, 3SS 0.2 L/min N_2.

	Glucose/ Glycerol	Acetic acid	Lactic acid	NO3	NO2	Fermentor ID (Duplicate description with steady states)
	[g/L]	[g/L]	[g/L]	[g/L]	[g/L]	
Glucose	2,16	8,85	15,1	0,04	0,11	FM19 1SS
	0,00	11,7	11,4	0,05	0,09	FM20 1SS
	3,43	8,45	12,8	0,04	0,20	FM19 2SS
	0,00	11,3	11,8	0,03	0,09	FM20 2SS
	21,1	0,43	6,23	1,22	0,35	FM19 3SS*
	0,00	1,54	29,3	0,13	0,09	FM20 3SS*
	25,7	0,20	2,08	2,29	0,10	Media average (n=6)
Glycerol	15,9	8,08	1,23	0,11	0,03	FM19 1SS
	19,7	8,13	1,16	0,11	1,51	FM20 1SS
	18,3	8,98	1,79	0,11	0,03	FM19 2SS
	18,4	7,47	2,51	0,11	0,03	FM20 2SS
	32,8	2,85	3,78	0,11	0,03	FM19 3SS*
	32,8	2,78	3,85	0,11	0,03	FM20 3SS*
	36,1	0,20	2,09	2,21	0,10	Media average (n=6)

Another factor that may have influenced sedimentation where that the medium was first UHT treated and then autoclaved for 60 min at 121°C (described in section 3.4). The extensive heat treatment of the medium may have caused several components to denature and/or precipitate. This should however be equal for each of the feed bottles since all were treated identically but as the medium looked different (uneven sedimentation) the effects of the medium treatment might have enhanced the divergence in composition between feed bottles.
The samples taken at the different steady states were analyzed quantitatively via HPLC for acids and carbohydrates which can be seen in table 4.4. As can be observed the total acid production corresponds with data shown in table 4.2 in regards to the amount of base used as mentioned before. The amount of carbon source together with

the concentration of NO_3 left at the steady states further strengthens the suggested hypothesis of KNO_3 to be the limiting substrate. This observation is however not the case for FM20 on Glucose which for steady states one & two in comparison to the other fermentor, FM19, on glucose. An interesting view on the acid production on the fermentations on glucose is that the anaerobic fermentation did not, as theory on *S. aureus* would suggest, yield an increase in acetic acid production and lowering of lactic acid production but the opposite. This looks to be the similar for the case of glycerol. This could however be supported by the theory that *S. carnosus* are working similarly and together with lactic acid bacteria in the application environment as described in section 1.
A further note on the concentrations revealed by the quantitative HPLC is that the glucose concentration in each of the media feeds seems to have been lower than the planned concentration. As mentioned in section 3.4 the preparation and addition of the carbon source was performed identically for all the media feeds but the concentration of glucose is according to the results at a lower concentration than what would be expected where a more probable would be a higher concentration as water may evaporate during separate autoclavation of the carbon source. This could be caused by a micro precipitation of the glucose which was halted in the sterile filtering process of adding the carbon source to the media feed.
For further analysis of the data shown in table 4.4 the normalized values for rates of conversion based on quantitative HPLC results are shown in table 4.5. The negative values are referring to if the substance is consumed as the positive values are for production of each substance. The table is displaying a comparison between aerobic and anaerobic steady states and is showcasing the relative increase/decrease when switching to anaerobic fermentation, hence all aerobic points in the table are shown as 100%. The normalization for biomass have been made using the OD_{600} values.

Table 4.5: Relative specific conversion rates for the data collected from the quantitative HPLC displaying aerobic and anaerobic conditions. The values are normalized against OD_{600} and 100% represents the expression for aerobic steady states. Negative values indicate that the conversion have changed direction when changed to anaerobic conditions. The fermentor ID is shown to indicate duplicates and the steady state settings corresponds as 1SS - 0.5 L/min Air and 3SS - 0.2 L/min N_2.

			Glucose/Glycerol	Acetic acid	Lactic acid	NO_3	NO_2
Glucose	FM19	1SS	-100%	100%	100%	-100%	100%
		3SS	-166%	27%	182%	-229%	1788%
	FM20	1SS	-100%	100%	100%	-100%	N.A
		3SS	-168%	22%	500%	-156%	N.A
Glycerol	FM19	1SS	-100%	100%	-100%	-100%	N.A
		3SS	-122%	233%	1279%	-668%	N.A
	FM20	1SS	-100%	100%	-100%	-100%	100%
		3SS	-24%	88%	487%	-257%	0%

What can be seen in the table for growth on glucose is that there is for glucose, lactic acid, acetic acid and nitrate a consistency of switching to anaerobic conditions in

regards to these substances. For growth on glycerol there are significant differences where no clear trend can be seen for either the carbon source or acetic acid. Nitrate is showing an increased consumption and there is a trend suggesting a switch to production instead of consumption for lactic acid. As seen in table 4.4 nitrite levels were in many cases too low to measure. For data analysis of please see appendix A.7

4.2.2.1 Chemostat set-up at Chr. Hansen

As described in section 3.4 the set-up of the media preparation evolved to fit the in-house logistical requirements. This also was the case for the reactor set-up. In this section a description of the limitations and issues when running chemostat in corner-lab at Chr. Hansen will be presented. Looking at figure 3.1 the external pumps used were, as previously mentioned, controlled by the scale before the pump (in the flow direction). Firstly this meant that there was a high sensitivity of the system to outside actions. As an example there was a maintenance work performed during a run on tubing behind the set-up, this meant moving the bench where the set-up was placed which resulted in a disturbance for the scale subsequently stopping the pumping and disruption of the time towards assuming a steady state. Further, as the pump was PID controlled by the scale the replacement of feed reservoir meant disruption of flow. The choice of using a PID controlled external scale was due to that there was an already functioning programming for such a device. There was consideration of instead using a fixed RPM of the pump corresponding to a specific flow rate but the experiments to determine flow rates at a range of RPMs was not performed. The working volume of 0.75 L was chosen as the fermentor had a total volume of 1 L.
There was at each assumed steady state sampling point an issue with withdrawing a large amount of the fermentation broth. The total volume withdrawn could be considered significant, this to the affect that the reactor might be needing time after each sampling point to recuperate in biomass present after sampling an refilling of media from reservoir. This could especially be important for the switch from aerobic to anaerobic conditions as growth rate experiments showed difficulties to yield biomass under anaerobic conditions growing on glycerol. A larger working volume using for example a 2 L fermentor might be needed if the sampling size at each assumed steady state would be remained as high as it was during this project.

To study the mechanism present for nitrogen in *S. carnosus* this study aimed at using the complex production medium to yield results that could be comparable to the production medium. However the production medium is far from optimal when studying the mechanism. This as it is easily causing sediments due to the complex component of yeast extract. Using defined media with amounts of needed or excess amounts of amino acids could better study where the nitrogen would end up from addition of nitrate or from amino acids used in the medium. Further, using a defined media could have been made media preparation easier. As depicted in figure 3.2 the UHT treated medium went through extensive autoclavation. This could have caused crystallization of nutrients in the medium and as it could be considered a randomized process discrepancies between batches of media feed could have occurred. By instead using a defined media the UHT treated medium could

have been sterile-filtered into flasks working as reservoirs, something not possible for the complex production medium that contains particles clogging the filter. Defined media could thereby be used in larger volume to not risk running out of media in reservoir prior to an assumed steady state.

In summary the setting up of chemostat using production medium is not trivial as unknown effects may occur, particularly as problems never seen on defined media can occur on production medium and are therefore hard to predict. It is therefore necessary that enough time is planned to use the chosen set-up and optimize the procedure of running chemostat with production medium at Chr. Hansen.

4.3 Nitrate Reductase

This section will describe the results of the nitrate reductase, the enzyme of interest in this physiology study. A description of the analysis performed can be found in section 3.5.6 and 3.5.7. For all the following analysis the samples from the chemostat fermentation from section 3.3.2 are considered and a disclaimer on units is issued stating that the results are to be viewed as relative measurements to each other if not otherwise specified.

4.3.1 Enzyme activity

As descried in section 3.5.6 the activity, here denoted as the conversion rate of nitrate to nitrite, of nitrate reductase was analysed for each of the sampling points from the chemostat experiments. The results of absorbance using a Griess reagent to quantitatively determine generation of NO_2^- over time. It should be noted that all the samples over time were taken using a constructed anaerobic environment for the cells and thereby the enzyme to work in. Shown in figure 4.2 are the conversion rates of nitrate reductase denoted per active cell or per volume fermentate.

Firstly the when viewing the results in figure 4.2 A) & B) it can be seen that there again is a large discrepancy between what should be duplicates as mentioned previously. If looking at figure 4.2 A) on glucose there seems to be decreasing trend of conversion rates when switching from aerobic to anaerobic conditions. A reference to this, for fermentor 19, can be found in table 4.4 where nitrate and nitrite concentrations are increasing over the SS points (0.04→0.04→1.22 and 0.11→0.20→0.35 [g/L] respectively). This is in line with the theory of a higher nitrate concentration represses the nitrite reductase from nitrite to be further reduced to ammonia. However it somewhat contradicts the theory of conversion rates would increase at anoxic conditions (for normalized HPLC data against cell count please see appendix A.6 table A.2). The trend of descending conversion rate at the anaerobic sampling point seems to also to be present in FM20 on glucose. On glycerol the interpretations are more difficult to distinguish any trends for the conversion rates.

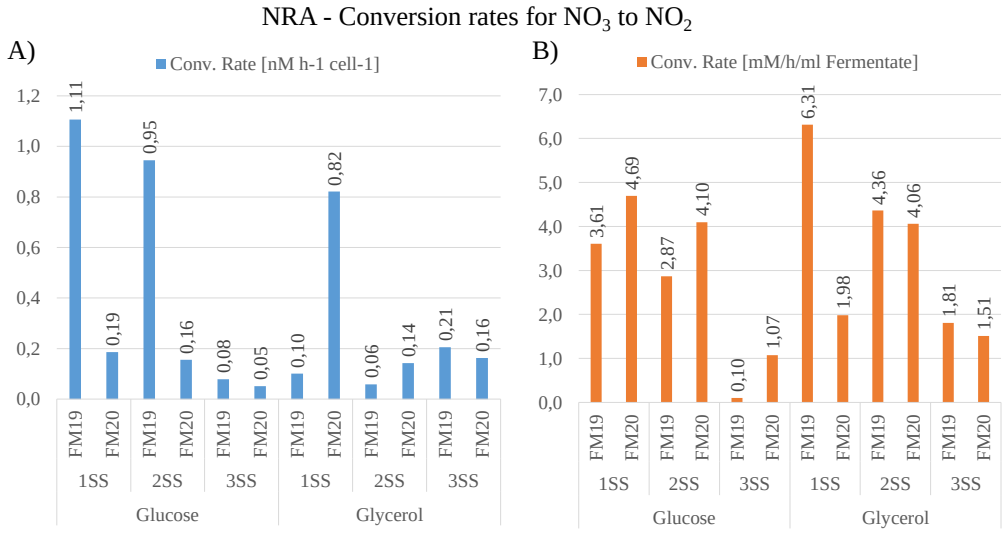

Figure 4.2: Conversion rates of nitrate. A) shows the conversion rates calculated on the basis of results per active cell from table 4.3, B) shows conversion rates on the basis of volume fermentate used for analysis. The fermentor ID is shown to indicate duplicates and the steady state settings as follows: 1SS; 0.5 L/min Air, 2SS; 0.2 L/min Air, 3SS 0.2 L/min N_2. For data displaying coefficient of determination, standard deviation and standard error please see appendix A.8

A remark on the results presented in table 4.2 is that the cellular determination of the sought after cell concentration was based on flow cytometry values and the chosen parameter was the active cell count [active cells/g]. What is not considered then is that the enzyme of interest may still maintain its conversion function even though cells are not viable, *i.e.* cells having an enzymatic activity might not be accounted for when the data is performed. However when adjusting for this the difference does not impact the final outcome considerably but should still be considered when studying the figure. Because of this the results are presented based on the activity per mL fermentate in figure 4.2 B). The two different unit alternatives is presented as results from the flow cytometry could have been non-representative, a result found when performing up-concentration of fermentate. The pellet size for this procedure did not correspond well with the calculations based on flow (data not shown) and suspicions of variations in cell count was raised. The 'per volume of fermentate' are shown to give an impression of the activity in the set of a production where the volumetric activity might be of interest to study. Studying instead 4.2 B), the decrease in conversion is again present when switching to anaerobic fermentation but here seemingly for all samples.

As a complement for the data shown in in figure 4.2 table 4.5 can be used. Looking at the nitrate rates of consumption normalized to biomass and relative to aerobic conditions there is a trend for cells grown on glucose and glycerol both. The trend suggests that there is a relative increase in the consumption of nitrate thus contradicting the NRA enzymatic analysis.

4.3.2 Gene expression of narG and nirB

The quantitative determination of mRNA expressed for two of the genes responsible for nitrate and nitrite reductase relative to four 'housekeeping' genes are shown in figure 4.3. As the results are relative measurements the unit of quantification does not include the biomass as offsets during the methodology of the analysis.

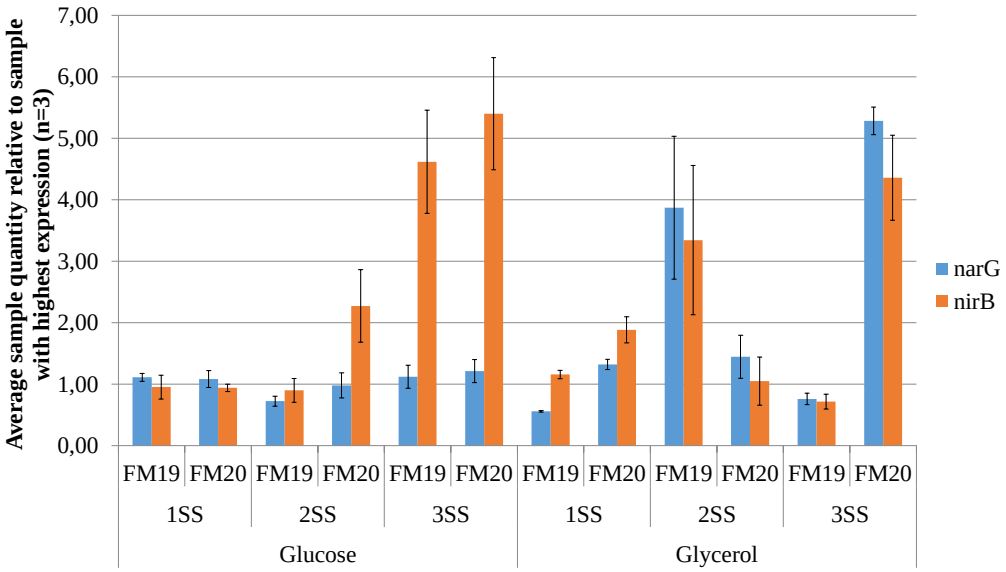

Figure 4.3: qPCT CT values normalized with four reference genes for the genes narG and nirB coupled generating the enzymes nitrate reducrase and nitrite reductase respectively. The fermentor ID is shown to indicate duplicates and the steady state settings as follows: 1SS; 0.5 L/min Air, 2SS; 0.2 L/min Air, 3SS 0.2 L/min N_2. Data shown here is based on triplicate RNA extractions from identical samples, triplicate qPCR samples using an external standard curve for each qPCR plate to generate quantities from CT (threshold cycle)-values using an arbitrarily chosen threshold of 0.1 for all plates (error bars represent SEM).

As can be seen in the figure the error bars presented are high and overlapping for several of the data points. The error bars are presented as the standard error of mean (SEM). This because the average displayed is a sample mean of calculated quantities. SEM describes how far the sample mean is likely to deviate from the population mean. The population here meaning the 12 different sets of samples analyzed to sum up to the three quantities of which the averaged value is displayed in the figure.
While investigating the results on glucose there cannot be any significant determination to say if there is an increase or decrease in the narG expression except perhaps for FM19 where a decrease when transfer to anaerobic phase is shown. This finding is contradicting the theory of nitrate reduction being a key mechanism for coping with anoxic conditions to recycle NADH as mentioned in section 2.1. nirB does not have any significant change over switching from aerobic to anaerobic but only a difference in FM20 between 0.5 and 0.2 L/min air. The theory on nitrite reduction being hindered by nitrate could be cemented here as nitrate is almost depleted in these

samples from HPLC analysis. Looking at fermentation on glycerol and particularly over the change from aerobic to anaerobic conditions show an increase in relative expression of narG. narG increase, as previously mentioned, could be synonym with the need to use nitrate as an 'electron dump' to be able to maintain redox balance for reducing agents such as NADH. The increase of narG when grown on glycerol, in contrast to glucose, is an interesting finding. This because at the time this project was conducted the production of *S. carnosus* was using glycerol as the carbon source with investigations if higher activity could be achieved using other carbon sources. The data in figure 4.3 indicates a higher expression of narG under anaerobic conditions when grown on glycerol which might suggest that a shift to glucose might not be desirable. However the results are not in line with the ones presented in figure 4.2 where no consistent trend of increasing activity difference was shown between the carbon sources but an indication of decrease was shown. Further, nirB also seems to increase over the 'anoxic switch' for results on glycerol. This could be a result of the growth requirements as mentioned previously in section 4.2. The levels of nitrogen could be the substrate limiting *S. carnosus* in these experiments. Considering this the higher levels of nirB under anoxic conditions might be in response to anoxic conditions maintaining stress on the cell population to have to use the assimilatory pathway to be able to satisfactory utilizing the nitrogen available.

5

Conclusion & Future Outlook

Growth of *S. carnosus* was shown to be limited by concentrations of nitrogen, more specifically KNO_3. An increase of nitrate is therefore suggested as an alteration to the production medium of *S. carnosus*.In regards to the carbon source used in the medium this project found that *S. carnosus* might have difficulties utilizing glycerol under anaerobic conditions. If not completely at least with a downgraded efficiency of metabolite utilization.

The experiments performed yielded results with issues of consistency of biomass accumulation for duplicate runs. Further issues stated are the number of duplicates for some experiments can be argued to be too low. The reason to not perform further repetitions of chemostat runs or a verification of growth rates for this project was the constraint of time that was dedicated to this particular project. Further, we could not distinguish any significant differences in the nitrate reductase enzyme activity between the two carbon sources of choice. Results from expression levels for the enzymes of interest, nitrate and nitrite reductase did however show a difference between the carbon sources. There was an increase of narG and nirB on glycerol when switching from oxic to anoxic conditions. The results could be viewed as a motivation to further study the effects on glycerol as the indication found in this project is that glycerol yielded a higher expression of both the enzymes responsible for the nitrogen assimilatory pathway. *S. carnosus* was found to work differently from *S. aureus* for anaerobic fermentation regarding acid production. In *S. aureus* anaerobic metabolism shifts to acetic acid production instead of lactic acid. *S. carnosus* instead shifted towards greater lactic acid production under anaerobic metabolism of on both glucose and glycerol.

Further experiments and validation of data is vital for the future implementation of results yielded from this project. This may include a validation of findings of KNO_3 increase to the medium to gain higher biomass yields as well as determining upper limits of toxicity not the least in the application. The results of qPCR showed a higher expression level on glycerol for anoxia than aerobic conditioned cells and the activity somewhat contradicted this. Hypothetically, the availability of enzyme co-factors my limit the activity of the enzymes produced by *S. carnosus*. An interesting further study would be to investigate the enzymatic activity of nitrate reductase when adding such enzyme co-factors such as perhaps ferredoxins [30] or Molybdenum [2]. Further understanding of the metabolic pathway for both glucose and glycerol in *S. carnosus* is needed to decide on what carbon source is preferable to use in the production medium. This as there seem to be a difference between for example

the acid production in comparison to *S. aureus*. Basic metabolic networks and flux analysis for different physiological states are needed in the future.

One of the major take home message of this project is that the metabolic fluxes through the different pathways *S. carnosus* could possibly display, in regards to nitrate, could not be determined. Whether it acts as electron acceptor under anaerobic conditions or if it can be used as a way to assimilate nitrogen. The inconclusiveness stems from that no analysis of further reduced nitrite was performed. This could have been known by testing for example ammonia levels quantitatively to see how it corresponds to the increase of nitrite. Simply it could be argued that if ammonia was accumulated under anaerobic conditions it could be a higher likelihood of nitrate reduction being used as an electron dump and if no or low amounts of ammonia were to be found under anoxia the nitrate is most probably incorporated in the biomass as for example amino acids as shown in figure 2.1. Another way of studying the nitrogen is to use labeled nitrogen and study, through *e.g.* NMR analysis, in what molecules the nitrogen is ending up. The yeast extract is a complex medium component and therefore contains unknown amounts and species of nutrients. There could be a need to instead use a defined medium to evaluate the assimilatory ways for nitrogen in *S. carnosus*.

A question that could be raised for the application purposes is what effects increasing nitrate reductase activity, both in the production of *S. carnosus* as well as their performance in the application, would have. The selling point for the strain in terms of beating its competitors can come in many different units of measurements. Using viable or colony forming units of cells is often used as a standard for the customer buying cells. However it could be desirable to be able to point to specific advantages to the cellular mechanics of the cell, *e.g.* enzymatic activity. However, this could further be argued to not be coupled directly to the performance and ability to form red color in the application environment. The application environment in itself is often very varying in concentrations of nutrients but there is still a need to develop a standardized application assay to yield a consistent results to be able to argue the organisms ability to yield the desired red color. Chr. Hansen does have such an application test, but its requirements for downstream processed *S. carnosus* biomass are very high. A need for a high throughput assay for lower biomass should therefore be explored to be able to determine performance both for research and customer purposes.

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A

Appendix

Here follows the appendices connected to this project for further understanding and explanation

A.1

In this appendix detailed information regarding the procedures of Nitrate Reductase Activity, NRA, is described. Presented is the still in progress SOP from Chr. Hansen developed by the *Enzymes* group that was used for performing the NRA assay. In this project section 9 of the SOP was used.

Due to confidential content, this appendix has been removed from the public version of the report.

A.2

In this appendix unofficial SOPs and infomation sheets from Chr. Hansen are presented, a combination of which where used to perform qPCR in this project. Detailed information regarding materials and proceedings of RNA extraction and RT-qPCR is described.

Due to confidential content, this appendix has been removed from the public version of the report.

A.3

In this appendix the data for media determination is desplayed.

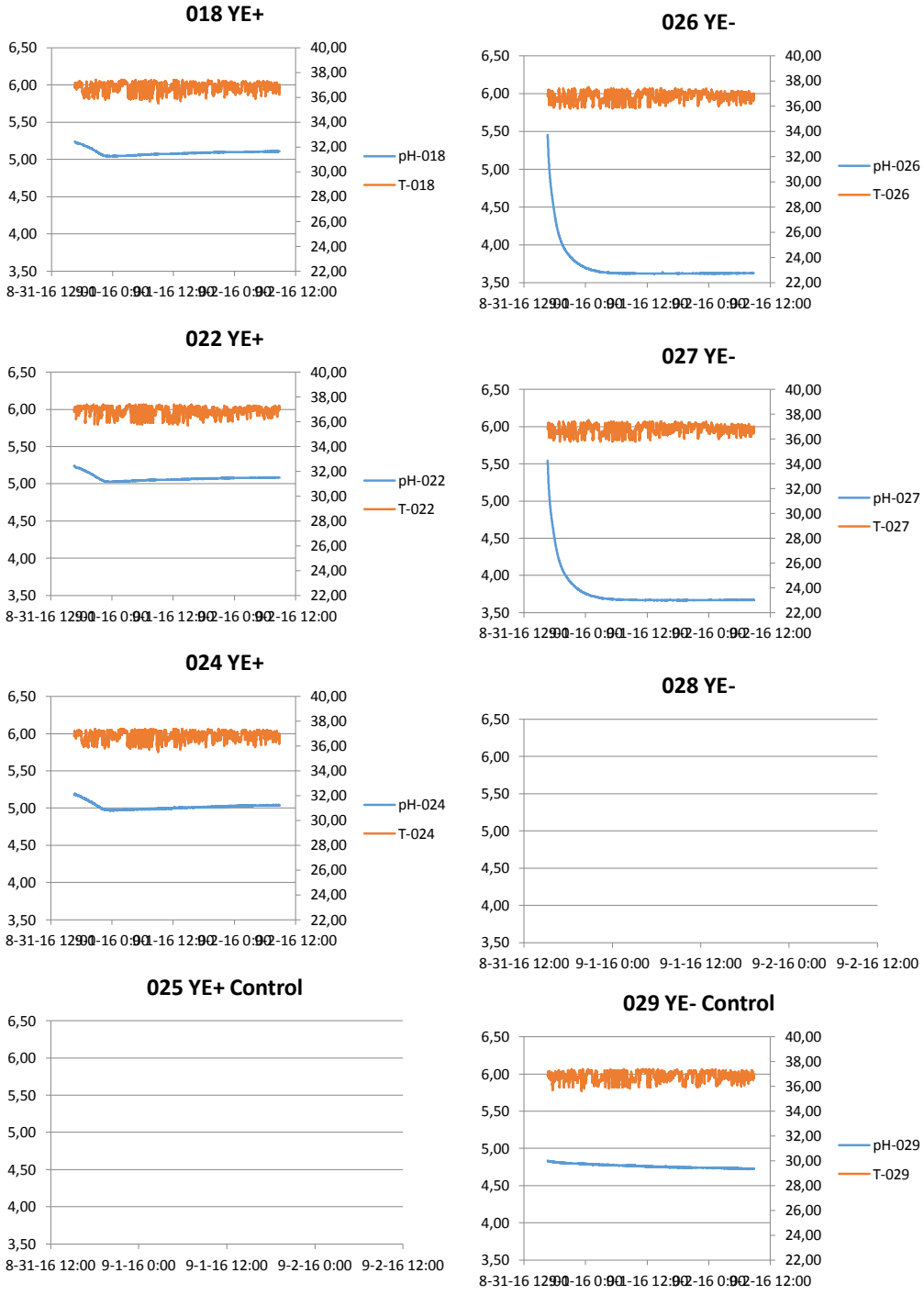


Figure A.1: Graphical representation of the set up experiments displaying pH and temperature over time.

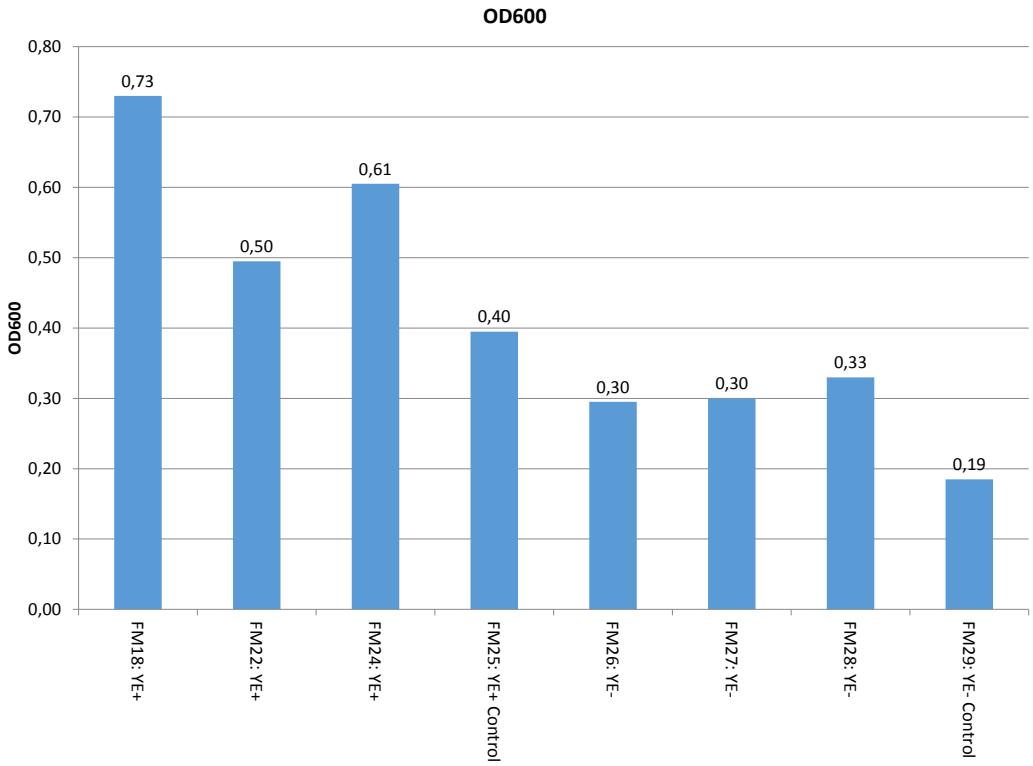


Figure A.2: Graphical representation of gathered OD_{600} values for set up experiments to evaluate nitrogen assimilation by removing yeast extract.

A.4

In this appendix the graphical data for maximum growth rate determination under anaerobic conditions is described.

Table A.1: OD_{600} values gather experimentally through batch fermentations.

	Anaerobic μ OD_{600} **values - Waange, M. Results**			**Aerobic μ** OD_{600} **values - Baroi, G.N. Results**			
	time [h]	FM19 Glucose	FM20 Glycerol	Time [h]	FM11 Glucose	FM12 Glucose	FM13 Gycerol
Legend	0,00	3,85	4,67	0,00	1,39	1,54	0,99
Included	3,50	3,42	0,83	4,00	2,23	2,69	0,26
values	4,00	4,26	0,71	5,00	7,31	8,43	1,21
for	4,42	5,06	1,23	6,00	18,77	21,79	3,29
calculations	5,00	5,78	1,33	7,00	34,41	34,89	15,49
Omitted	5,50	6,00	1,01	8,00	49,17	38,61	17,45
values	6,00	6,54	0,89	9,00	60,61	45,81	32,73
for	6,50	7,28	0,69	10,00	44,21	44,21	38,13
calculations	7,00	7,96	1,25	11,00	42,45	69,33	57,41
R^2>0.9	μ = d(ln x)/dt) [h^{-1}]	0,22	0,03	μ = d(ln x)/dt) [h^{-1}]	0,63	0,71	0,33
R^2<0.9	R^2 of regression	0,95	0,02	R^2 of regression	0,96	0,96	0,92

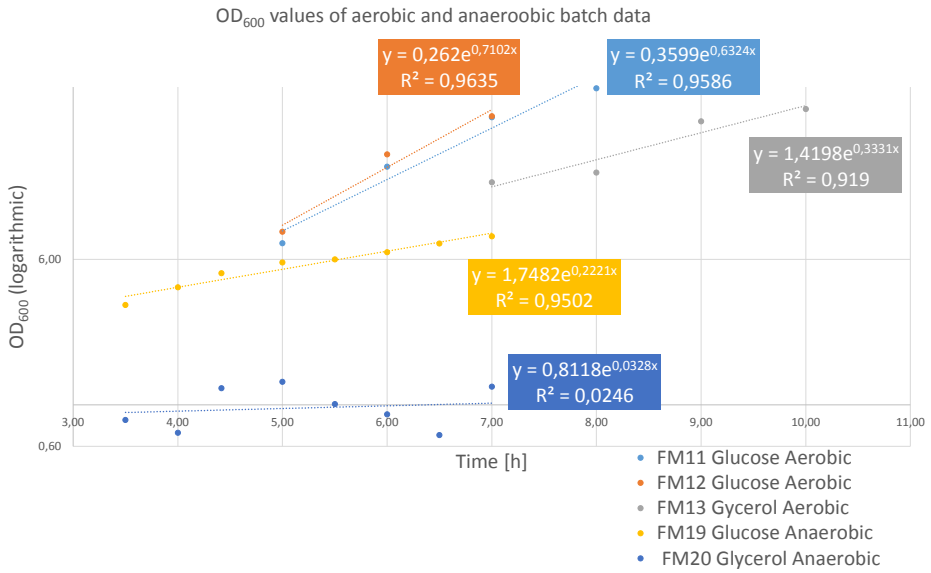
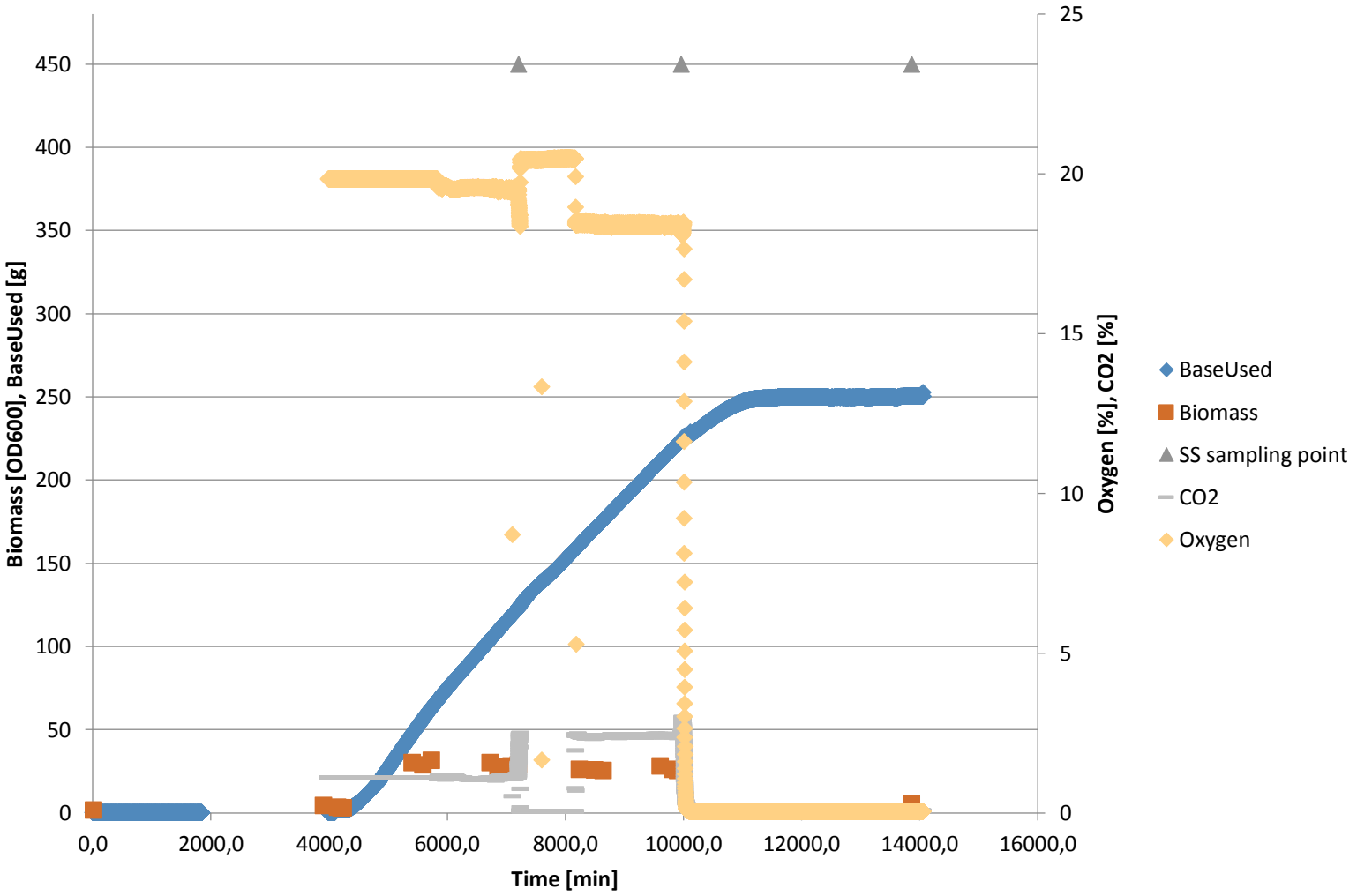


Figure A.3: Graphical representation of gathered OD_{600} values used to calculate μ_{max} for MIII

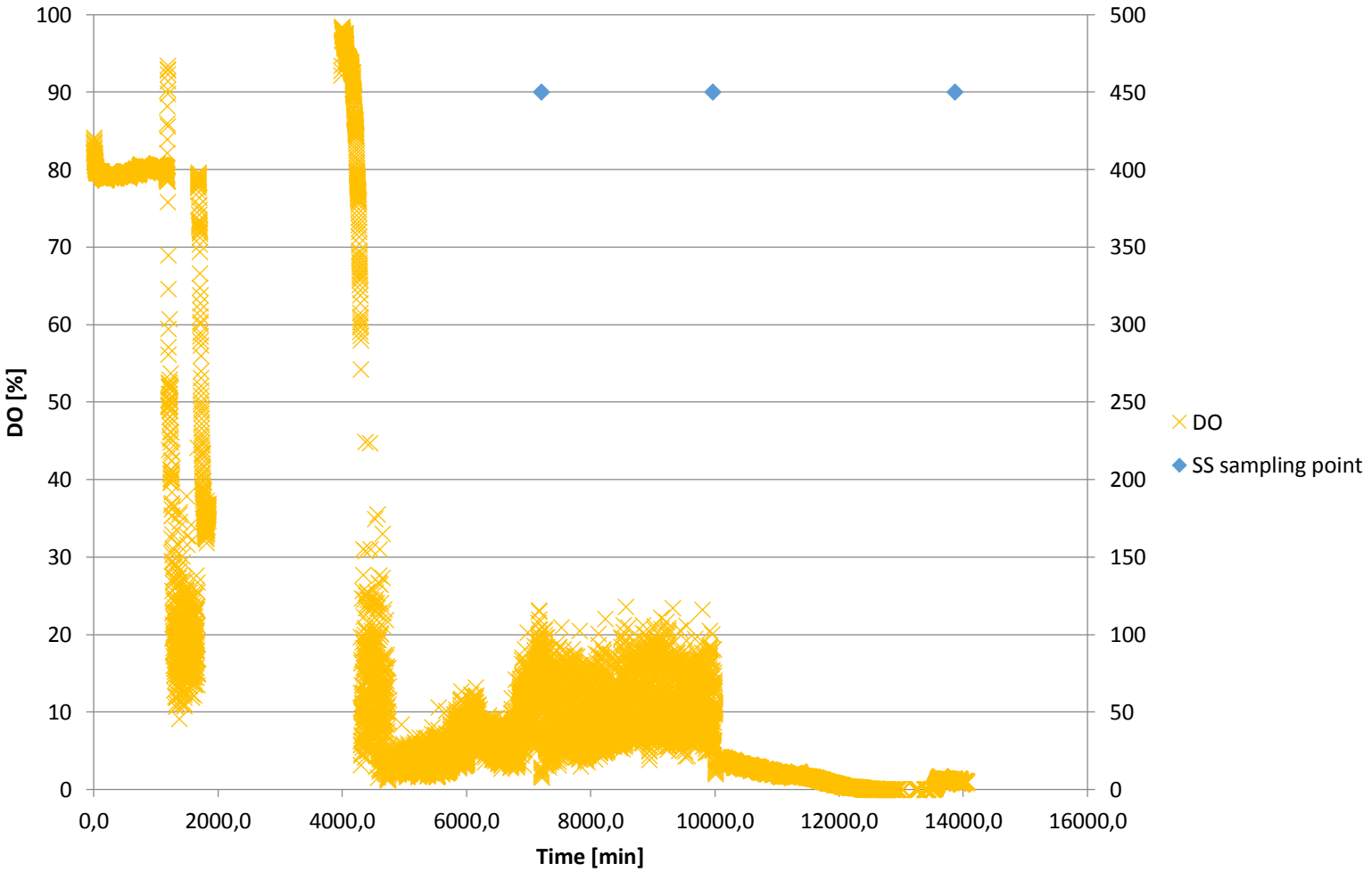
A.5

In this appendix the physiology state of of the chemostat fermentations will be presented in graphs. The graphs displayed below are the raw data presented graphically. Because of this some gaps or missing data points are occurring. These figures are used as reference for some of the interpretation discussed in 4.2

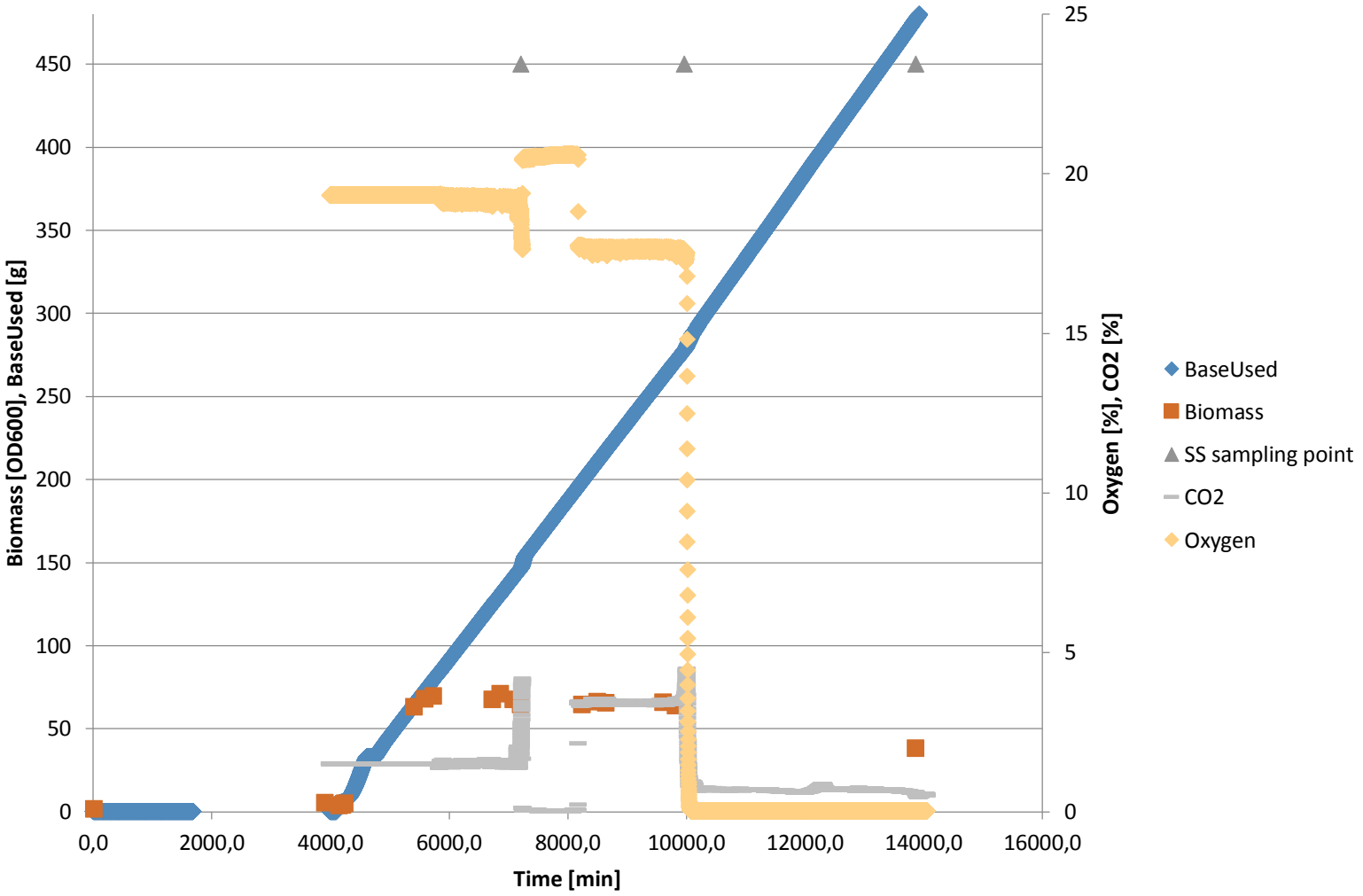
FM19 Glucose



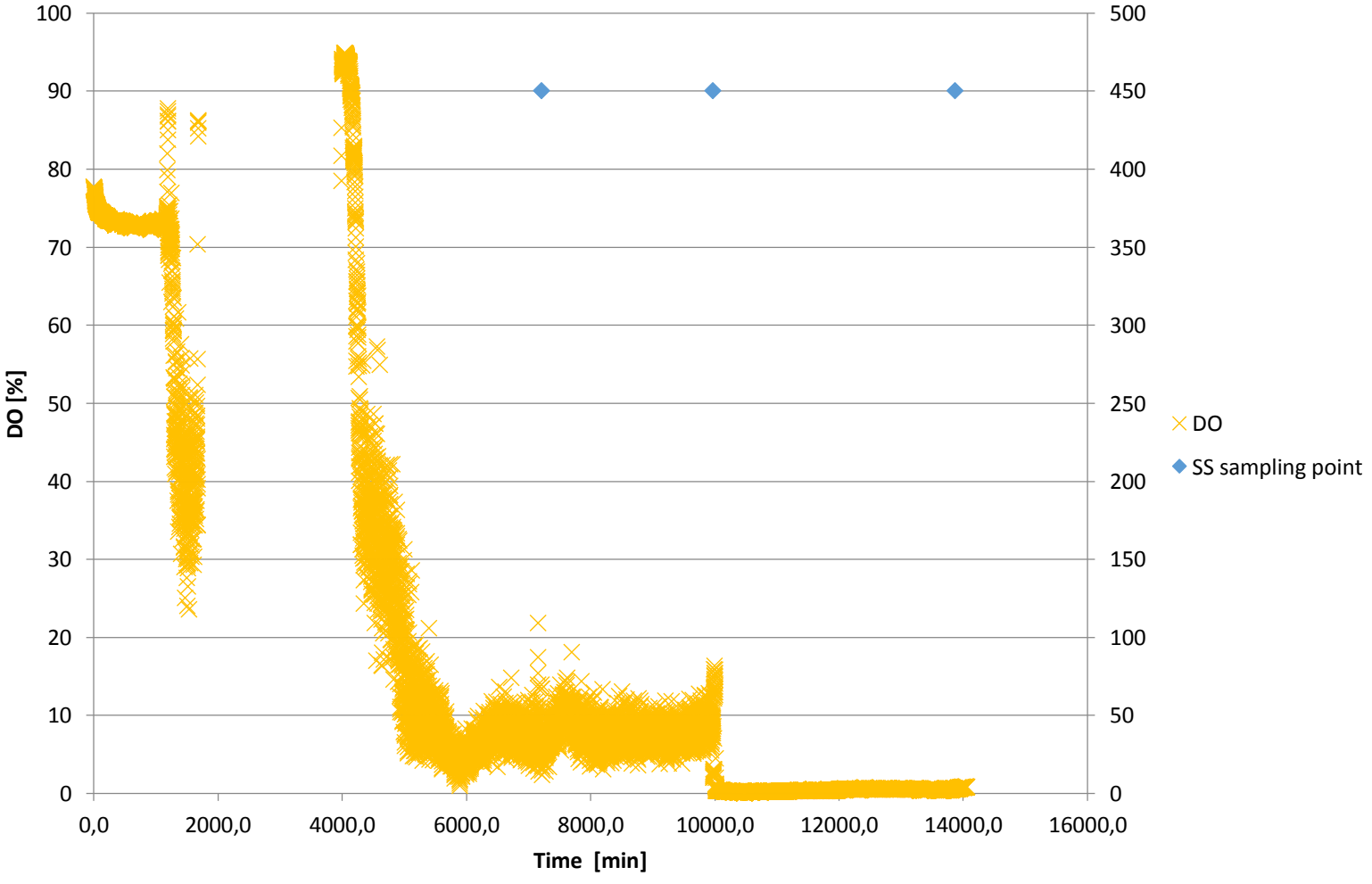
FM19 Glucose

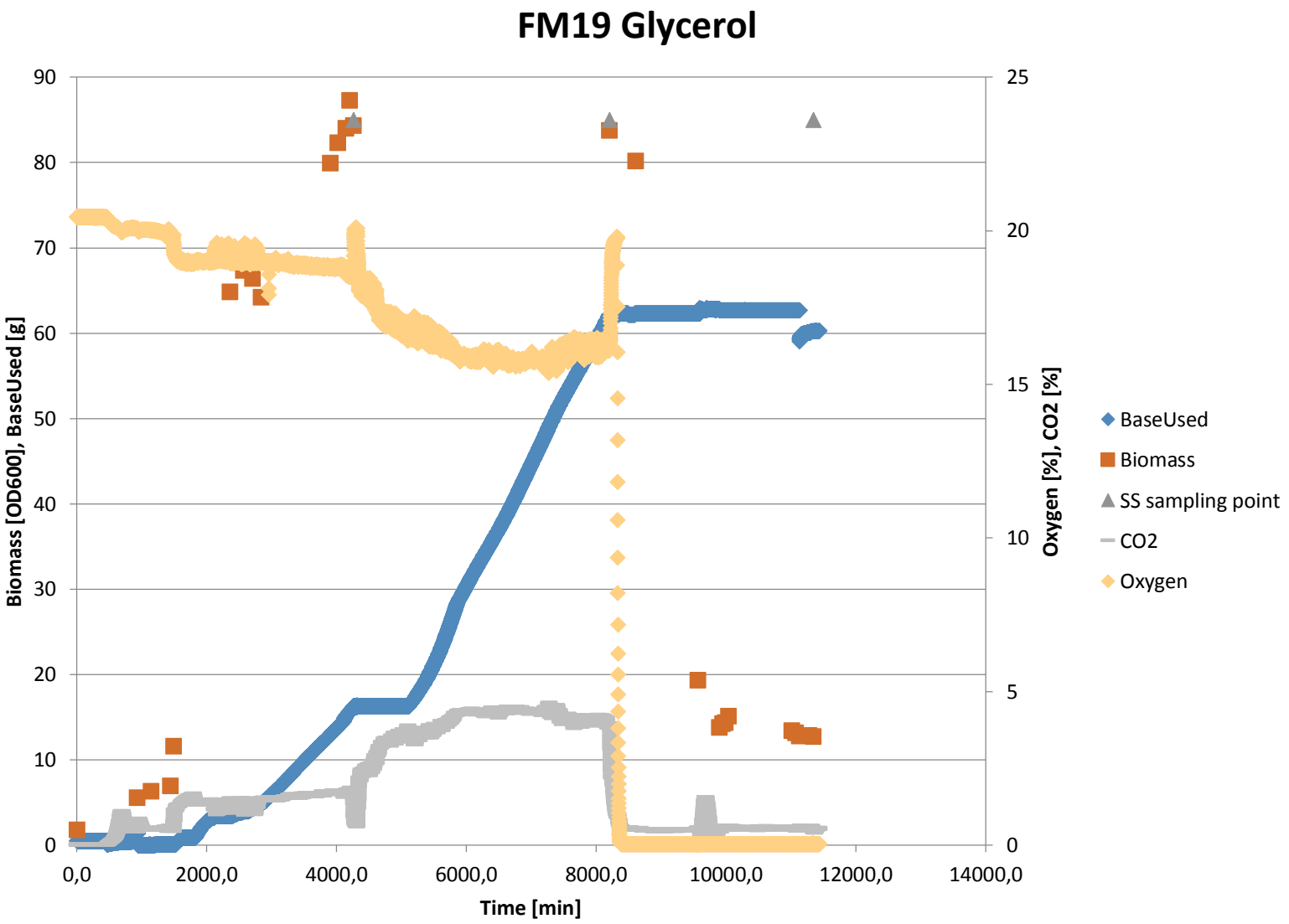


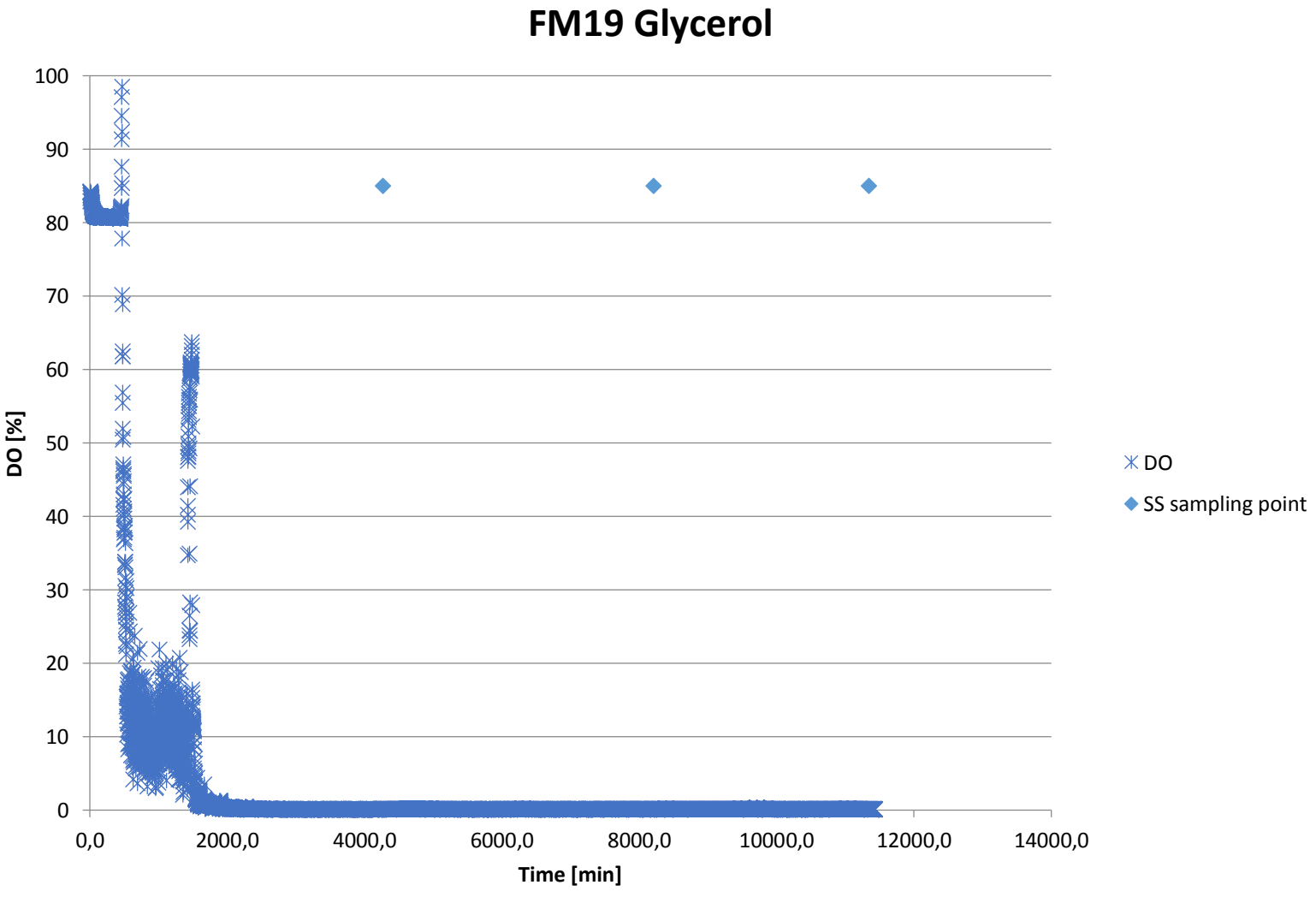
FM20 Glucose

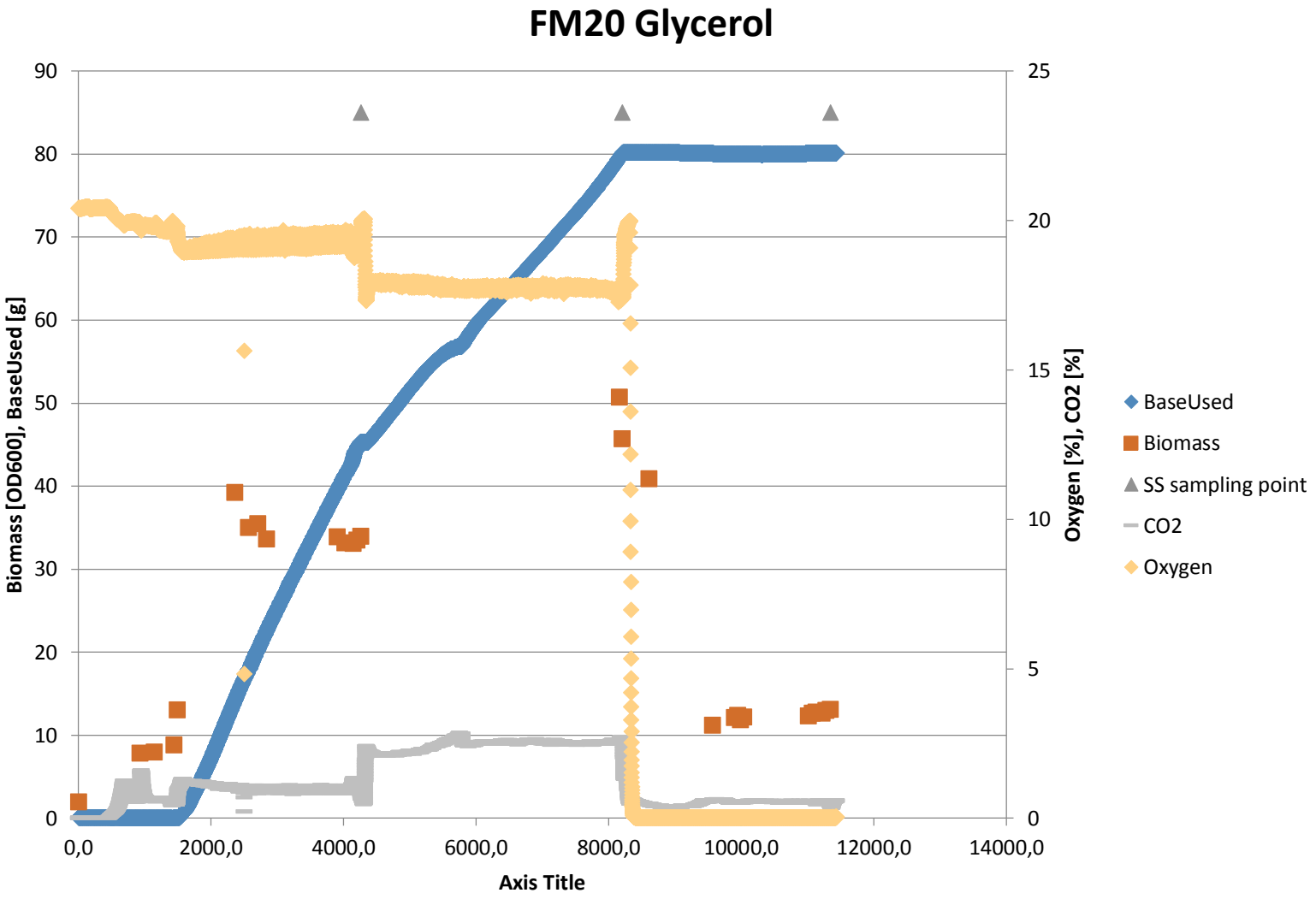


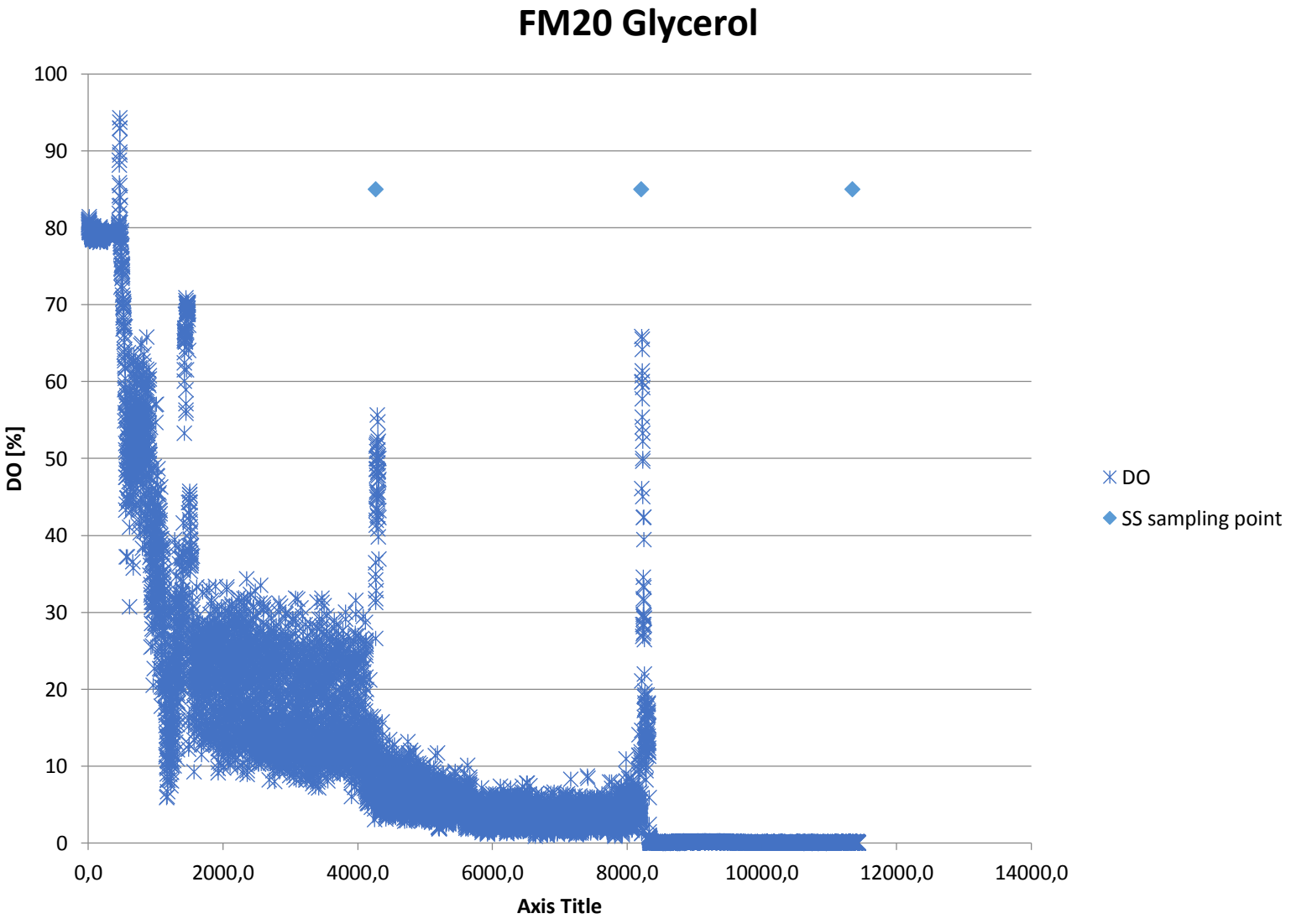
FM20 Glucose




FM19 Glycerol
Biomass [OD600], BaseUsed [g]
Oxygen [%], CO2 [%]
Time [min]
0,0
2000,0
4000,0
6000,0
8000,0
10000,0
12000,0
14000,0
BaseUsed
Biomass
SS sampling point
CO2
Oxygen


FM19 Glycerol
DO [%]
Time [min]
0,0
2000,0
4000,0
6000,0
8000,0
10000,0
12000,0
14000,0
DO
SS sampling point


FM20 Glycerol
Biomass [OD600], BaseUsed [g]
Oxygen [%], CO2 [%]
Axis Title
BaseUsed
Biomass
SS sampling point
CO2
Oxygen


FM20 Glycerol
DO [%]
Axis Title
DO
SS sampling point

A.6

In this appendix the an extension of the data gathered from the quantitative HPLC is presented.

Table A.2: HPLC data normalized with the total cell count of 1E+09 cells to accomodate arguments of normalization araginst the total biomass.

		HPLC normalized against total cell count of 1E+09 cells				
		Glucose/Glycerol	Acetic acid	Lactic acid	NO3	NO2
		g/L/cell(tot)	g/L/cell(tot)	g/L/cell(tot)	g/L/cell(tot)	g/L/cell(tot)
Glucose	FM19 1SS	3,05	12,49	21,30	0,05	0,15
	FM19 2SS	5,52	13,59	20,55	0,07	0,32
	FM19 3SS	47,68	0,98	14,08	2,77	0,79
	FM20 1SS	0,04	2,29	2,25	0,01	0,02
	FM20 2SS	0,04	2,14	2,22	0,01	0,02
	FM20 3SS	0,05	0,35	6,72	0,03	0,02
Glycerol	FM19 1SS	1,27	0,64	0,10	0,01	0,00
	FM19 2SS	1,21	0,59	0,12	0,01	0,00
	FM19 3SS	18,28	1,59	2,10	0,06	0,02
	FM20 1SS	40,06	16,54	2,36	0,22	3,07
	FM20 2SS	3,21	1,30	0,44	0,02	0,01
	FM20 3SS	17,48	1,48	2,05	0,06	0,02

A.7

In this appendix the raw data for the analysis of relative conversion rates are displayed. The data originates from appendix A.6 and have been modified as to be normalized against biomass and then calculated to relative measurements when switching from aerobic to anaerobic conditions,

g/L

	Biomass (g/L cell weight based)			Biomass (g/L, OD-based)		GLC/G LY	Lactic Acid		Acetic Acid		NO3		NO2		Carbon dioxide		Oxygen		Nitrogen INERT	Dilution rate h-1	Growth rate gL-1h-1	Feed Flow [L/h]	Gas Flow in[L/h]	Gas Flow out [L/h]	Qout [m³/h]	Qin [m³/h]
	Xin	X g/ml	X g/L	Xin	X	S in	S	LAin	LA	AAin	AA	NO3in	NO3	NO2in	NO2	CO2in	CO2	O2in	O2	Nitrogen			FEED	FLOW	FLOW	FLOW	FLOW
FM19 ISS 0.5 L/min Air	0,00	5,3E-04	0,53	0,00	10,01	24,68	2,16	2,12	15,09	0,00	8,85	2,38	0,04	0,00	0,11	0,00	0,01	0,21	0,20	0,79	0,79	0,10	0,00	30,00	29,86	0,030
FM19 ISS 0.5 L/min Air	0,00	4,7E-04	0,47	0,00	8,94	25,51	3,43	2,12	12,78	0,00	8,45	2,23	0,04	0,00	0,20	0,00	0,02	0,21	0,18	0,79	0,79	0,10	0,00	30,00	29,86	0,030
FM19 ISS 0.2 L/min N2	0,00	3,3E-04	0,33	0,00	1,81	27,84	21,10	1,95	6,23	0,00	0,43	2,19	1,22	0,00	0,35	0,00	0,00	0,00	0,00	1,00	1,00	0,10	0,00	0,08	12,00	12,02	0,012
FM20 ISS 0.5 L/min Air	0,00	3,8E-03	3,82	0,00	22,59	25,35	0,00	2,17	11,44	0,00	11,67	2,34	0,05	0,00	0,00	0,00	0,02	0,21	0,19	0,79	0,79	0,10	0,00	0,08	30,00	29,89	0,030
FM20 ISS 0.2 L/min N2	0,00	3,3E-03	3,27	0,00	13,33	25,06	0,00	1,95	29,29	0,00	1,54	2,24	0,13	0,00	0,00	0,00	0,01	0,00	0,00	1,00	0,99	0,10	0,00	0,08	12,00	12,08	0,012
FM19 ISS 0.5 L/min Air	0,00	9,4E-03	9,40	0,00	29,51	38,79	15,90	2,07	1,23	0,00	8,08	2,24	0,00	0,00	0,00	0,00	0,02	0,21	0,19	0,79	0,80	0,10	0,00	0,08	30,00	29,81	0,030
FM19 ISS 0.2 L/min Air	0,00	1,1E-02	11,33	0,00	29,30	34,23	18,30	2,10	1,79	0,00	8,98	2,23	0,00	0,00	0,00	0,00	0,04	0,21	0,16	0,79	0,80	0,10	0,00	0,08	12,00	11,89	0,012
FM19 3SS 0.2 L/min N2	0,00	1,3E-03	1,35	0,00	4,46	37,05	32,83	2,16	3,78	0,00	2,85	2,26	0,00	0,00	0,00	0,00	0,01	0,00	0,00	1,00	0,99	0,10	0,00	0,08	12,00	12,07	0,012
FM20 ISS 0.5 L/min Air	0,00	3,7E-04	0,37	0,00	11,87	37,59	19,70	2,06	1,16	0,00	8,13	2,23	0,00	0,00	1,51	0,00	0,01	0,21	0,19	0,79	0,80	0,10	0,00	0,08	30,00	29,76	0,030
FM20 ISS 0.2 L/min Air	0,00	4,3E-03	4,30	0,00	15,99	34,59	18,41	2,00	2,51	0,00	7,47	2,09	0,00	0,00	0,00	0,00	0,03	0,21	0,18	0,79	0,80	0,10	0,00	0,08	12,00	11,89	0,012
FM20 3SS 0.2 L/min N2	0,00	1,4E-03	1,41	0,00	4,58	34,43	32,79	2,16	3,85	0,00	2,78	2,21	0,00	0,00	0,00	0,00	0,01	0,00	0,00	1,00	0,99	0,10	0,00	0,08	12,00	12,07	0,012

mmol/L

	Biomass (cell weight based)		Biomass (OD) based	GLC/G LY	Lactic Acid		Acetic Acid		NO3		NO2															
	Xin	X	Xin	S in	S	LAin	LA	AAin	AA	NO3in	NO3	NO2in	NO2													
FM19 ISS 0.5 L/min Air				137,12	12,01	23,51	167,68	0,00	########	38,33	0,61	0,00	2,35													
FM19 ISS 0.2 L/min Air				141,71	19,08	23,57	142,02	0,00	########	35,98	0,65	0,00	4,34													
FM19 3SS 0.2 L/min N2				154,67	117,20	21,71	69,20	0,00	7,24	35,37	19,74	0,00	7,60													
FM20 ISS 0.5 L/min Air				140,84	0,00	24,16	127,11	0,00	########	37,72	0,85	0,00	0,00													
FM20 ISS 0.2 L/min Air				141,66	0,00	24,23	131,02	0,00	########	38,27	0,44	0,00	0,00													
FM20 3SS 0.2 L/min N2				139,22	0,00	21,63	325,50	0,00	25,64	36,06	2,18	0,00	0,00													
FM19 ISS 0.5 L/min Air				421,59	172,84	22,96	13,62	0,00	########	36,14	0,00	0,00	0,00													
FM19 ISS 0.2 L/min Air				372,11	198,95	23,34	19,87	0,00	########	36,01	0,00	0,00	0,00													
FM19 3SS 0.2 L/min N2				402,67	356,80	23,94	42,00	0,00	47,50	36,46	0,00	0,00	0,00													
FM20 ISS 0.5 L/min Air				408,59	214,09	22,89	12,89	0,00	########	35,93	0,00	0,00	32,86													
FM20 ISS 0.2 L/min Air				375,95	200,07	22,21	27,90	0,00	########	33,64	0,00	0,00	0,00													
FM20 3SS 0.2 L/min N2				374,21	356,36	23,97	42,75	0,00	46,25	35,61	0,00	0,00	0,00													

Compand conv. Rates

	Volumetric conv. r mmol/(L*h)			Specific conv. q mmol/(g cells*h) OD based						Normalized against OD				Relative against 1SS (aerobic)											
	GLC/G LY			Lactic Acid			Acetic Acid			NO3			NO2			Carbon dioxide		Oxygen							
	S on O	S on OD	LA (mm	LA on C	LA on O	AA (mm	AA on C	AA on O	NO3 (mm	NO3 on C	NO3 on O	NO2 (mm	NO2 on C	NO2 on O	CO2in	CO2	O2in	O2							
FM19 ISS 0.5 L/min Air	1,25	100%	-14,42	-27,14	-1,44	100%	-14,75	-27,77	-1,47	100%	3,77	7,10	0,377	100%	-0,23	-0,44	-0,02	100%	751,17	9,19	########	13,36				
FM19 ISS 0.2 L/min Air	1,37	110%	-11,85	-25,39	-1,325	92%	-14,09	-30,20	-1,58	107%	3,53	7,57	0,395	105%	-0,43	-0,93	-0,05	207%	677,74	9,29	736,38	10,09				
FM19 3SS 0.2 L/min N2	2,07	166%	-14,31	-2,623	182%	-0,72	-2,18	-0,4	27%	1,56	4,71	0,864	229%	-0,76	-2,29	-0,42	1788%	30,46	2,06	12,65	0,86					
FM20 ISS 0.5 L/min Air	0,62	100%	-10,68	-2,69	-0,469	103%	-18,91	-4,75	-0,83	96%	3,78	0,95	0,166	102%	0,00	0,00	0,00	#######	965,52	5,19	944,30	5,08				
FM20 ISS 0.2 L/min N2	0,62	100%	-30,39	-2,28	500%	-2,56	-0,78	-0,19	22%	3,39	1,04	0,254	156%	0,00	0,00	0	#######	175,37	1,61	7,18	0,07					
FM19 ISS 0.5 L/min Air	0,84	100%	0,93	0,0317	100%	-13,47	-1,43	-0,46	100%	3,61	0,38	0,122	100%	0,00	0,00	0	#######	1128,24	4,68	########	6,50					
FM19 ISS 0.2 L/min Air	0,59	70%	0,03	0,0118	37%	-14,97	-1,32	-0,51	112%	3,60	0,32	0,123	100%	0,00	0,00	0	#######	1117,44	4,67	########	5,74					
FM19 3SS 0.2 L/min N2	1,03	122%	-1,81	-1,34	-0,405	-12,79%	-4,75	-3,53	-1,07	233%	3,65	2,71	0,818	668%	0,00	0	#######	147,28	4,05	11,92	0,33					
FM20 ISS 0.5 L/min Air	1,64	100%	1,00	2,71	0,0843	100%	-13,55	-36,75	-1,14	100%	3,59	9,74	0,303	100%	-3,29	-8,91	-0,28	100%	647,93	6,69	########	12,63				
FM20 ISS 0.2 L/min Air	1,1	67%	-0,57	-0,13	-0,036	-42%	-12,46	-2,90	-0,78	68%	3,36	0,78	0,21	69%	0,00	0,00	0	0%	703,67	5,39	972,76	7,45				
FM20 3SS 0.2 L/min N2	0,39	24%	-1,88	-1,34	-0,41	-487%	-4,63	-3,29	-1,01	88%	3,56	2,53	0,778	257%	0,00	0,00	0	0%	153,59	4,11	8,87	0,24				

A.8

In this appendix the processed raw data for the analysis assay of nitrate reductase activity (NRA) is presented. The tables consists of the absorbency results of the plates, conversion using the external standard curve, corrections for dilutions and determinations of conversion rates using either a 'per cell'- or 'per mL fermentate' basis.

Absorbancy values with plotted standard curves for each plate

PLATE1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,136	0,176	0,261	0,378	0,55	0,649	0,677	0,627	0,936	1,183	1,172	1,624
B	0,096	0,137	0,223	0,251	0,345	0,424	0,514	0,463	0,696	0,836	0,849	1,021
C	0,033	0,038	0,052	0,05	0,054	0,065	0,084	0,088	0,093	0,112	0,124	0,12
D	0,033	0,043	0,05	0,061	0,062	0,112	0,112	0,127	0,135	0,196	0,188	0,229
E	0,027	0,034	0,056	0,052	0,04	0,091	0,113	0,113	0,09	0,16	0,183	0,191
F	0,027	0,033	0,043	0,038	0,037	0,045	0,054	0,058	0,053	0,068	0,075	0,077
G	0,055	0,239	0,446	0,638	0,735	0,895	0,976	1,036	0,306	0,025	0,026	0,026
H	0,057	0,27	0,455	0,62	0,752	0,847	0,975	1,025	0,029	0,45	0,028	0,026
s.c 1	0,056	0,2545	0,4505	0,629	0,7435	0,871	0,9755	1,0305				
[S.C]	0	0,075	0,15	0,225	0,3	0,375	0,45	0,5				

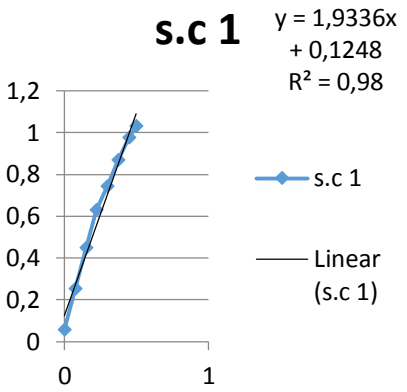


PLATE2

	1	2	3	4	5	6	7	8	9	10	11	12
A	1,262	1,458	1,426	2,011	1,592	2,702	N.A	2,894	3,681	4,105	4,083	4,251
B	0,95	1,083	1,058	1,391	1,222	1,764	2,086	1,864	2,768	3,43	3,114	3,633
C	0,069	0,102	0,127	0,127	0,132	0,15	0,158	0,203	0,208	0,23	0,251	0,269
D	0,149	0,24	0,217	0,295	0,243	0,346	0,363	0,412	0,592	0,679	0,638	0,812
E	0,108	0,213	0,2	0,237	0,183	0,315	0,332	0,347	0,463	0,601	0,625	0,656
F	0,048	0,067	0,069	0,082	0,068	0,094	0,109	0,124	0,142	0,172	0,176	0,189
G	0,05	0,204	0,448	0,432	0,715	0,863	0,774	1,132	0,027	0,026	0,027	0,594
H	0,057	0,208	0,449	0,554	0,689	0,873	0,978	1,059	0,222	0,033	0,034	0,036
s.c 2	0,0535	0,206	0,4485	0,493	0,702	0,868	0,876	1,0955				
[S.C]	0	0,075	0,15	0,225	0,3	0,375	0,45	0,5				

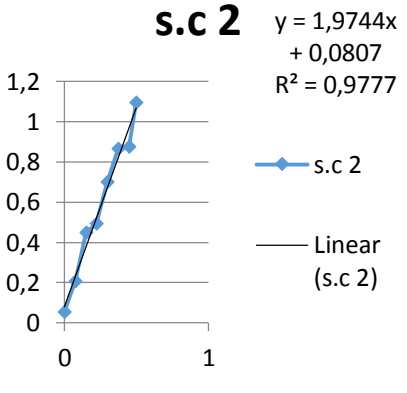


PLATE3

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,034	0,031	0,032	0,034	0,047	0,047	0,043	0,056	0,08	0,101	0,112	0,099
B	0,036	0,031	0,036	0,031	0,037	0,039	0,039	0,044	0,057	0,062	0,065	0,062
C	0,052	0,049	0,055	0,066	0,091	0,092	0,098	0,1	0,183	0,209	0,193	0,18
D	0,273	0,218	0,283	0,271	0,783	0,484	0,576	0,514	1,111	1,08	1,093	1,164
E	0,043	0,042	0,046	0,052	0,087	0,09	0,091	0,107	0,162	0,151	0,235	0,185
F	0,051	0,053	0,063	0,06	0,1	0,093	0,115	0,113	0,179	0,194	0,234	0,178
G	0,261	0,23	0,344	0,336	0,808	0,543	0,526	0,555	0,952	0,818	1,344	1,017
H	0,06	0,274	0,415	0,58	0,708	0,83	0,947	1,026	0,438	0,032	0,295	0,031
s.c 3	0,06	0,274	0,415	0,58	0,708	0,83	0,947	1,026				
[S.C]	0	0,075	0,15	0,225	0,3	0,375	0,45	0,5				

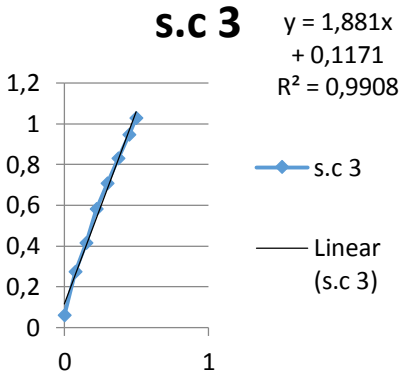
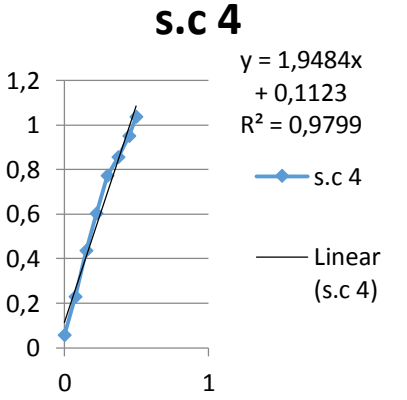


PLATE4

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,133	0,094	0,149	0,136	0,186	0,201	0,202	0,199	0,379	0,38	0,405	0,369
B	0,076	0,087	0,092	0,096	0,119	0,133	0,142	0,147	0,216	0,228	0,224	0,208
C	0,29	0,315	0,319	0,292	0,425	0,459	0,463	0,484	0,793	0,79	0,772	0,809
D	1,098	1,373	1,273	1,096	1,604	1,977	2,268	1,668	2,694	3,008	3,542	3,092
E	0,206	0,223	0,227	0,258	0,309	0,362	0,345	0,367	0,602	0,616	0,621	0,613
F	0,247	0,255	0,283	0,249	0,379	0,386	0,42	0,38	0,657	0,67	0,738	0,678
G	1,191	1,285	1,266	0,941	1,533	1,905	2,194	1,741	3,491	3,368	3,745	3,68
H	0,056	0,23	0,436	0,603	0,772	0,856	0,951	1,037	0,029	0,036	0,036	0,055
s.c 4	0,056	0,23	0,436	0,603	0,772	0,856	0,951	1,037				
[S.C]	0	0,075	0,15	0,225	0,3	0,375	0,45	0,5				



Average absorbance values (n = 4)

PLATE1

	Abs	t	stdv	std error	Abs	t	stdv	std error	Abs	t	stdv	std error
1	0,23775	10	0,107046	0,053523	0,62575	20	0,054488	0,027244	1,22875	30	0,287076	0,143538
2	0,17675	10	0,072463	0,036232	0,4365	20	0,071267	0,035634	0,8505	30	0,133108	0,066554
3	0,04325	10	0,009215	0,004608	0,07275	20	0,016029	0,008014	0,11225	30	0,013769	0,006884
4	0,04675	10	0,011786	0,005893	0,10325	20	0,028395	0,014197	0,187	30	0,038944	0,019472
5	0,04225	10	0,013961	0,006981	0,08925	20	0,034432	0,017216	0,156	30	0,04592	0,02296
6	0,03525	10	0,00685	0,003425	0,0485	20	0,009399	0,004699	0,06825	30	0,010874	0,005437

PLATE2

	Abs	t	stdv	std error	Abs	t	stdv	std error	Abs	t	stdv	std error
1	1,53925	40	0,326008	0,163004	2,396	50	0,702871	0,351436	4,03	70	0,244319	0,12216
2	1,1205	40	0,189344	0,094672	1,734	50	0,366901	0,18345	3,23625	70	0,378221	0,18911
3	0,10625	40	0,027488	0,013744	0,16075	50	0,030192	0,015096	0,2395	70	0,026363	0,013181
4	0,22525	40	0,060456	0,030228	0,341	50	0,071073	0,035536	0,68025	70	0,09475	0,047375
5	0,1895	40	0,056454	0,028227	0,29425	50	0,07531	0,037655	0,58625	70	0,085195	0,042598
6	0,0665	40	0,014012	0,007006	0,09875	50	0,02388	0,01194	0,16975	70	0,019873	0,009936

PLATE3

	Abs	t	stdv	std error	Abs	t	stdv	std error	Abs	t	stdv	std error
7	0,03275	10	0,0015	0,00075	0,04825	20	0,0055	0,00275	0,098	30	0,013292	0,006646
8	0,0335	10	0,002887	0,001443	0,03975	20	0,002986	0,001493	0,0615	30	0,003317	0,001658
9	0,0555	10	0,007416	0,003708	0,09525	20	0,004425	0,002213	0,19125	30	0,013074	0,006537
10	0,26125	10	0,029307	0,014654	0,58925	20	0,134728	0,067364	1,112	30	0,036923	0,018462
11	0,04575	10	0,0045	0,00225	0,09375	20	0,008995	0,004498	0,18325	30	0,037295	0,018647
12	0,05675	10	0,005679	0,002839	0,10525	20	0,010532	0,005266	0,19625	30	0,026209	0,013105

PLATE4

	Abs	t	stdv	std error	Abs	t	stdv	std error	Abs	t	stdv	std error
7	0,128	40	0,023707	0,011853	0,197	50	0,007439	0,003719	0,38325	70	0,015327	0,007663
8	0,08775	40	0,008655	0,004328	0,13525	50	0,012285	0,006142	0,219	70	0,008869	0,004435
9	0,304	40	0,015122	0,007561	0,45775	50	0,024432	0,012216	0,791	70	0,015166	0,007583
10	1,21	40	0,136721	0,068361	1,87925	50	0,306089	0,153044	3,084	70	0,350097	0,175049
11	0,2285	40	0,021672	0,010836	0,34575	50	0,026247	0,013124	0,613	70	0,008042	0,004021
12	0,2585	40	0,016683	0,008342	0,39125	50	0,019414	0,009707	0,68575	70	0,035892	0,017946

Calculated concentrations with translated numbers to names for with linear regression for rate determination, corrected for dilutions ad basis of unit that is to be presented

TIME	FM19 1 SS GLC	FM19 2SS GLC	FM19 3SS GLC	FM20 1SS GLC	FM20 2SS GLC	FM20 3SS GLC	FM19 1SS GLY	FM19 2SS GLY	FM19 3SS GLY	FM20 1SS GLY	FM20 2SS GLY	FM20 3SS GLY
10	0,058	0,027	-0,042	-0,040	-0,043	-0,046	-0,045	-0,044	-0,033	0,077	-0,038	-0,032
20	0,259	0,161	-0,027	-0,011	-0,018	-0,039	-0,037	-0,041	-0,012	0,251	-0,012	-0,006
30	0,571	0,375	-0,006	0,032	0,016	-0,029	-0,010	-0,030	0,039	0,529	0,035	0,042
40	0,739	0,527	0,013	0,073	0,055	-0,007	0,008	-0,013	0,098	0,563	0,060	0,075
50	1,173	0,837	0,041	0,132	0,108	0,009	0,043	0,012	0,177	0,907	0,120	0,143
70	2,000	1,598	0,080	0,304	0,256	0,045	0,139	0,055	0,348	1,525	0,257	0,294
LINREG		**0,361**	**0,287**	**0,024**	**0,063**	**0,055**	**0,018**	**0,034**	**0,019**	**0,072**	**0,264**	**0,054**	**0,060**
R2		**0,975**	**0,956**	**0,995**	**0,955**	**0,955**	**0,979**	**0,939**	**0,955**	**0,965**	**0,968**	**0,964**	**0,963**

CORRECTED PER CELL

up-conc.	[active/g]		4,9E+09	4,6E+09	4,5E+09	5,0E+09	5,3E+09	5,2E+09	5,0E+09	4,9E+09	5,3E+09	4,8E+09	5,7E+09	5,6E+09
Diluted 1:10			4,9E+08	4,6E+08	4,5E+08	5,0E+08	5,3E+08	5,2E+08	5,0E+08	4,9E+08	5,3E+08	4,8E+08	5,7E+08	5,6E+08
InDeepWPlt		0,4	2,0E+08	1,8E+08	1,8E+08	2,0E+08	2,1E+08	2,1E+08	2,0E+08	2,0E+08	2,1E+08	1,9E+08	2,3E+08	2,2E+08
Conv. Rate	**[nM/h/cell]**		**1,11**	**0,95**	**0,08**	**0,19**	**0,16**	**0,05**	**0,10**	**0,06**	**0,21**	**0,82**	**0,14**	**0,16**

CORRECTED PER ML FERMENTATE

ml fermentate			15,00	15,00	35,00	2,00	2,00	2,50	0,80	0,66	6,00	20,00	2,00	6,00
InDeepWPlt		0,4	6,00	6,00	14,00	0,80	0,80	1,00	0,32	0,26	2,40	8,00	0,80	2,40
Conv. Rate	**[mM/h/ml Fermentate]**		**3,61**	**2,87**	**0,10**	**4,69**	**4,10**	**1,07**	**6,31**	**4,36**	**1,81**	**1,98**	**4,06**	**1,51**

A.9

In this appendix the processed data for the analysis assay of qPCR of the two genes; narg and nirB is presented.

Table A.3: Processed data for qPCR, normalized against reference genes; rpoB, rplD, GyrB, recA. The CT-threshold was set to 0.1 and all plates used en external standard curve using a reference DNA sample at a 10-fold range of 5 dilutions to generate efficiency values to convert CT-values to quantities relative to the highest expressing target.

	Sample#	# of duplicate	timepoint	Flowcyt. (active cells/g)	Average normalized expression level (n=3) narG	stdev narG	Standard error	Average normalized expression level nirB	stdev nirB	Standard error
GLC FM19 1SS	1	A-B-C	1SS	6,52E+08	1,11	0,22	0,13	0,95	0,67	0,39
GLC FM19 2SS	2	A-B-C	2SS	6,07E+08	1,08	0,48	0,28	0,94	0,21	0,12
GLC FM19 3SS	3	A-B-C	3SS	2,58E+08	0,72	0,28	0,16	0,90	0,68	0,39
GLC FM20 1SS	4	A-B-C	1SS	5,04E+09	0,98	0,71	0,41	2,27	2,04	1,18
GLC FM20 2SS	5	A-B-C	2SS	5,27E+09	1,12	0,65	0,37	4,62	2,91	1,68
GLC FM20 3SS	6	A-B-C	3SS	4,19E+09	1,21	0,65	0,38	5,40	3,16	1,82
GLY FM19 1SS	7	A-B-C	1SS	1,25E+10	0,56	0,05	0,03	1,16	0,24	0,14
GLY FM19 2SS	8	A-B-C	2SS	1,48E+10	1,32	0,29	0,17	1,88	0,74	0,43
GLY FM19 3SS	9	A-B-C	3SS	1,76E+09	3,87	4,03	2,33	3,34	4,20	2,43
GLY FM20 1SS	10	A-B-C	1SS	4,82E+08	1,44	1,21	0,70	1,05	1,35	0,78
GLY FM20 2SS	11	A-B-C	2SS	5,70E+09	0,76	0,32	0,19	0,72	0,42	0,24
GLY FM20 3SS	12	A-B-C	3SS	1,86E+09	5,28	0,78	0,45	4,36	2,40	1,38