Bachelor thesis

Upscaling the extracellular glucose oxidase production in *Aspergillus niger* var *tubingensis* strain Ed8

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Declaration of Authorship

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Abbreviations

Units

s	Seconds
min	Minutes
h	Hours
g	Gram
mg	Milligram
μmole	Micromole
L	Liter
μL	Mikroliter
mL	Milliliter
g/L	Gram per liter
M	Molar; [M] = $\mathrm{mol/L}$
mM	Millimolar; [mM] = $\mathrm{mmol/L}$
ppm	Parts per million
U	Activity Unit
U/ml	Activity Units per milliliter solution
cm	Centimeter
nm	Nanometer
°C	Degrees Celsius
%	Percent; solutions: 1 % w/v = 1 g / 100 ml, solids: 1 % w/w = 1 g / 100 g
pH	Negative logarithm of the molar concentration of hydrogen ions
rpm	rounds per minute

Substances / Mediums

$(NH_4)_2SO_4$	Ammonium sulfate
ABTS	2,2'-Azino-bis(3-Ethylbenzthiazoline-6-Sulfonic Acid)
DNS	3,5-dinitrosalicylic acid
FAD	Flavin adenine dinucleotide

$FADH_2$	Flavin adenine dinucleotide (hydroquinone form)
GOX	Glucose oxidase
H_2O_2	Hydrogen peroxide
H_2O_{dest}	Distilled water
H_2SO_4	Sulfuric acid
$K_2Cr_2O_7$	Potassium dichromate
KH_2PO_4	Monopotassium phosphate
LMM	Lee's Minimal Medium
$MgSO_4$	Magnesium sulfate
Na_2S	Sodium sulfide
$NaCl$	Sodium chloride
$NaOH$	Sodium hydroxide
PDA	Potato Dextrose Broth
POX	Peroxidase

Other Abbreviations

^{TM}	Registered trademark
Cr(III)	Trivalent chromium
Cr(VI)	Hexavalent chromium
DNA	Deoxyribonucleic acid
Ed8	*Aspergillus niger* var. *tubingensis* strain Ed8
OD_{420}	Optical density at 420 nm
OD_{540}	Optical density at 540 nm
$Y_{A/\Delta S}$	Yield; activity / total substrate consumption
Y_{A/S_0}	Yield; activity / starting substrate concentration

1 Task | Aufgabenstellung

1.1 Task

1.1.1 Objective

The aim of this work is scaling-up the GOX (Glucose oxidase) production via *Aspergillus niger* var. *tubingensis* strain Ed8, whose fermentation process has been previously carried out in flask systems. The task of this project was to explore the GOX production in systems that are similar to industrial ones like the stirred tank reactor.

1.1.2 Justification

The idea of producing GOX in the Ed8 is a consequence of a study that has been carried out to do chromium reduction with that strain. It was found that GOX is expressed during the fermentation and plays an important role in it [1]. To develop the process of GOX production in a large scale, it is necessary to evaluate the performance in different systems that are similar to industrial ones. Giving a mathematical model will help to get deeper insights to the fermentation behavior.

1.1.3 Hypothesis

The high growth rate in the flask system can be maintained in the industrial scale by finding effective process conditions. Optimizing the process conditions will lead to better yields of formed product referred to the consumed substrate. With the mathematical model, a connection between biomass, substrate and product concentration can be found. Optimizing the fermentation will lead to higher product formation and besides higher rates of Cr-reduction.

1.1.4 Strategy

Towards the scaling-up of GOX production, fermentation processes where carried out in systems that are similar to industrial ones and the performance was evaluated through frequent measurements along the process to by obtaining the variables biomass and substrate concentration as well as GOX activity. On the basis of the experimental

data a mathematical model was constructed to further descriptions and understandings of the process. By similarity it is meant that the reactors are mechanically stirred jacketed tanks, like the tank stirred by an impeller and the Airlift reactor.

1.2 Aufgabenstellung

1.2.1 Zielsetzung

Ziel dieser Arbeit ist es, eine Maßstabsvergrößerung der GOX Produktion in *Aspergillus niger* var. *tubingensis* Stamm Ed8 durchzuführen, dessen Fermentationsprozess bisher in Fläschchen durchgeführt wurde. Die Aufgabe dieses Projektes ist, die GOX-Produktion in Systemen zu testen, die denen der Industrie ähnlich sind, wie etwa der Rührkessel.

1.2.2 Begründung

Die Idee, GOX im Stamm Ed8 zu produzieren, resultiert aus einer Studie, die zur Reduktion von Chrom mit diesem durchgeführt wurde. Man fand heraus, dass während der Fermentation GOX exprimiert wird und in dieser eine wichtige Rolle spielt. Um den Prozess der Produktion von GOX im großen Maßstab zu entwickeln, ist es notwendig, die Eignung in verschiedenen Systemen zu bewerten, die industriell nutzbar sind. Die Ermittlung eines mathematischen Modells wird helfen, tiefere Einblicke in die Dynamik der Fermentation zu erhalten.

1.2.3 Hypothese

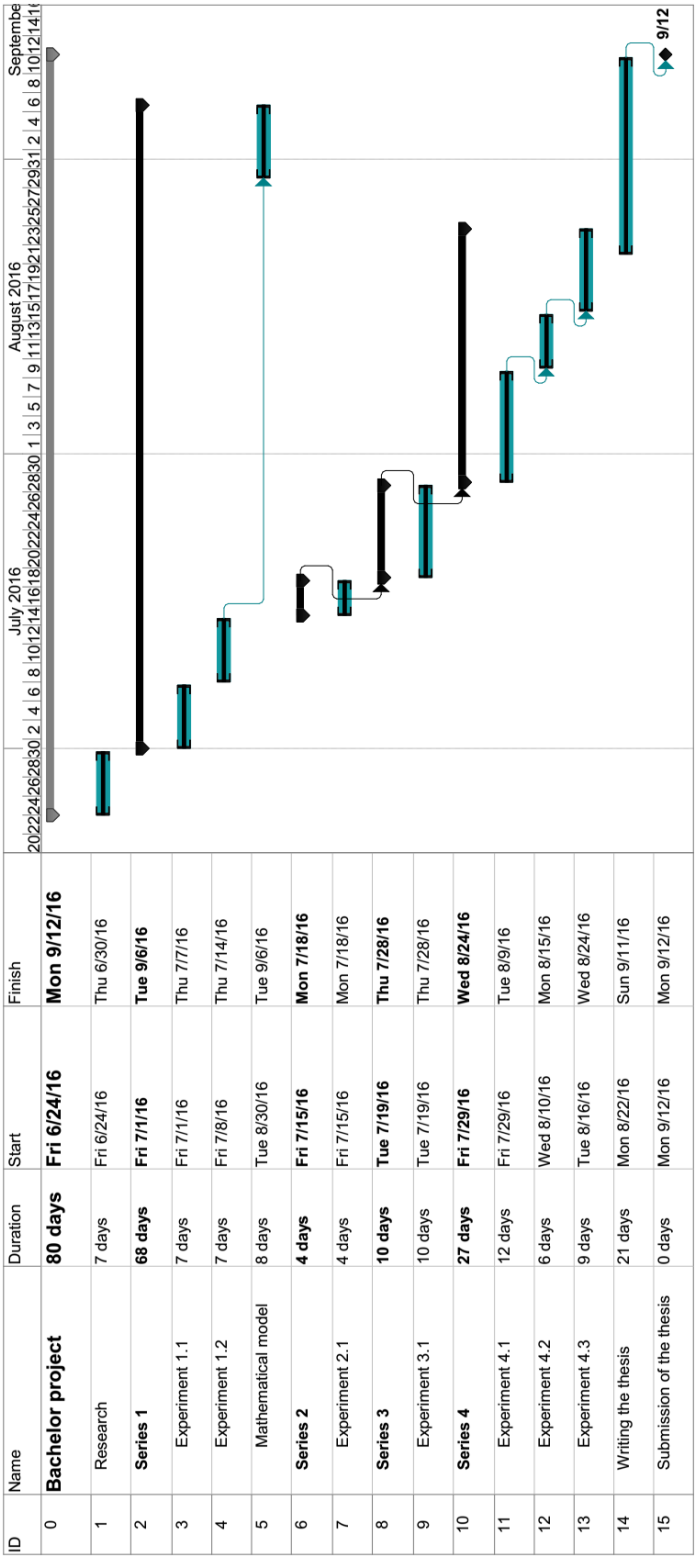
Die hohe Wachstumsrate im Fläschchen kann im industriellen Maßstab durch das Finden effektiver Prozessbedingungen beibehalten werden. Das Optimieren der Prozessbedingungen wird zu größeren Produktausbeuten bezogen auf das verbrauchte Substrat führen. Mit dem mathematischen Modell kann eine Verbindung zwischen Biomasse-, Substrat- und Produktkonzentration hergestellt werden. Die Optimierung der Fermentation wird zu einer erhöhten Reduktionsrate von Chrom führen.

1.2.4 Strategie

Die Maßstabsvergrößerung der GOX-Produktion anstrebend, wurden Fermentationsprozesse durchgeführt, die denen der Industrie ähnlich sind, wobei die Leistung durch regelmäßige Messungen während der Fermentation durch die Bestimmung der Variablen Biomasse- und Substratkonzentration, sowie GOX-Aktivität, ermittelt wurde. Auf Basis der experimentellen Daten wurde zur weiteren Beschreibung und Verständnis des Prozesses ein mathematisches Modell erstellt. Mit Ähnlichkeit ist gemeint, dass die Reaktoren mechanisch gerührte und bemantelte Reaktoren sind, wie der durch Propeller gerührte Reaktor und der Airlift-Reaktor.

2 Timeline

The project was organized in series and experiments, represented as working packages in the following Gantt chart.



ID	Name	Duration	Start	Finish
0	**Bachelor project**	**80 days**	**Fri 6/24/16**	**Mon 9/12/16**
1	Research	7 days	Fri 6/24/16	Thu 6/30/16
2	**Series 1**	**68 days**	**Fri 7/1/16**	**Tue 9/6/16**
3	Experiment 1.1	7 days	Fri 7/1/16	Thu 7/7/16
4	Experiment 1.2	7 days	Fri 7/8/16	Thu 7/14/16
5	Mathematical model	8 days	Tue 8/30/16	Tue 9/6/16
6	**Series 2**	**4 days**	**Fri 7/15/16**	**Mon 7/18/16**
7	Experiment 2.1	4 days	Fri 7/15/16	Mon 7/18/16
8	**Series 3**	**10 days**	**Tue 7/19/16**	**Thu 7/28/16**
9	Experiment 3.1	10 days	Tue 7/19/16	Thu 7/28/16
10	**Series 4**	**27 days**	**Fri 7/29/16**	**Wed 8/24/16**
11	Experiment 4.1	12 days	Fri 7/29/16	Tue 8/9/16
12	Experiment 4.2	6 days	Wed 8/10/16	Mon 8/15/16
13	Experiment 4.3	9 days	Tue 8/16/16	Wed 8/24/16
14	Writing the thesis	21 days	Mon 8/22/16	Sun 9/11/16
15	Submission of the thesis	0 days	Mon 9/12/16	Mon 9/12/16

3 Introduction

3.1 The project

The superordinated topic is the reduction of chromium Cr(VI) to Cr(III). It was investigated in previous studies that GOX is playing an important role in reducing chromium in the Ed8 fermentation [1]. This work follows a master thesis that was carried to investigate the GOX production in *Aspergillus niger* var. *tubingensis* strain Ed8 [2]. The study was carried out based on 50 mL flasks to analyze and optimize the GOX expression of Ed8 by parameter variation. The used medium was Lee's Minimal Medium with glucose as the main substrate. It was found that the maximum GOX activity could be achieved with 50 g/L glucose concentration. In this work a deeper analysis of the fermentation and its parameters, like biomass and substrate concentration as well as product activity, had been done. For the stirred tank system, the behavior of the fermentation towards agitation and aeration had been investigated. The aim is to determine the feasibility of scaling-up the GOX production by producing as much GOX as possible.

3.2 Microorganism

The strain Ed8 of *Aspergillus niger* var. *tubingensis* is a filamentous mold which had been isolated from a Cr(VI) polluted soil in San Francisco del Rincón (Guanajuato, Mexico) [3]. *Aspergillus niger* is widely used in the food industry to produce citric acid. Also, some secondary metabolites are produced, like glucoamylase for the high fructose corn syrup production and pectinases for whine clarification. It also naturally produces the enzyme GOX. In previous studies it was discovered that the Ed8 strain is doing that remarkably well compared to other strains like NRRL593 and FGSCA732 [2]. The optimum conditions for biomass growth are 28°C constant temperature, a starting pH of 5.3 and 45 mM citric acid. The Ed8 is stored as spores which have to go through a long lag phase to adapt to the surrounding medium and to initiate the mitosis back to cells with active metabolism and reproduction. It is very adherent and is therefore growing in agglomerates that are forming thick spheres during the fermentation. The agitation has to be set in a way that the Ed8 spheres are not getting too big because the cells in the center of the sphere get inactive and degenerate back to spores. The

Ed8 also tends to grow on the wall of the reactor. That is why the usage of baffles and sufficient agitations is recommended.

3.3 Carbon source

The mostly used carbon source for *Aspergillus niger* is glucose. Also maltose, lactose, sucrose, starch and fructose had been used. Sucrose seems to have a positive effect on biomass growth where glucose was determined to cause maximum GOX production [2]. In these studies industrially purified sugars had been used. They are very expensive and one of the reasons for the high prices of the products by microorganisms. An aim could be to replace the expensive purified sugar with a cheaper alternative.
The Department of Chemical Engineering at the University of Guanajuato is trying piloncillo as an economic replacement for industrial purified glucose. Piloncillo is unrefined whole cane sugar containing 72-78 % sucrose, 1.5-7 % fructose and 1.5-7 % glucose [4]. According to a previous study, the biomass concentration with pure sucrose should be about equal to the concentration with glucose [5]. There was even an increase of GOX production detected. Though, another strain was used (*Aspergillus niger* BTL) and there might be a different behavior with piloncillo because it contains only 72-78 % sucrose and additionally some minerals.

3.4 Glucose oxidase

3.4.1 Characterization and usage

The Enzyme Commission (EC) number of GOX is 1.1.3.4. GOX catalyzes the oxidation of glucose to gluconolactone by reducing the cofactor FAD (Flavin adenine dinucleotide) to $\mathrm{FADH_2}$ (Flavin adenine dinucleotide (hydroquinone form)). The half-velocity constant is about 30 mM. The pH range of maximum stability is $4\sim8$. [6]
GOX is widely used in the industry as an additive to preserve food. The conversion of glucose can prevent caramelization and avoid deterioration by removing oxygen. It can also act as a preservative for milk because of the produced hydrogen peroxide which reduces microbial growth. It can be used in the clinical area as well to determine glucose in biological fluids and to monitor glucose in fermentation processes. Due to its antimicrobial activity, GOX is also used in tooth paste, lotions, shampoos, cosmetics,

etc. The main product of the GOX catalyzation, gluconic acid, is used in chemical industries as a whitener and cleaner. [2, 7]

3.4.2 Production

GOX is expressed by filamentous fungi like *Aspergillus*, *Penicillium* and *Saccharomyces*. The most common microorganism used for GOX production is *Aspergillus* [7]. Fungi like *Aspergillus niger* produce GOX to protect themselves from bacterial contamination because of the produced hydrogen peroxide [8]. The production was found to be affected by substrate inhibition as well as competitive inhibition by gluconic acid, the main product of GOX catalyzation. The by-product hydrogen peroxide can inhibit the activity by inactivating the enzyme. This way, a maximum amount of H_2O_2 in the medium will not be exceeded [9].

3.4.3 Assay of GOX enzymatic activity

Enzymatic activity measurement of GOX can be done with an ABTS-peroxidase-linked assay (see Figure 3.1). The procedure was published by Sigma-Aldrich [10]. It was varied in a way that the originally used o-dianisidine was replaced by ABTS (2,2'-Azino-bis(3-Ethylbenzthiazoline-6-Sulfonic Acid)). The volumes and the wavelength had also been adjusted.

GOX: $\mathrm{beta-D-glucose} \; + \; H_2O \; + \; O_2 \rightarrow \mathrm{D-glucono-1,5-lactone} \; + \; H_2O_2$

POX: $H_2O_2 \; + \; 2 \; \mathrm{ABTS_{red}} \rightarrow 2 \; H_2O \; + \; 2 \; \mathrm{ABTS_{ox}}$

Figure 3.1: Reaction of ABTS/peroxidase solution

GOX catalyzes the oxidation of D-glucose to D-glucono-1,5-lactone, which is forming an equilibrium of gluconolactone and gluconic acid in aqueous buffers. A by-product of the GOX reaction is hydrogen peroxide which is needed for the peroxidase (POX) catalyzed reaction. In this reaction, hydrogen peroxide is getting reduced to water. The ABTS therefore gets oxidated, giving the solution a dark green color. The color shift is measurable by spectrophotometry at 420 nm wavelength.

3.5 Chromium

3.5.1 Characterization

Chromium is a hard metal that is widely used in chemical industry such as chrome plating, leather tanning, paint pigments and wood treatment. Its importance as an ambient contaminant is getting bigger and bigger due to the extensive use in the industry and the high toxicity of hexavalent chromium. Cr(VI) can be detected in ground water, soil and industrial effluents. [11] Where the trivalent chromium Cr(III) is relatively harmless, the Cr(VI) shows high solubility and mobility. Therefore it can pass through the membrane of all types of (micro-)organisms and bind to DNA and protein molecules due to its high oxidative potential [12].

3.5.2 Aim of chromium reduction

The reduction of toxic Cr(VI) to Cr(III) is in the interest of several studies in Mexico. There also have been studies carried out regarding chromium reduction specifically in Ed8. They showed that the Ed8 has a high efficiency of chromium reduction in the extracellular medium. The microorganism can as well absorb chromium into the mycelial biomass. [13]

3.5.3 Assay of hexavalent chromium concentration

The method used to determine the concentration of hexavalent chromium in the samples was a varied version of method '3500-Cr' by the American Public Health Association [14]. The diphenylcarbazide will form a Cr(III)-diphenylcarbazone complex in aqueous solution with added sulfuric acid. The complex is of a reddish purple color, measurable by spectrophotometry at 540 nm wavelength.

3.6 Mathematical model

3.6.1 Mass balance of a fermentation

The standard differential equation of a component i in a fermentation is expressed as a general mass balance:

$$V_R \cdot \frac{dc_i}{dt} = \dot{V}_I \cdot c_{i,I} - \dot{V}_O \cdot c_{i,O} + R_i \cdot V_R \tag{3.1}$$

V_R: Working volume of reaction
c_i: Concentration of component i in the working volume, in-(I) and out-(O) flow
$\dot{V}$: Volume in-(I) and out-(O) flow
R_i: Reaction term of component i

For a batch fermentation which does not have in- and outflow, the rate of change of a component i is only depending on the corresponding reaction term (Equation 3.2).

$$\begin{aligned} \frac{dc_i}{dt} &= R_i \\ &= \mu \cdot c_i \end{aligned} \tag{3.2}$$

μ: Specific reaction rate (h^{-1})

3.6.2 Monod kinetics

The specific reaction rate μ was obtained by applying the Monod kinetics [15] on the component i (see Equation 3.3 and Equation 3.4). It is depending on n limiting factors. Each limiting factor is calculated from the current concentration of the limiting component c_j and a corresponding half-velocity constant k_j.

$$\mu_i = \mu_{max,i} \cdot \prod_{j=1}^{n} \frac{c_j}{k_j + c_j} \tag{3.3}$$

$$\frac{dc_i}{dt} = \mu_{max,i} \cdot \prod_{j=1}^{n} \frac{c_j}{k_j + c_j} \cdot c_i \tag{3.4}$$

μ_{max}: Maximum specific reaction rate (h^{-1})
c_j: Concentration of limiting component j (g/L)
k_j: Half-velocity constant for the limiting component j (g/L)

3.6.3 Adjusted model

The fermentation model was created with the following differential equations:

$$\frac{dX}{dt} = \mu_{max} \cdot \frac{S}{k_S + S} \cdot X - m_X \cdot X \tag{3.5}$$

$$\frac{dS}{dt} = -Y_{S/X} \cdot \mu_{max} \cdot \frac{S}{k_S + S} \cdot X - m_S \cdot S \tag{3.6}$$

$$\frac{dP}{dt} = v_{max} \cdot \frac{S}{k_{p,1} + S} \cdot \frac{X}{k_{p,2} + X} - m_P \cdot P \tag{3.7}$$

X, S, P:	Biomass, substrate and product concentration	(g/L)
k_S, $k_{p,1}$, $k_{p,2}$:	Half-velocity constants of corresponding limiting components	(ppm)
$Y_{S/X}$:	Reciprocal yield of biomass formed due to substrate consumption	(g/g)
v_{max}:	Maximum product formation velocity	($mUmL^{-1}h^{-1}$)
$m_{X,S,P}$:	Correction factor of corresponding component	(h^{-1})

The biomass growth (Equation 3.5) is assumed as linear exponential. It is determined according to the Monod kinetics. The substrate concentration S is assumed as the only limiting factor. Substrate and product inhibition are neglected.
The substrate consumption (Equation 3.6) is, according to Monod [15], directly proportional to the biomass growth.
The product formation (Equation 3.7) is proportional to the two limiting factors S and X. A formation velocity v_{max} is introduced to describe the rate of product secretion.
The correction factors m_X, m_S and m_P are used to compensate deviations from linearity. The biomass growth can deviate due to an additional death rate and unknown dependencies on other substrates. The substrate consumption can deviate due to the additional consumption to form by-products (mainly citric acid or other products like enzymes). The product formation can deviate due to unknown additional dependencies (e.g. oxygen concentration; rate of agglomeration...) or feedback inhibition.
The yield $Y_{S/X}$ used is the average yield taken from the experimental data.
The maximum specific growth rate μ_{max} is obtained using the logarithmic slope of the exponential biomass course according to Equation 3.8.

$$\mu_{max} = \frac{\ln \frac{X(t)}{X(t_0)}}{t - t_0} \tag{3.8}$$

3.6.4 Chromium reduction

To describe the concentration of chromium in the medium, a first order nonlinear differential equation can be used (see Equation 3.9). This model is used for logistic growth.

$$\frac{dCr}{dt} = -r \cdot Cr \cdot \left(1 - \frac{Cr}{K}\right) \tag{3.9}$$

Cr:	Cr(VI) concentration	(ppm)
r:	Degradation rate	(h^{-1})
K:	Carrying capacity	(ppm)

3.7 Stirred tank reactors

The stirred tank reactor is the most commonly used reactor for the GOX production in *Aspergillus* [7]. Because oxygen transfer is a limiting factor for the aerobic GOX fermentation, a sufficient aeration has to be supplied. In the small scale (50 mL flasks) there is no further aeration needed. The agitation of 150 rpm translatory shaking is causing a big specific power entry (P/V). Therefore there is a high turbulence on the liquid-gas interface on the mediums surface, causing, together with the big specific surface (A/V), a sufficient oxygen transfer. By doing a scale-up, one has to consider less turbulence, less specific surface, less specific power entry and an overall decreased oxygen transfer due to the increase of reaction volume. For an aerobic fermentation, the oxygen transfer rate (OTR) has to reach the oxygen uptake rate (OUR) to have constant exponential growth without cell dying. This is why active aeration is needed for bigger working volumes than the small analytic scale. The active aeration has to fulfill more and more demands with increasing working volume. The air bubbles need to be as small as possible to achieve a maximum specific surface of gas-liquid oxygen transfer and a maximum residence time in the medium. The agitation can help in a way that it is causing further reduction of the air bubble size and as well an even longer residence time. The agitation is also significantly important to prevent early cell growth on the reactors wall. Additionally, agitation is needed to keep the forming Ed8 spheres small to prevent cell deactivation and even cell dying inside the spheres.

4 Materials and methods

In this section the materials and methods used in this project are described. After the general description of the microorganism handling, a detailed look on the fermentation analysis, including sample measurements and the mathematical model, is given.

4.1 Devices

The used devices are listed in Table 4.1.

Table 4.1: List of Devices

Type	Name	Manufacturer
Flask (250 mL)	-	Corning
Shaking water bath	SW22	Julabo
Stirred tank reactor (3.6 L)	Labfors 5	Infors HT
Stirred tank reactor (0.5 L)	-	SEV
Airlift reactor (1.5 L)	-	SEV
Spectophometer	Genesys 10S UV-VIS	Thermo Scientific
Microcentrifuge	LSE 6765-HS	Corning
Precision scale	PL403	Mettler Toledo
Micropipettes 10$\sim$1000 μL	Pipetman	Gilson
Microscope	Primo Star	Zeiss
Autoclave	CV300	AESA
Recirculator	FC-10	PRENDO

4.2 Storage and subcultivation

The Ed8 spores are stored in distilled water at 4°C in a sterile 50 mL tube. To guarantee constant spore viability throughout all experiments, the Ed8 has to be subcultivated every month.

4.2.1 PDA agar plates

The PDA (Potato Dextrose Broth) medium is prepared according to the table Table 4.2.

Table 4.2: Composition of PDA medium in H_2O_{dest}

Substance	Amount
Potato	200 g per Liter medium
Dextrose	2 % w/v
Bacterial agar	1.5 % w/v

The steps of medium preparation are as follows:

- Boil sliced potato pieces for 20 min in the desired volume of water
- Fill the broth in a measuring cylinder and add dextrose and agar
- Fill up with H_2O_{dest} to the desired volume
- Sterilize in the autoclave for 15 min

For 20 agar plates, 0.5 L of PDA medium is prepared.

4.2.2 Subcultivation

The steps of subcultivation are as follows:

- Spread the spores out on PDA-Plates with a grease pencil
- Cultivate 4-6 days in an incubator at room temperature
- Resuspend the spores with distilled water
- Wash 2 times with distilled water by centrifugation (1500 g, 15 min)
- Determine cell concentration by cell counting according to Section 4.2.3

4.2.3 Spore counting

The Ed8 spores are counted in a Neubauer Improved^{TM} counting chamber. The chamber is filled with an Ed8 spore solution that was previously diluted with a dilution factor $\mathrm{df} = 0.01$. The counting chamber gets inserted to the microscope and a suitable magnifying factor has to be chosen to have a close-up view to the top left big square (see Figure 4.1).

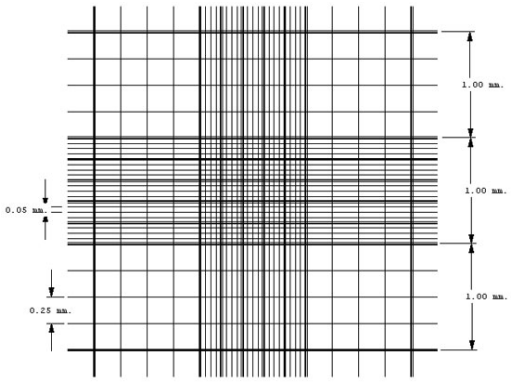


Figure 4.1: Neubauer improved [source: Dhurba Giri / laboratoryinfo.com]

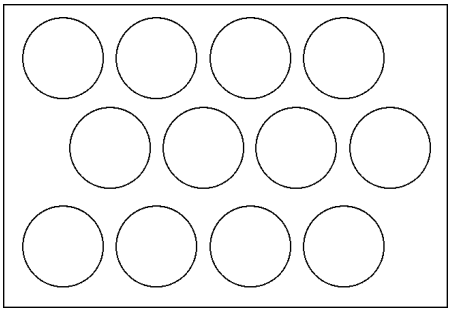
After counting the cells in all four big squares, Equation 4.1 is used to calculate the spore concentration of the undiluted Ed8 parent solution.

$$\text{Concentation in spores/mL} = \frac{\mathrm{n_{spores}}}{\mathrm{n_{squares}} \cdot \mathrm{df}} \cdot 10,000\ \mathrm{mL}^{-1} \tag{4.1}$$

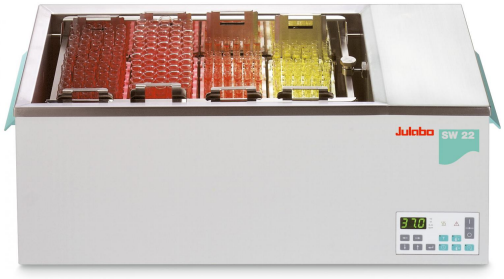
4.3 Fermentation systems

4.3.1 250 mL flasks

The 250 mL flasks are placed in a shaking water bath with 28°C temperature. 50 mL are used as working volume for each flask. The agitation is translatory at 150 rpm. There is no active aeration used. Twelve flasks are used to determine cell growth by taking samples every 6 h. The configuration of the 12 flasks in the shaking water bath is shown in Figure 4.2a. The Julabo SW22 is used (see Figure 4.2b).



(a) Flask configuration



(b) Julabo SW22 [source: julabo.com]

Figure 4.2: Julabo shaking water bath

4.3.2 Labfors 5 bench top reactor

The Labfors 5^{TM} is a stirred tank bench top reactor with a reactor volume of 3.6 L and a working volume of 2 L. For agitation, a helical ribbon impeller powered by a motor running at 150 or 250 rpm was used. Aeration is supplied with 5 L/min of sterile dry air. Due to a lack of original sparging equipment, a simple thin metal tube was used. The reactor works with an automatic control system of temperature and pH. The Labfors 5 control system has to be connected to a recirculator supplying water of constant temperature (28°C). The reactor (Figure 4.3a) is connected to a control station (Figure 4.3b) that is supplying water for the temperature and a base for the pH control.

(a) Labfors 5 3.6 L bench top reactor (b) Complete setup

Figure 4.3: Labfors 5 and its setup [source: infors-ht.com]

4.3.3 Airlift reactor

The airlift reactor (shown in Figure 4.4) has a working volume of 1.5 L. Aeration is supplied with 2∼4 L/min of sterile dry air. Agitation is the result of reactor design and buoyancy of air bubbles. The reactor is connected to a recirculator supplying water of constant temperature (28°C).

Figure 4.4: Airlift reactor

4.3.4 0.5 L stirred tank reactor

The stirred tank reactor (shown in Figure 4.5) has a working volume of 0.5 L. Aeration is supplied with 3 L/min of sterile dry air. For agitation, a 4-bladed impeller running at 100 rpm is used. The reactor is connected to a recirculator supplying water of constant temperature (28°C).

Figure 4.5: SEV 0.5 L stirred tank reactor

4.4 Scale-up procedure

After analyzing the fermentation in the small scale, a scale-up is done with the aim to produce GOX in an industrial-like scale. The factors affecting the fermentation have to be taken in consideration. The main factors are glucose concentration, aeration, agitation, temperature and pH. The temperature and the pH where kept constant throughout all experiments. At first, the response of the system to lower oxygen transfer (aeration) and specific power entry (agitation) due to an increase of working volume is investigated (Series 2). Then, experiments in the Labfors 5 reactor are carried out with different aeration and agitation setups to see how these parameters affect the growth and the product formation (Series 3). At last, fermentations in a 0.5 L reactor are carried out to see the response to the variation of substrate concentrations (Experiment 4.1). In Experiment 4.2, the response of the system to piloncillo as a substrate replacement is investigated. Finally, in Experiment 4.3, the system gets tested on its performance in reducing chromium.

4.5 Medium

As a medium, a modified variant of LMM (Lee's Minimal Medium) [16] with varied glucose concentration, is used (for composition see Table 4.3). This medium is used in all previous studies with Ed8 [13, 2, 17]. Citric acid is added to the original LMM mixture because it was found that it has a positive impact on the biomass growth [2]. Where 0.25 % glucose is the standard concentration of LMM, it was found that the optimum GOX production was achieved with 5 % glucose [2].

Table 4.3: Composition of LMM medium in H_2O_{dest}

Substance	Amount
KH_2PO_4	0.25 % w/v
$MgSO_4$	0.2 % w/v
$(NH_4)_2SO_4$	0.5 % w/v
NaCl	0.5 % w/v
Glucose	0.25 | 1 | 3 | 5 | 7 % w/v
Citric acid	45 mM

The pH is adjusted to 5.3 with NaOH after complete dissolution. Then the medium gets sterilized in an autoclave at 121°C for 15-30 min.

4.6 Inoculation and sample taking

To start the fermentation, the inoculation is done with $5 \cdot 10^5$ spores/mL. The fermentation is carried out at 28°C under most sterile conditions possible. The samples of 1 mL are taken in equidistant time intervals of 6 or 12 hours. They are stored in 1.5 mL microtubes at -4°C.

4.7 Biomass concentration measurement

To determine the dry biomass concentration in g/L, the fermentation broth first gets filtered. The filtrated biomass is stored in a drying oven until it reaches constant weight. The dry biomass gets weighted and the result is divided by the total working volume (see Equation 4.2).

$$X = \frac{m_{total} - m_{filter}}{V_{reaction}} \tag{4.2}$$

4.8 Substrate concentration measurement

4.8.1 Mechanism

The glucose concentration measurement is done with the DNS test [18] which is used to detect reducing sugars. Reducing sugars are monosaccharides that have a free aldehyde group (aldoses) or ketone group (ketoses). Glucose is a reducing sugar because it has an aldehyde group on the first C-atom. Therefore DNS test is suitable for glucose concentration measurement. During the reaction, the sugar itself gets oxidized from D-Glucose (Figure 4.6a) to D-Gluconic acid (Figure 4.6b). More specifically, the aldehyde group gets oxidated to a carboxyl group. [18]

(a) D-Glucose [Source: Ben; Yikrazuul / Wikimedia Commons]

(b) D-Gluconic acid [Source: NEUROtiker / Wikimedia Commons]

Figure 4.6: D-Glucose and D-Gluconic acid

The DNS (3,5-dinitrosalicylic acid) gets reduced to 3-Amino-5-nitrosalicylic acid when reducing sugars are present (see Figure 4.7). DNS changes its color from a light yellow to a dark reddish brown which is measurable by spectrophotometry at 540 nm wavelength.

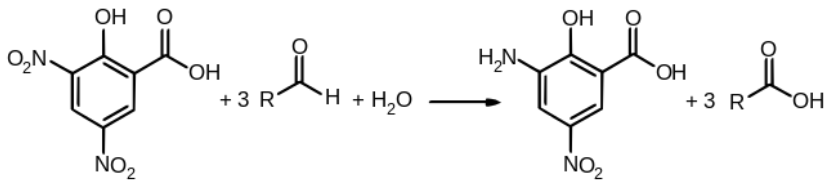


Figure 4.7: Reaction of DNS [source: Krakatit / Wikimedia Commons]

4.8.2 Composition of DNS solution

Table 4.4: Composition of DNS solution in H_2O_{dest} according to Miller [18]

Substance	Amount
3,5-Dinitrosalicylic acid	1 % w/v
Phenol	0.2 % w/v
Na_2S (Sodium sulfide)	0.05 % w/v
$NaOH$ (Sodium hydroxide)	1 % w/v
Potassium sodium tartrate	20 % w/v

- Dissolve $NaOH$ and 3,5-dinitrosalicylic acid in H_2O_{dest} (5min).
- Add phenol, potassium sodium tartrate and Na_2S until dissolved.

4.8.3 Procedure

The DNS-test is done according to Miller [18]. The used procedure is described as follows:

- 50 µL sample + 50 µL DNS (+ 1 blanc (50 µL H_2O_{dest} + 50 µL DNS)) in 1.5 mL tubes. The samples have to be diluted to have a maximum glucose concentration of 2 g/L.
- Centrifuge 5 s at 3,000 rpm.
- 5 min in heat bath (100°C).
- 5 min in ice bath (0°C).
- Centrifuge 5 s at 3,000 rpm.
- Add 500 µL H_2O_{dest}.
- Transfer the samples (+blanc) to cuvettes.
- Photometric measurement at OD_{540}.

4.8.4 Calibration

The calibration curve is done by doing the DNS-test first with glucose solutions of the predefined concentrations 0.5, 1.0, 1.5 and 2.0 g/L. The test is done in triplicate to ensure an exact conversion factor. The conversion factor is the slope of the best-fit straight line (calibration curve) going through $(0, 0)$ and is defined by Equation 4.3. It was calculated using Microsoft Excel^{TM}.

$$m = \frac{d\, c_{cal}}{d\, Abs_{cal}} \tag{4.3}$$

m:	Conversion factor
$d\, c_{cal}$:	Change in concentration of calibration solutions
$d\, Abs_{cal}$:	Change in Absorption of calibration solutions

4.8.5 Measurement and evaluation

The testing of the samples is done at the same time as the calibration to ensure equal conditions for the calibration solutions and test samples during the procedure. For every DNS-test a new calibration was done. The test is only accurate in the range between 0-2 g/L glucose concentration. The dilution factor was obtained with Equation 4.4.

$$df = \frac{c_{cal}}{c_{max}} \tag{4.4}$$

df:	Dilution factor
c_{cal}:	Maximum concentration allowed by the calibration resolution
c_{max}:	Maximum (starting) concentration in the Medium

The OD_{540} absorbance values of the test samples are inserted in Equation 4.5 to calculate the glucose concentration.

$$S = \frac{Abs_{test} \cdot m}{df} \tag{4.5}$$

S:	Glucose concentration
Abs_{test}:	Absorption of the sample
m:	Conversion factor
df:	Dilution factor

4.9 Product activity measurement

4.9.1 Composition of the ABTS solution

The ABTS master-mix is prepared from the following three solutions and ABTS as a solid:

Table 4.5: Composition of ABTS solution in H_2O_{dest}

Substance	Amount in pre-solutions	Percentage in master-mix
Phosphoric acid	50 mM, pH6.3	90 % v/v
Glucose	20 % w/v	5 % v/v
Peroxidase	50 U	5 % v/v
ABTS		0.05 % w/v

The peroxidase solution and the master-mix have to be prepared freshly for each analysis. The master-mix has a durability of one day. All components are mixed at 200 rpm agitation by magnetic stirring. The usual end volume used is 20 mL, respectively 18 mL phosphoric acid, 1 mL glucose, 1 mL peroxidase and 10 mg ABTS.

4.9.2 ABTS procedure and evaluation

The procedure of the GOX activity test is as follows:

- In 3 mL cuvettes, fill 1.4 mL ABTS master-mix and 100 µL undiluted sample.
- Wait 30min.
- Spectrophotometric measurement at 420 nm.

The OD_{420} absorbance values of the test samples are inserted in Equation 4.6 to calculate the corresponding Activity. The equation was derivated from the Beer-Lambert law [19]. One activity unit U is defined as µmole of oxidated glucose per minute.

$$A = \frac{[\text{Abs} / t] \cdot V_{react}}{\varepsilon \cdot d \cdot V_{sample} \cdot df}; \quad [A] = \text{U/ml} \tag{4.6}$$

Abs:	Absorption
t = 30 min:	Test time
V_{react} = 1.5 mL:	Reaction volume
ε = 36 M^{-1} cm^{-1}:	Molar extinction coefficient of $ABTS_{ox}$ at 420 nm (for activity expressed as U/ml) [20]
d = 1cm:	Cuvette width
V_{sample} = 0.1 mL:	Sample volume
df:	Dilution factor

4.10 Chromium reduction assay

4.10.1 Procedure

The procedure of chromium reduction measurement is as follows:

- Prepare test tubes for all fermentation samples, calibration solutions and the blank.
- Add 4.8 mL H_2O_{dest}.
- Add 100 µL sample.
- Add 50 µL 21 mM diphenylcarbazide.
- Add 100 µL H_2SO_4 and mix the solution by vortexing.
- Spectrophotometric measurement at 540 nm.

4.10.2 Calibration

The calibration curve is done by running the procedure with Cr(VI)-containing solutions of the concentrations 10, 20, 30, 40 and 50 ppm. As a source of hexavalent chromium, $K_2Cr_2O_7$ (Potassium dichromate) is used. The conversion factor is the slope of the best-fit straight line (calibration curve) going through $(0, 0)$ and is defined by Equation 4.3.

4.10.3 Measurement and evaluation

The testing of the samples is done at the same time as the calibration to ensure equal conditions for the calibration solutions and test samples during the procedure. For every assay a new calibration has to be done. To fit the samples into the range of 0-50 ppm, the samples have to be diluted. The dilution factor is obtained with Equation 4.4. The OD_{540} absorbance values of the test samples are inserted in Equation 4.7 to calculate the Cr(VI) concentration.

$$c_{Cr(VI)} = \frac{Abs_{test} \cdot m}{df} \tag{4.7}$$

$c_{Cr(VI)}$: Cr(VI) concentration
Abs_{test}: Absorption of the sample
m: Conversion factor
df: Dilution factor

4.11 Mathematical model

A table is created in Microsoft Excel 2016 to implement the Euler algorithm on the equations 3.5, 3.6 and 3.7. 720 iterations with 0.05h step size are implemented. As starting conditions, the values X_0, S_0 and P_0 obtained from the experiment, are used. The yield $Y_{S/X}$ is obtained from the average yield of the fermentation. The rate of product formation v_{max}, the half-velocity constants k_S, $k_{p,1}$ and $k_{p,2}$ and the correction factors m_X, m_S and m_P are set to 0. The variance of each component i (X, S and P) is calculated by comparing the experiment values with the iterated values of the model (see Equation 4.8).

$$S_i^2 = \frac{1}{n-1} \cdot \sum_{j=1}^{n} (c_{i,exp,j} - c_{i,model,j})^2 \tag{4.8}$$

The sum of the three partial variances S_X^2, S_S^2 and S_P^2 is calculated with Equation 4.9.

$$S^2 = \sum_{i=1}^{3} S_i \tag{4.9}$$

The Excel-add-in 'Solver' is used to determine the optimum set of the seven parameters to minimize the variance S^2 by an iterative converging evolutionary algorithm. The

algorithm parameters are:

Convergence =	0.0001
Mutation rate =	0.1
Size of statistical population =	100
Maximum time without improvement =	120 s

The relative standard deviation of each component is calculated with Equation 4.10 after the optimization.

$$S_i = 100\% \cdot \frac{\sqrt{S_i^2}}{n_i^{-1} \cdot \sum_{j=1}^{n} c_{i,exp,j}} \tag{4.10}$$

The Excel file (with all calculations and results included) is enclosed as an electronic attachment.

5 Results

5.1 Series 1: Small scale fermentation

5.1.1 Experiment 1.1: Small scale wit 2.5 g/L

A fermentation in a small scale was done to evaluate the fermentation behavior. The fermentation was carried out for 72 h in twelve 250 mL flasks in a shaking water bath. The water temperature was controlled to 28°C. 150 rpm where used for agitation. Every 6 h one flask was removed for analysis. This system was chosen because there was no possibility of sterile sample taking. The biomass concentration measurement was done directly after removing the flask. From the filtrated broth a sample of 1 mL was taken in a 1.5 mL tube. All samples where analyzed for GOX activity and glucose concentration at once after the complete fermentation. The fermentation was carried out as duplicate. The results are shown in Figure 5.1.

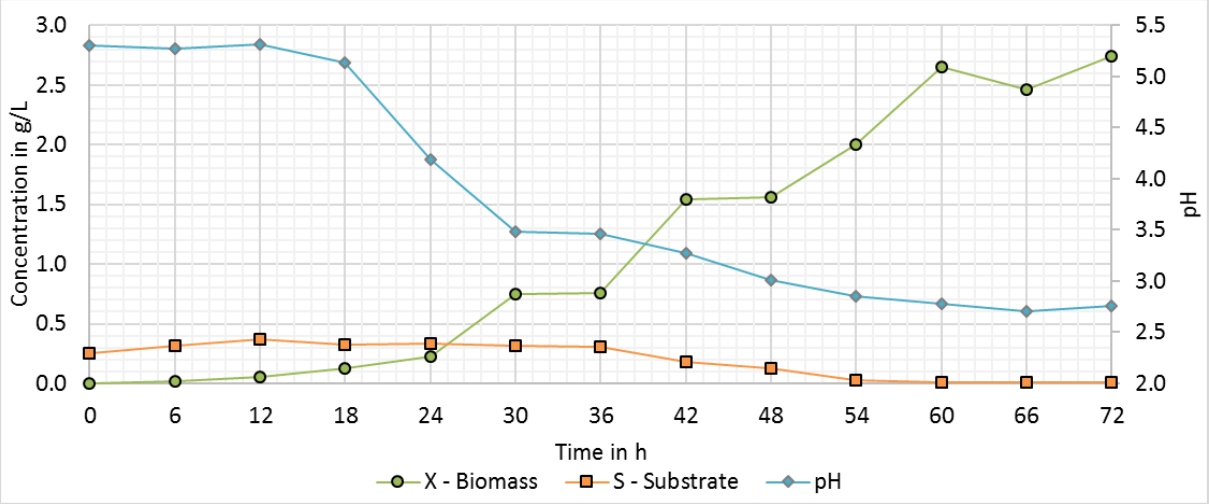


Figure 5.1: Small scale fermentation: 2.5 g/L glucose

The biomass growth shows a lag-phase from 0 to ∼18 h, an exponential log-phase from ∼18 to ∼60 h and a stationary (or dying) phase after ∼60 h. There is a big pH-drop measurable because of the produced citric acid as a main primary metabolite of *Aspergillus niger*. The substrate was completely consumed after 60 h. Also, no further citric acid was produced after that time.

5.1.2 Experiment 1.2: Small scale wit 50 g/L

Experiment 1.1 was repeated under the same conditions but with manual sequential pH control and 50 g/L glucose concentration. The pH of all flasks had been adjusted every 6 h. The glucose concentration had been increased because the standard glucose concentration in LMM (2.5 g/L) was not sufficient to produce GOX. Because 50 g/L was suggested as the optimum concentration for the GOX production in Ed8 [2], the LMM composition was adjusted in that matter. The results are shown in Figure 5.2.

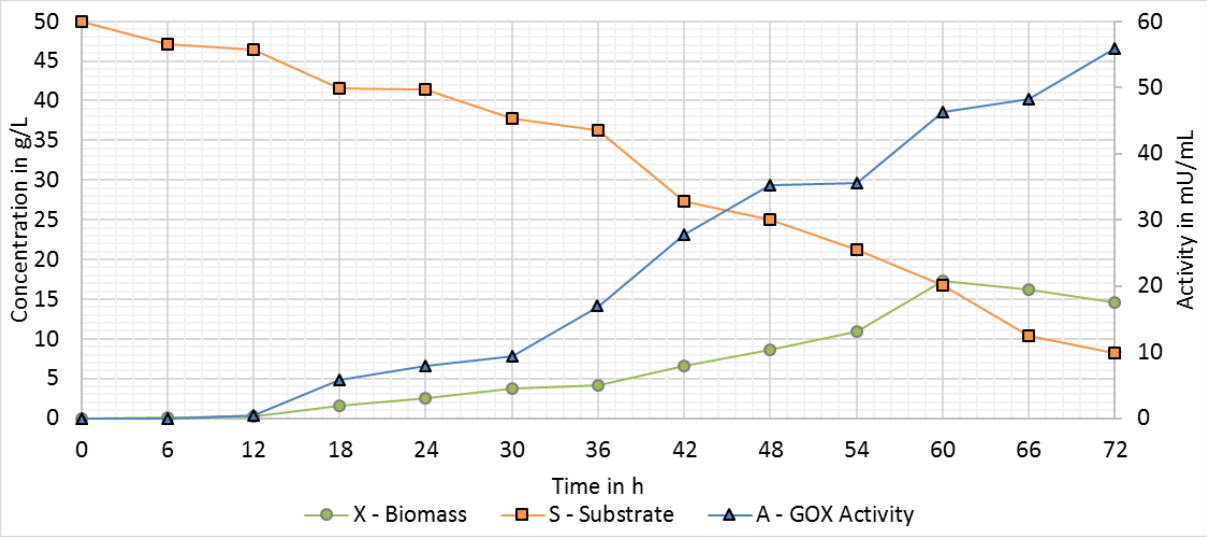


Figure 5.2: Small scale fermentation: 50 g/L glucose

83,60 % of the substrate was consumed and the activity curve looks similar to the previous study [2]. The log-phase was determined to start at about 24 h and to end at about 60 h. In contrast to Experiment 1.1, a high GOX activity was achieved due to the use of more substrate. This shows the high dependency of the GOX production on substrate concentration. Also the end biomass concentration (at 60 h) has increased by 553,96 % compared with 2.5 g/L. In the future experiments, 50 g/L glucose had been used instead of the low concentration suggested by Lee [16].

5.1.3 Mathematical model

The mathematical model described in Section 4.11 was supplied with the data of Experiment 1.2. The used interval was the time of exponential growth which was determined as 24 - 60 h. It has to be mentioned that there is a fluent transition between the growth phases. Especially the start of exponential growth is hard to determine which

affects the accuracy of the model. The comparison of experimental data and the model is shown in Figure 5.3.

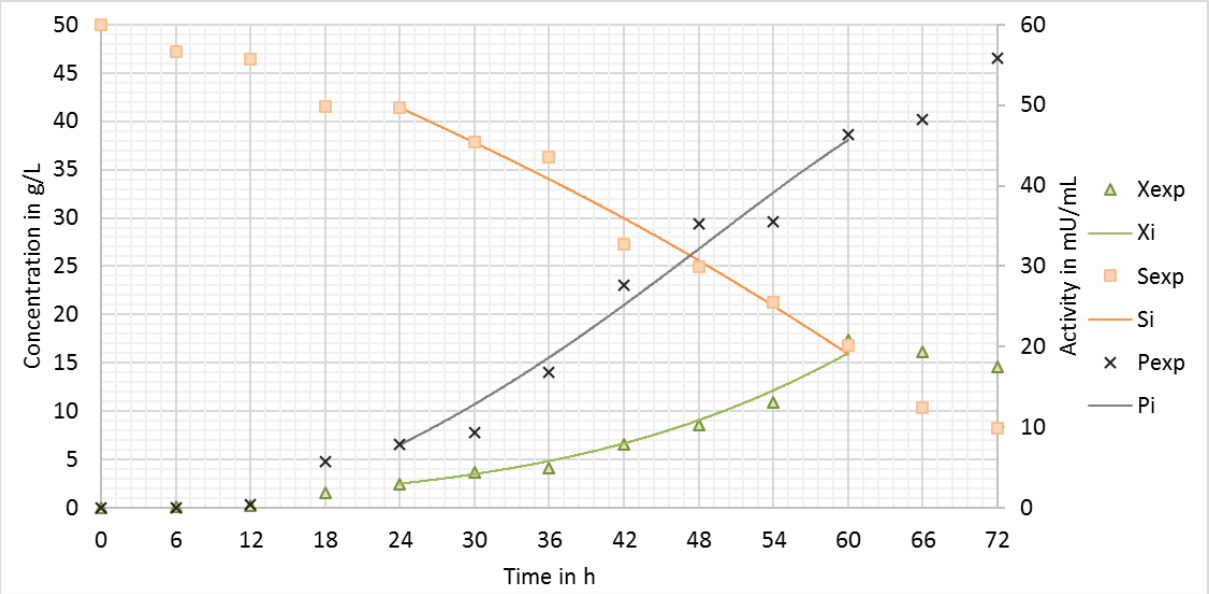


Figure 5.3: Solved differential equations for all components

The parameters introduced in Section 3.6.3 where calculated with the Euler method by running an optimization algorithm as described in Section 4.11. The calculated parameters are shown in Table 5.1.

Table 5.1: Parameters of the mathematical model

Parameter	Value	Unit
μ_{max}	0.05378	1/h
k_S	25.165	g/L
$Y_{S/X}$	2.001	$\frac{g/L}{g/L}$
v_{max}	5.26524	$\frac{U}{L \cdot h}$
$k_{p,1}$	10.798	g/L
$k_{p,2}$	8.384	g/L
m_X	0.023	1/h
m_S	0.010	1/h
m_P	0.023	1/h

The calculated relative standard deviations according to Equation 4.10 are:

$$S_X = \pm 10.709\ \%$$
$$S_S = \pm 5.127\ \%$$
$$S_P = \pm 10.699\ \%$$

5.2 Series 2: Small scaling-up fermentation

Because of failed experiments in a magnetically stirred 0.5 L reactor where the same conditions as in Section 5.1 but with 50 g/L had been used, a new fermentation series was started again in 250 mL flasks. This time, a gradient of working volumes had been created with the volumes 50, 100, 150 and 200 mL to show the dependency of growth rate and GOX activity on aeration. The results are shown in Figure 5.4

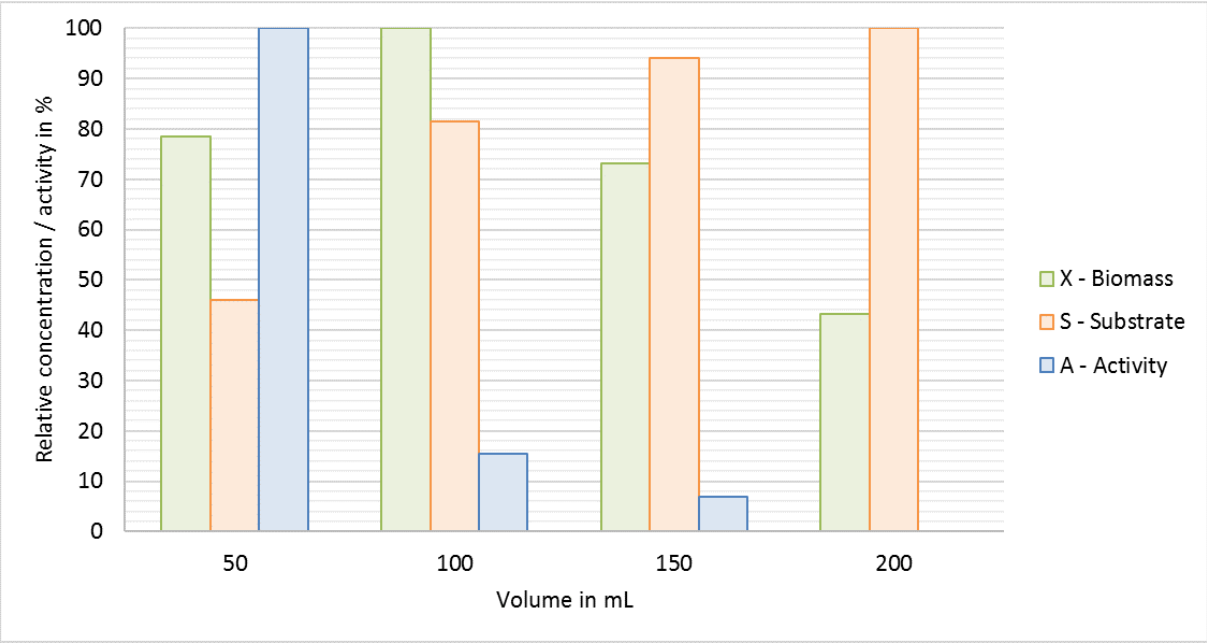


Figure 5.4: Small upscale with a working volume gradient

From 200 mL up there is no GOX activity detectable. A maximum biomass concentration of 24.220 g/L has been achieved at 100 mL, a maximum activity of 25.021 mU/ml at 50 mL. The substrate consumption seems to be directly related to the product formation. It was found that the fermentation is highly depending on oxygen supply. Where

the specific area of oxygen transfer is suitable for small reaction volumes, it will decrease with higher volumes and one has to supply additional air by active aeration. It could be shown that the specific surface of oxygen transfer and the specific power entry are having a big impact on the Ed8 to set the focus on biomass growth (see 100 mL) or product formation (see 50 mL). High aeration and agitation seem to force the Ed8 to produce more GOX.

5.3 Series 3: Labfors 5 fermentation

The Labfors 5 is a stirred tank bench top reactor with 2 L working volume. First, a fermentation was carried out without aeration to ensure that the behavior of the small up-scaling fermentation was not due to the changing power entry per volume. Then aeration was supplied with 150 and 250 rpm. The temperature was controlled to 28°C by the Labfors controller as well as the pH which was controlled to 5.3 by an automated pump feeding a 20 % NaOH solution. The starting concentration of glucose was 50 g/L. The results are shown in Figure 5.5 - 5.7.

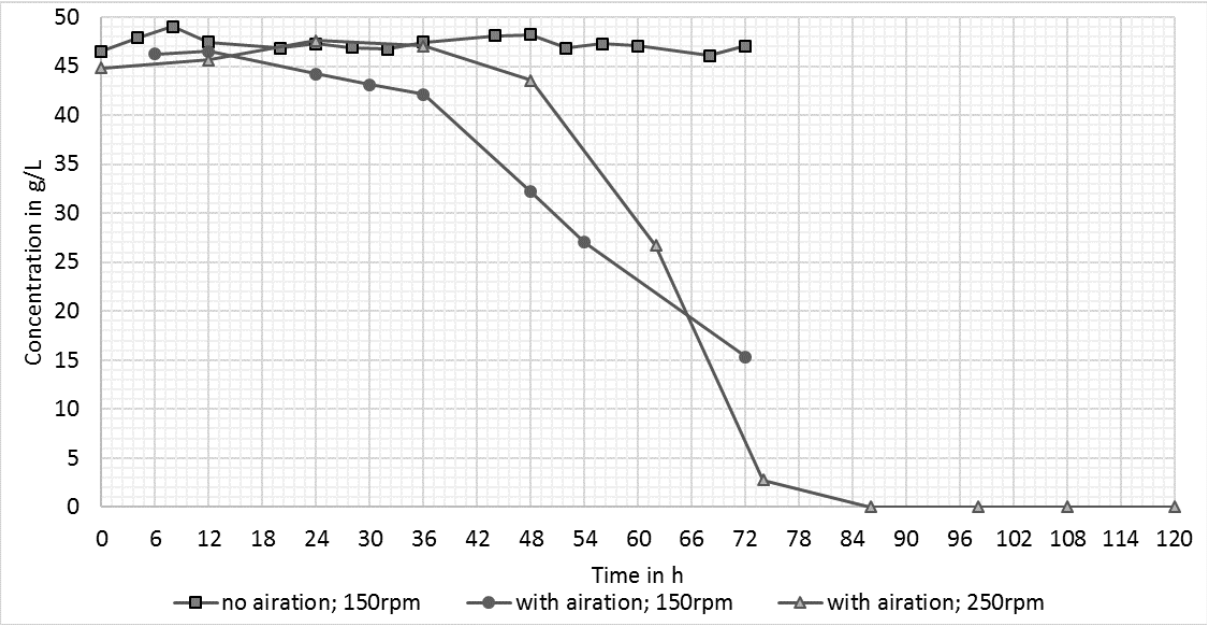


Figure 5.5: Labfors fermentation: Substrate

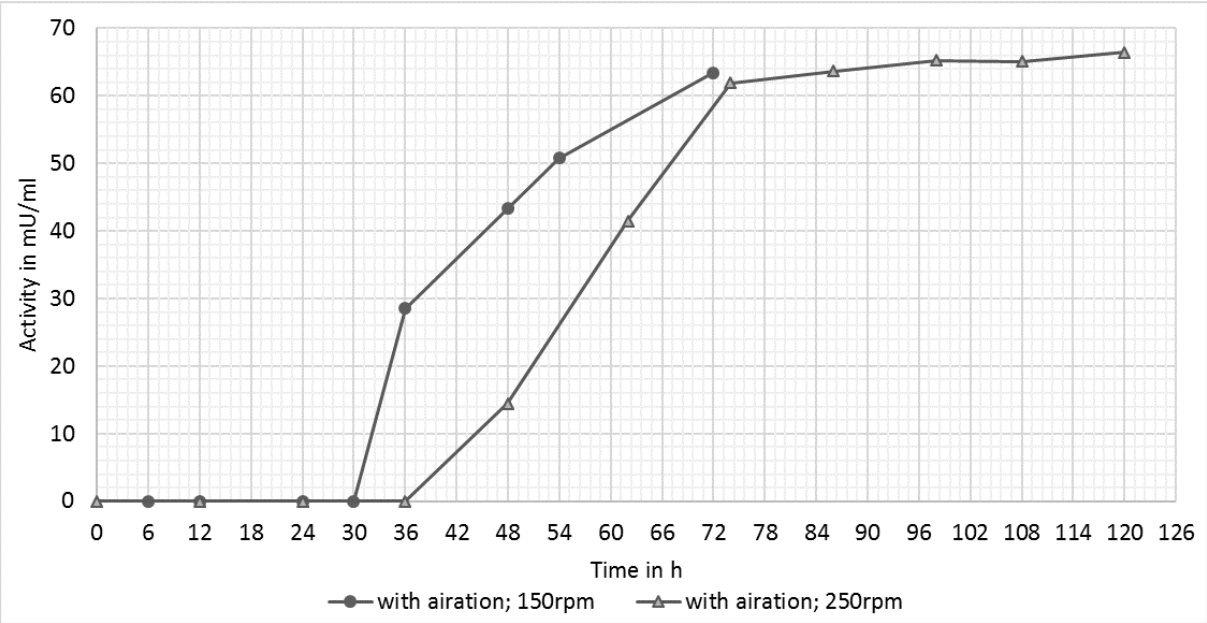


Figure 5.6: Labfors fermentation: Activity

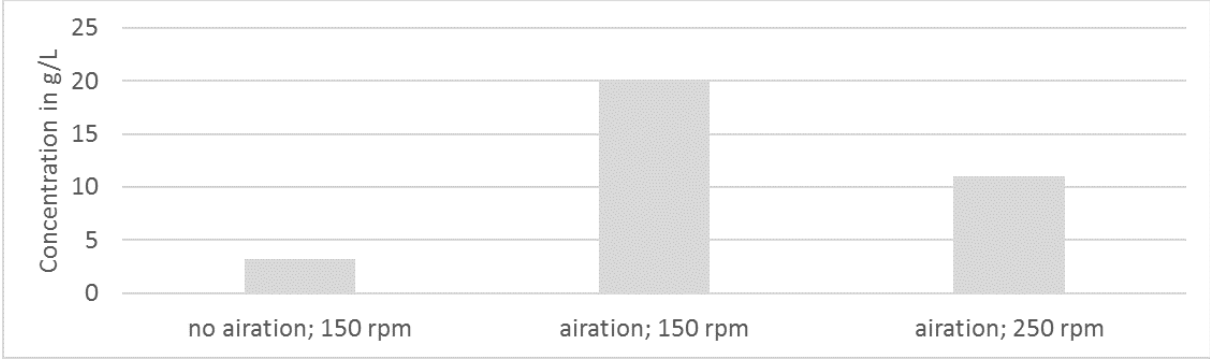


Figure 5.7: Labfors fermentation: Biomass

Without aeration there was no substrate consumption. The end biomass concentration was only 3.20 g/L. There was no GOX detectable. This shows the high dependency of the fermentation on aeration. With aeration there was a drop of substrate concentration starting at 36 h. The drop got bigger with higher agitation. After 48 h the substrate consumption of the 250 rpm fermentation got higher than the 150 rpm fermentation. Also the substrate was nearly fully consumed at 72 h.

With 150 rpm there was a higher consumption in the first 48 h which caused higher GOX production (Figure 5.6) and a higher growth rate (Figure 5.7). Because of the increased substrate consumption and GOX production in the beginning, both decreased later on. Also, high amounts of citric acid where produced. About 111 mL of 20 % $NaOH$ solution needed to be added to maintain the pH of 5.3. The productivity of the

fermentation might increase if no pH control is used because then the logistic secretion of citric acid will end up in a maximum, allowing the Ed8 to focus on GOX production. The 250 rpm fermentation showed less substrate consumption and GOX production until 48 h but then both constantly increased until the fermentation reached a state of maximum GOX activity (∼62 mU/ml) and completely used substrate after about 72 h.

5.4 Series 4: Stirred tank reactor fermentation

To investigate the response of the fermentation on glucose variation and the replacement with piloncillo, a stirred tank reactor with a working volume of 0.5 L was used. Due to difficulties with the Labfors 5 and the airlift reactor[1], this easier environment was chosen to get consistent data and to have a prevention against fermentations going wrong. It was tried to maintain the aeration and agitation conditions. The dependency of the fermentation on glucose concentration had been analyzed. A comparison was made between glucose and the more economical piloncillo. The summarizing results are shown below. The experiments are described in the following subsections.

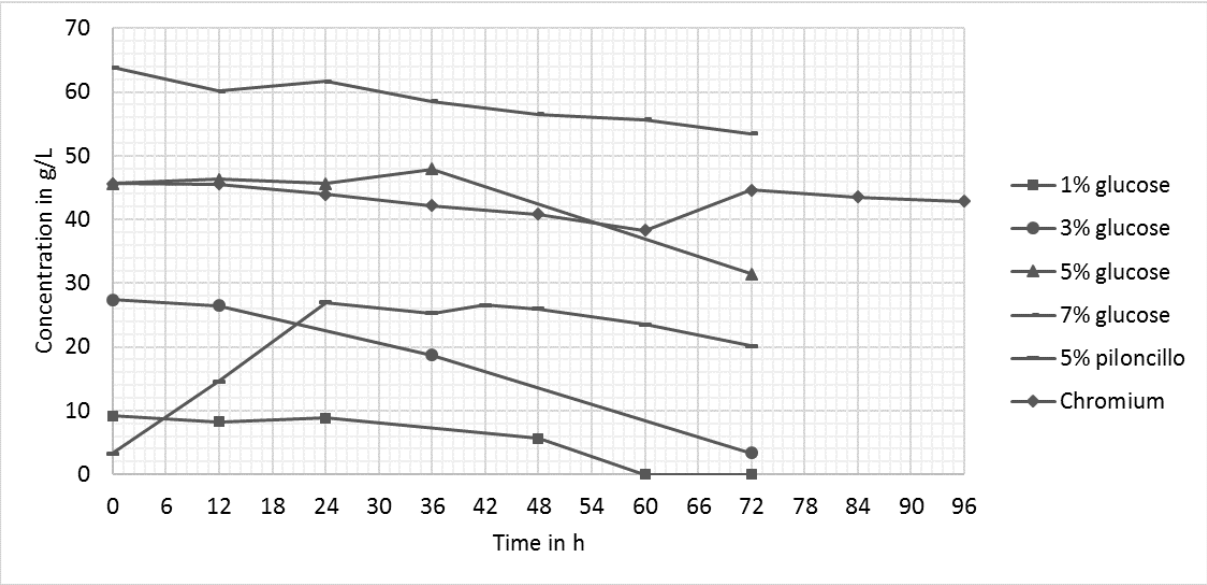


Figure 5.8: Stirred tank: Substrate

[1] The motor of the Labfors 5 got damaged; Using the airlift reactor was not suitable because of insufficient agitation causing the Ed8 spheres growing too big and blocking the circulation.

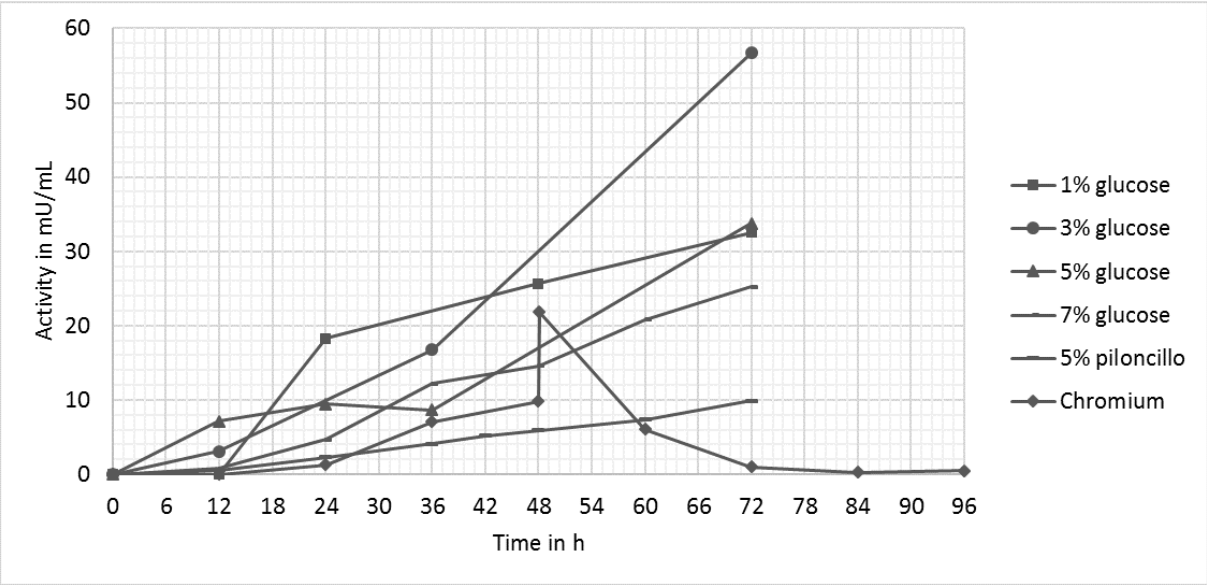


Figure 5.9: Stirred tank: Activity

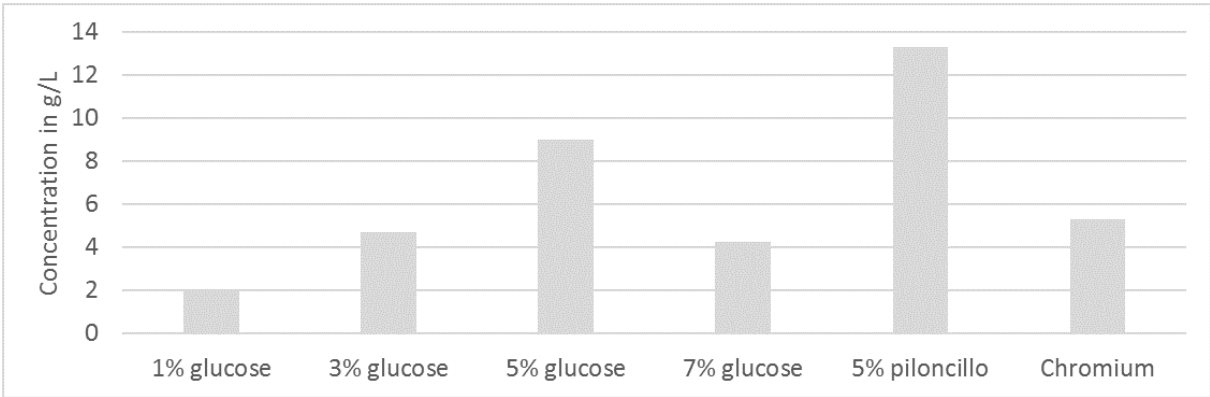


Figure 5.10: Stirred tank: Biomass

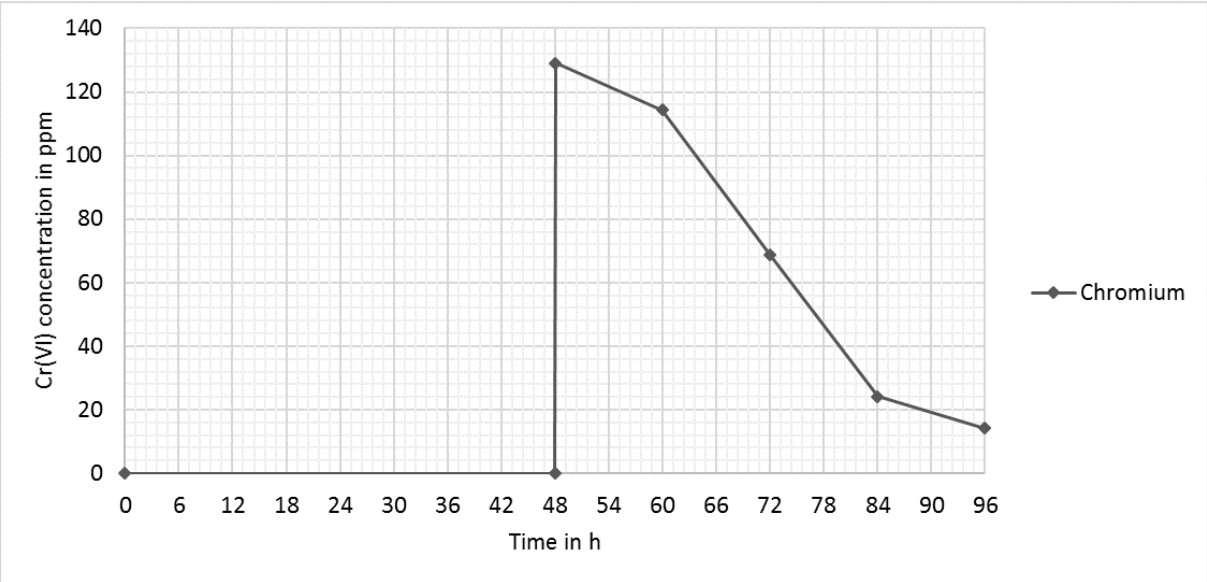


Figure 5.11: Stirred tank: Chromium reduction

5.4.1 Experiment 4.1: Comparison of glucose concentrations

According to Figure 5.10, the best glucose concentration for optimum biomass growth is 5 %. The reached biomass concentration is about twice as big compared to 3 and 7 % glucose. The concentration of 7 % seems to be too high and to inhibit the biomass growth; resulting in a decrease of GOX activity by 33.8 % compared with 5 % glucose (see Figure 5.9). The fastest increase of activity was reached with 1 %, the highest total amount with 3 % glucose. There was about the same ending activity reached with 1 (32.5 mU/ml) and 5 % (33.7 mU/ml) glucose.

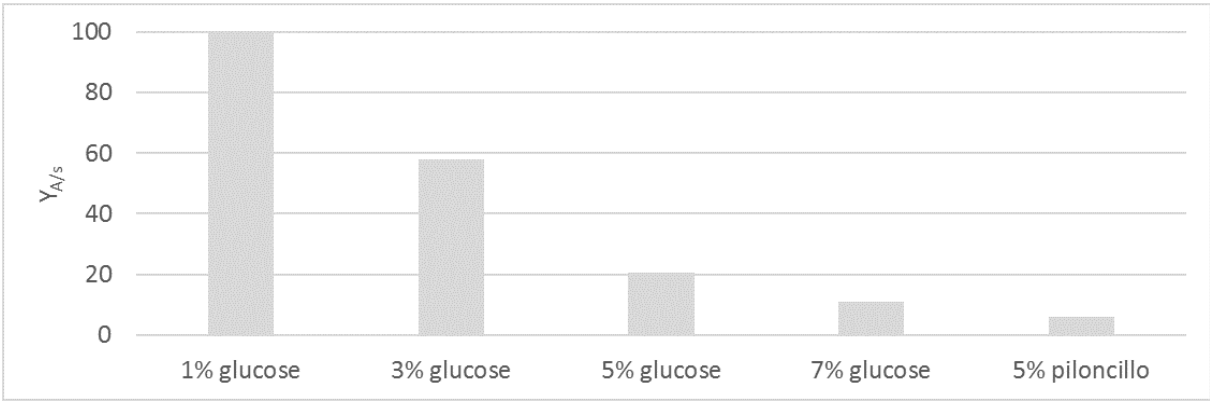


Figure 5.12: Stirred tank: Yield based on starting substrate concentration (normalized)

Figure 5.12 shows the course of Y_{A/S_0} (Yield; activity / starting substrate concentration). It shows that the highest yield is reached with 1 % glucose concentration. It

gets worse with higher concentrations of glucose, meaning that a higher substrate concentration will not lead to equivalently more GOX activity. Though, there has to be a minimum substrate concentration to get a sufficient secondary metabolism (see Experiment 1.1).

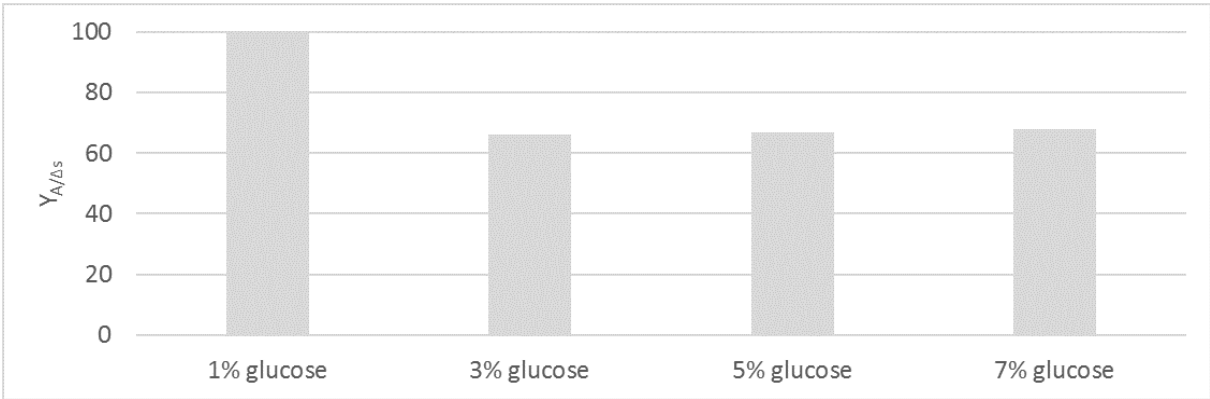


Figure 5.13: Stirred tank: Yield based on total substrate consumption (normalized)

Figure 5.13 shows the course of $\mathrm{Y_{A/\Delta S}}$ (Yield; activity / total substrate consumption). It shows that the effectiveness of GOX production is nearly independent on the starting glucose concentration. Only with 1 % glucose there is a deviation of 49.5 % compared with the average yield of the other fermentations. 1 % glucose seems to be the optimum concentration to economically produce GOX with the Ed8 microorganism.

5.4.2 Experiment 4.2: Piloncillo

The concentration curve of piloncillo (Figure 5.8) shows a maximum at 24 h because only reducing sugars (the glucose and fructose parts of piloncillo) can be detected by the DNS test. The undetectable sucrose part gets split to monosaccharides through invertase enzymes first, what explains the positive slope until 24 h. After that, substrate consumption outweighs glucose formation and the activity level increases (see Figure 5.9). The end activity is reduced by 70.73 % compared to pure glucose.

5.4.3 Experiment 4.3: Chromium reduction

The glucose concentration curve of the chromium reduction experiment shows a period of increase from the hours 60 to 72. This could be justified with cell dying which could release intracellular glucose or with the reduction of already oxidated glucose due to

higher reducing activity. The end biomass concentration (Figure 5.10) is reduced by 41.48 % compared to the standard 5 % fermentation. The end concentration of Cr(VI) is 14.17 ppm, so 95.28 % of the chromium had been reduced. The Activity graph (Figure 5.9) shows a big step from 48 h to five minutes after the addition of chromium. This could be caused by the highly oxidizing Cr(VI) affecting the ABTS reaction. Later on, the GOX level falls to zero.

5.4.4 Mathematical model of chromium reduction

The mathematical model was created according to the procedure described in Section 3.6.4 and Section 4.11. The parameters of the differential equation (Equation 5.1) had been found:

$$\frac{dCr}{dt} = -0.12184\ h^{-1} \cdot Cr \cdot \left(1 - \frac{Cr}{135.570\ ppm}\right) \tag{5.1}$$

The calculated relative standard deviation according to Equation 4.10 is:

$$S_{Cr} = \pm 5.774\ \%$$

A comparison of the experimental data and the model is shown in Figure 5.14.

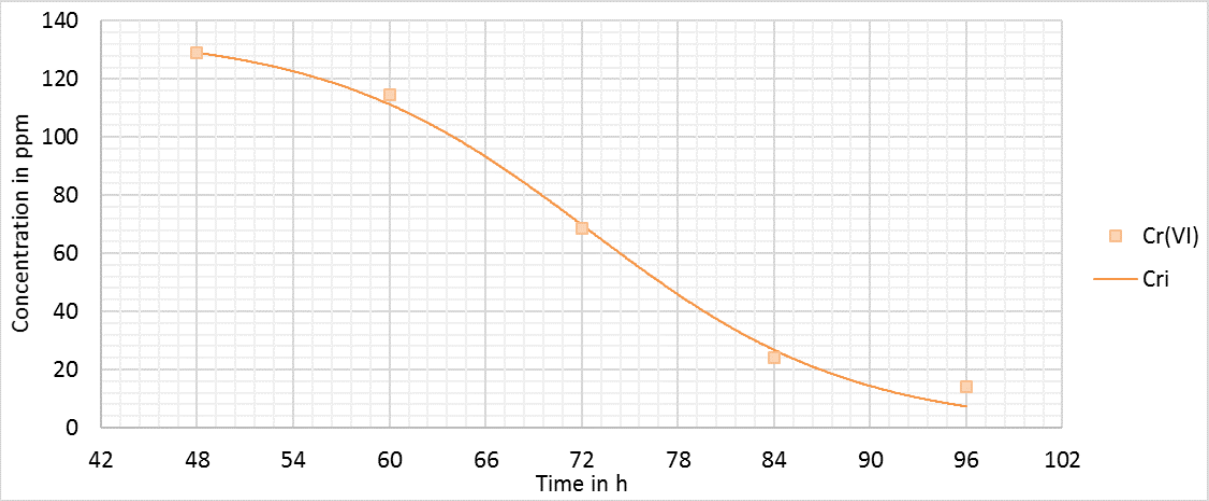


Figure 5.14: Stirred tank: Mathematical model of chromium reduction

The approximation curve does not fit perfectly to the data, especially in the last stage. Additional dependencies have to be investigated to obtain a better accuracy of the model. Nevertheless, it could be shown that the chromium reduction follows a logistic behavior with a degradation rate of about 0.12 h^{-1}.

6 Discussion

6.1 Dependency on aeration and agitation

The results confirm that aeration is a factor to be optimized and it was proven that it has a high impact on the fermentation. For example in the Labfors, the biomass concentration was increased up to 6.23 times compared to the aeration-less fermentation. Without aeration, GOX activity was not even detectable. It was also found that only increasing agitation and aeration is not sufficient. As shown in previous studies [21], there has to be done an experiment design to determine the optimum parameter set of agitation and aeration. This will have to be done in future investigations because the Ed8 strain behaves differently from other strains of *Aspergillus niger*.
Also the used equipment has a significant effect. For the agitation, a helical ribbon impeller was used, combined wit a flat wall. The usage of baffles will highly increase inner turbulence and oxygen transfer in the medium. The sparging equipment has to be chosen in a way that the residence time and specific surface of oxygen transfer are getting maximized by as small air bubbles as possible. It is expected that the growth rate and product expression of the small scale can be achieved or even exceeded with the right aeration.

6.2 Dependency on substrate concentration

The best substrate concentration for optimum biomass growth (9.01 g/L) is 5 % but there is more GOX activity measurable with lower starting concentrations. Probably the Ed8 sets the focus on growth at high substrate concentrations. In addition, it has to produce less GOX due to the big overshoot of substrate causing high rates of hydrogen peroxide formation. The best yield of product formation referred to substrate consumption was found to be at 1 % glucose. With that concentration the Ed8 seems to switch the focus from further biomass growth to better protection through GOX activity. Also a reason might be that with the low amount of glucose in the medium, more GOX molecules are needed to achieve the desired activity for optimum protection.

6.3 Piloncillo as a substrate

The biomass concentration increased by 35.89 % compared to the standard 5% glucose medium but the GOX production decreased heavily by 70.74 %. The Ed8 seems to set the focus mainly to biomass growth and to hydrolyze the sucrose part of the piloncillo. This might be a good strategy for reducing chromium because of intracellular reductive mechanisms but not for the aim of producing GOX. The high growth rate with piloncillo as a substrate might also be attractive for the production of citric acid which is the main primary metabolite of *Aspergillus niger*, a mold that is widely used in the industry of citric acid production. Especially the price makes piloncillo a very attractive alternative to glucose with about 1.9 USD (alibaba.com) against 154 USD (sigmaaldrich.com) per kilogram.

6.4 Chromium reduction

48 h after adding chromium, 95.28 % had been reduced. The reduction could be optimized by varying the substrate concentration as well as agitation and aeration conditions. For the chromium reduction it seems to be important to have a quick biomass growth in the first 48 h before the addition because after the addition of chromium the biomass growth decreases drastically because of the high concentration of Cr(VI) that is added at once. Because the Cr(VI) is a strongly inhibiting factor of biomass growth there has to be as much Ed8 as possible before the addition to manage the situation and rapidly reduce the chromium.

6.5 Reproducability

Every experiment was carried out only once due to time restriction. Only the experiments of Series 1 where carried out as duplicates. A reason for that had been a constant loose of time caused by contamination of four fermentations and the change of the fermentation system for several times. It was planned to run all fermentations in the Labfors 5 to keep a certain degree of reproducibility and comparability but the system needed to be changed after the first experiments due to a damaged motor. An experiment in the airlift reactor did not work because the Ed8 spheres got too big and blocked the circulation due to too much aeration and insufficient agitation. The system

had to be switched again to the 0.5 L stirred tank reactor where the last experiments could be done. Therefore, the data provided is not comparable with other studies but at least allows a limited comparability between experiments from within this thesis.

6.6 Mathematical model

The mathematical model shows a reasonable approximation of the real fermentation metabolism. Though, especially the activity is hard to approximate due to the high inaccuracy and deviation of the assay measurements. This is why the standard deviation from the activity results could not fall below $\pm 10.70\ \%$. The model with the introduced assumptions would not work well without the correction factors. It shows that the fermentation is depending on more limiting and inhibiting factors like dissolved oxygen, agitation or product concentration. The model requires high data quality and quantity. Further analysis with more data consistency is needed to obtain the exact metabolic behavior and to find a corresponding model. The preliminary work that was done shows that the very simple structure proposed for the model could be useful for a further general description of the fermentation, including all parameters that are involved in it.

6.7 Future prospects

6.7.1 Optimizing adjustments

In future investigations there has to be done an experiment design to optimize the fermentation in terms of maximizing the yield (product/substrate) by varying the fermentation conditions. It furthermore can be discovered how the mathematical model will change through parameter variation.

The main parameters discovered in this thesis affecting the fermentation performance are substrate concentration, agitation and aeration. It would be convenient to fix the rate of aeration by controlling the pO_2 during the fermentation. With that control, there would be a constant level of oxygen saturation throughout all experiments which would allow a focus on substrate concentration and agitation. With only these two parameters, an experiment design would be easy to do and would rapidly lead to the optimum

parameter set for the GOX production. These parameters could then be maintained for the continuous fermentation described in the following section.
An adjustment of the starting cell concentration could be done determine the starting concentration that leads to the fastest increase of biomass growth or product formation. Furthermore, a sequential fermentation can be tested by using a preculture that is incubated for e.g. 24 h before transferring the biomass to the reactor.

6.7.2 Continuous fermentation of GOX

Aspergillus niger produces GOX as a protection against bacteria because the catalytic reaction (see Figure 3.1) produces hydrogen peroxide which can oxidize and damage the cell wall of the bacteria [8]. The Ed8 adjusts the GOX production depending on the glucose and hydrogen peroxide concentration to save resources [7]. There is also a maximum GOX activity that the Ed8 is heading to (see Figure 5.6). Therefore a continuous reactor could be chosen to maximize the secretion of GOX. For example a perfusion reactor would have a constant dilution rate to maintain a constantly high substrate and low GOX/H_2O_2 concentration to force the Ed8 to permanently produce GOX with the maximum product formation velocity.

6.7.3 Airlift fermentation

Although the airlift reactor got blocked, 17.09 % more GOX activity was detectable compared with the 0.5 L reactor at the same substrate concentration of 50 g/L glucose. By widening the annular gap to prevent blocking, the airlift reactor could be a very usable system due to its naturally high oxygen transfer. Problematic for the optimization of the airlift process is the high diameter of the Ed8 spheres, causing cell deactivation and even cell death inside the spheres. The experiments have shown that a certain agitation is needed to keep the spheres at a restricted size. One could provide that agitation by using a small impeller.

7 Conclusion | Zusammenfassung

7.1 Conclusion

1. The experiments show a high dependency of GOX production on aeration. Where in the small scale of 50 mL working volume the oxygen transfer is sufficient, one has to use active aeration at higher working volumes. Also important is the agitation or more specifically the power entry per volume which affects the size of the Ed8 agglomerates. To run the fermentation system on a large scale, one has to find the best parameter set of agitation and aeration to achieve as much and as small Ed8 agglomerates as possible. The glucose concentration is another factor that has to be balanced with the other parameters. It should be optimized to get higher yields of formed product referred to the amount of used substrate. By finding the best parameter set of the up-scaled fermentation, one can reach or even exceed the product formation rate of the small scale.
2. A mathematical model could be created but the relative standard deviations are yet very high in the range of 5.13 % to 10.71 %. More experiment data would be needed to obtain a suitable and exact model. To provide more exactness in the model, one could add additional dependencies like inhibitions and limitations.
3. Due to time restriction, only one chromium reduction experiment could be performed. More experiments would be needed to show a dependency on GOX production and biomass growth. It could be shown that piloncillo is not the best carbon source for producing GOX with Ed8. With pure glucose the Ed8 can rapidly focus on the production of complex metabolites like GOX. The piloncillo forces the Ed8 to hydrolyze the sucrose part first and to focus on biomass growth.

7.2 Zusammenfassung

1. Die Experimente verdeutlichen die hohe Abhängigkeit der GOX-Produktion von Begasung. Wo im kleinen Maßstab mit 50 mL Arbeitsvolumen der Sauerstofftransfer ausreicht, muss bei größeren Arbeitsvolumen aktive Begasung angewandt werden. Ebenfalls wichtig ist die Durchmischung, bzw. genauer die eingetragene Leistung pro Volumen, die die Größe der Ed8 Agglomerate beeinflusst. Um das Fermentationssystem im größeren Maßstab zu betreiben, muss das beste Parameter-Set aus Durchmischung und Begasung gefunden werden, bei dem möglichst kleine Ed8-Agglomerate erzielt werden. Die Glucosekonzentration ist ein weiterer Faktor, der mit den anderen Parametern bilanziert werden muss. Sie sollte dahingehend optimiert werden, möglichst hohe Erträge an Produktbildung bezogen auf Substratverbrauch zu erzielen. Indem das beste Set aus Parametern für die Maßstabsübertragung gefunden wird, kann die Produktbildungsrate des kleinen Maßstabs erreicht oder sogar überschritten werden.
2. Ein mathematisches Modell konnte erstellt werden, jedoch sind die relativen Standardabweichungen im Bereich 5,13 % bis 10,71 % noch sehr hoch. Mehr experimentelle Daten wären notwendig, um ein geeignetes und exaktes Modell zu erhalten. Um erhöhte Exaktheit im Modell zu gewährleisten, könnten zusätzliche Abhängigkeiten, wie Inhibitionen und Limitationen, eingeführt werden.
3. Wegen der zeitlichen Begrenzung konnte nur ein Experiment zur Reduktion von Chrom durchgeführt werden. Mehr Experimente wären notwendig, um eine Abhängigkeit von GOX-Produktion und Biomassenwachstum nachzuweisen. Es konnte gezeigt werden, dass Piloncillo (Panela) nicht die beste Kohlenstoffquelle für den Stamm Ed8 ist, um GOX zu produzieren. Mit reiner Glucose kann der Stamm Ed8 schnell auf die Produktion von komplexen Metaboliten wie GOX fokussieren. Der Piloncillo zwingt den Stamm Ed8 zunächst zur Hydrolyse der Saccharose-Anteile und zum Fokus auf das Biomassenwachstum.

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