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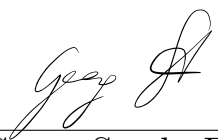
DIPLOMA THESIS

Impact of Carbon and Nitrogen Source on Erythritol Production in *Trichoderma reesei*

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Kurzfassung

Ernährungsbezogene Krankheiten sind eine zunehmende Bedrohung hinsichtlich öffentlicher Gesundheit. Übermäßiger Konsum von Zucker kann dabei einer Menge an verschiedenen Krankheiten zugeordnet werden. Daher ist es von großem Interesse, eine sichere Alternative zu Zucker bei gleichbleibender Süße zu finden. Eine solcher Alternativen ist Erythritol. Es hat keinen Einfluss auf den Blutzuckerspiegel, zeigt keine ungewollten Nebeneffekte und hat eine Süße von 60 - 80 % von Haushaltszucker [1]. Die meisten industriellen Prozesse verwenden mutierte Stämme von Hefen zur Produktion von Erythritol [2]. In dieser Diplomarbeit wird die Entwicklung eines Prozesses zur Produktion von Erythritol im filamentösen Pilz *Trichoderma reesei* diskutiert. Diese Arbeit ist Teil eines größeren Projekts, welches auf einen industriell nutzbaren Prozess zur Erythritolproduktion zielt. In diesem Organismus wäre es möglich, einen industriell brauchbaren Prozess zur Produktion von Erythritol zu entwickeln. Zwei modifizierte Stämme von *T. reesei* werden verglichen. Das Hauptziel der Arbeit lag darin, den Einfluss bestimmter Parameter auf die Ausschleusung von Erythritol ins Medium zu verstehen und gleichzeitig die Ausbeute zu erhöhen. Als Kohlenstoffquellen wurden Glukose, Xylose, und Laktose verglichen. Glukose wurde als einzige Option detektiert, welche zum sekretieren von Erythritol anregt. Als Stickstoffquellen wurden Ammonium, Nitrat, Hefeextrakt und Urea getestet. Urea stellte die optimale N-Quelle unter den Untersuchten dar. Mit einem Design of Experiments (DoE) Ansatz wurde weiters versucht, den Einfluss der Konzentrationen beider Parameter auf die Kultivierung besser zu verstehen. Schlussendlich wurde zuzüglich der Entdeckung des Einflusses vom pH-Wert die Kondition von 70 g/l Glucose und 80 mM Urea bei pH unter 5 als optimaler Prozess erklärt. Mit diesem verbesserten Prozess war es möglich, die volumetrische Produktion von Erythritol von 28 auf 978 mg/l (+ 3425 %), die Ausbeute bezogen auf Glucose von 0.3 auf 15.27 mg/g (+ 4990 %) und die Ausbeute bezogen auf das Myzel von 3 auf 80.08 mg/g (+ 2569 %) zu erhöhen.

Abstract

Dietary diseases are an increasing danger to public health. Excessive sugar consumption can be linked to a plethora of avoidable health conditions. Thus, finding a safe alternative to added sugars while keeping sweetness similar is of great interest. One possible alternative is erythritol. It does not affect blood glucose levels, shows no unwanted side effects, and has a sweetness of 60-80 % of sucrose [1]. Most industrial practices to produce erythritol include mutagenized strains of yeasts [2]. In this thesis, we will discuss the impact of both carbon and nitrogen sources on the production of erythritol in the filamentous fungus *Trichoderma reesei*. This thesis is part of a bigger project, aiming to develop an industrially viable process for erythritol production in *T. reesei*. *T. reesei* can grow on agricultural waste products. This could lead to an industrially viable process for erythritol production. Two modified strains of *T. reesei* were compared. The main research goals concerned understanding the impact of different compounds on the excretion of erythritol into the supernatant and improving volumetric yield. The tested carbon sources were glucose, xylose and lactose. Glucose was found to be the only viable option. Tested nitrogen sources were ammonium, nitrate, yeast extract and urea. The best source of nitrogen was found to be urea. Using a design of experiments (DoE) approach, it was possible to better understand the impact of different glucose and urea concentrations on the cultivation. Finally, after investigating the impact of pH, the optimal conditions of glucose and urea were found to be 70 g/l glucose and 80 mM urea while maintaining a pH below 5. Using this improved process, the volumetric erythritol production was increased from 28 to 978 mg/l (+ 3425 %), the yield of erythritol per g glucose from 0.3 to 15.27 mg/g (+ 4990 %) and the yield of erythritol per g mycelium from 3 to 80.08 mg/g (+ 2569 %).

Contents

1	Introduction	5
1.1	Erythritol	5
1.2	Biotechnological Production	5
1.3	Filamentous Fungi as Production Organisms	6
1.4	Impact of Process Parameters	7
1.4.1	Carbon sources	7
1.4.2	Nitrogen sources	9
1.5	Research Goals	10
2	Materials and Methods	11
2.1	Strains	11
2.2	Media composition	11
2.2.1	Carbon Sources	12
2.2.2	Nitrogen Sources	12
2.3	Cultivation Parameters	13
2.3.1	General Parameters	13
2.4	Evaluation Parameters	13
2.4.1	Biomass	13
2.4.2	Sugar consumption and Polyol Production	13
3	Results and Discussion	15
3.1	Carbon Source	15
3.1.1	Comparison of Xylose, Lactose and Glucose	15
3.2	Nitrogen Source	17
3.2.1	Comparison of Ammonium, Nitrate, Yeast Extract and Urea on Biomass	17
3.2.2	Comparison of Ammonium, Nitrate, Yeast Extract and Urea on Biomass and Polyols	18
3.3	DoE	19
3.3.1	DoE-1 Urea/Glucose	19
3.3.2	DoE-2 Urea/Glucose	21
3.3.3	DoE-3 Urea/Glucose	22
3.4	Further Optimization	24
3.4.1	Limit of Urea Concentration	24
3.4.2	Impact of pH	25
4	Conclusion and Outlook	26

Chapter 1

Introduction

Among the top ten leading causes of death worldwide, six relate to diet [3]. In high-income areas, the number goes up to nine [3]. Excessive sugar consumption is one of the main factors contributing to health deterioration and coincides with many diseases [4]. Replacing sugars with low-caloric, safe alternatives is thus one option to improve public health.

1.1 Erythritol

One possible sugar replacement is erythritol, a 4-carbon polyalcohol (polyol), naturally occurring in melons, peaches, pears, fermented foods and grapes [5]. Besides being low on calories, it is tooth-friendly and appears to have no toxic side effects [6, 7]. Erythritol has an approximate sweetness of 60-80 % compared to that of sucrose [1]. While it is part of the family of polyols, its most considerable distinction is the occurrence of only four carbon atoms. Since it is symmetrical, it only exists in one form, namely the meso-form (see figure 1.1) [2].

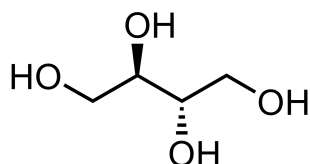


Figure 1.1: Chemical structure of meso-erythritol

With their many hydroxyl groups, polyols like mannitol or erythritol naturally tend to be radical scavengers and antioxidants [8, 9]. Hydroxyl groups can interact with free radicals to inhibit oxidation. In turn, erythritol can be considered a sweet antioxidant [10]. This antioxidant behavior is especially relevant for patients with lifestyle diseases accompanied by oxidative stress [11, 10]. The human gut can quickly absorb up to 90 % of the given erythritol. Due to this fast absorbance in the small intestine, erythritol gets discarded by urine within 24 hours and cannot metabolize to stored fat [12].

1.2 Biotechnological Production

The chemical synthesis of erythritol is economically irrelevant, as high temperatures, low yields, and the need for a nickel catalyst are costly [2]. Since chemical synthesis is economically irrelevant, industrial erythritol production happens by the yeast-like fungi *Moniliella*

pollinis, *Trichosporonoides megachiliensis* and *Yarrowia lipolytica* [2]. Besides *Y. lipolytica*, all industrially used organisms require glucose as the carbon source [13]. *Y. lipolytica* is able to produce erythritol using crude glycerol - a waste product in the biodiesel industry - as carbon source with yields as high as 180 g/l [14]. In fungi, erythritol likely forms in two steps by erythrose-4-phosphate, an intermediate of the non-oxidative pentose phosphate pathway (PPP), shown in figure 1.2.

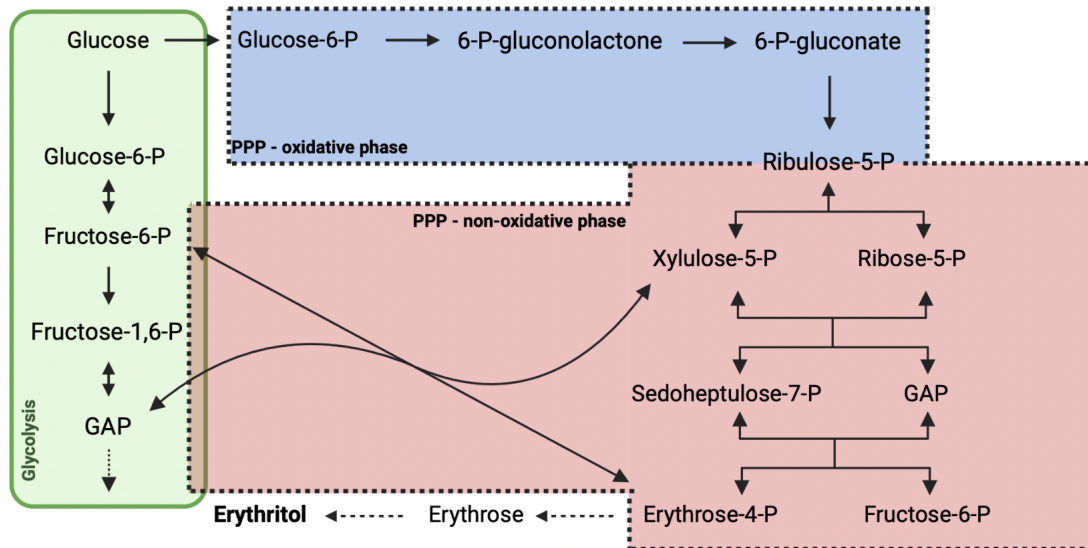


Figure 1.2: Erythritol synthesis in fungi

Erythritol is produced as the cell's response to counter external osmotic stress [13]. Also, low pH appears to be favourable for erythritol production in *Y. lipolytica*, with the optimum lying at a pH of 3 [15]. Even higher concentrations are observed when osmotic pressure and low pH are combined [16].

Trichoderma reesei, a filamentous ascomycota, is an interesting organism for industrial production of erythritol, since it is able to produce erythritol on agricultural biowaste material [17]. Currently, the approximate volumetric production of erythritol in such conditions has been reported to be 5 mg/l [17].

1.3 Filamentous Fungi as Production Organisms

Filamentous fungi can produce erythritol and other polyols, especially under influence of osmotic stress [13]. In *P. chrysogenum* and *A. niger*, the two most commonly found polyols are glycerol and erythritol, with a lower production when compared to yeasts [18]. *T. reesei* is a potential fungus for industrial applications due to the previously mentioned ability to grow on lignocellulosic material [2, 19]. The enzyme erythrose reductase (*err1*) is reported to catalyze the reduction of erythrose to erythritol [20] and naturally occurs in *T. reesei* [21]. Following their recent study, Jovanović *et al.* designed new strains of *T. reesei*, based on the strains QM6a Δ *tmus53* and Rut-C30 [17, 22]. When grown on xylose, an overexpression of *err1* in the QM6a Δ *tmus53* resulted in a 3.2 fold increase of intracellular erythritol, while yields in transformed Rut-C30 did not increase. Lack of extracellular erythritol was hypothesised to be due to it being the favorable carbon source over xylose or due to lack of a transporter. Forcing the cell to excrete erythritol with an introduced transporter system from yeasts was their suggestion to facilitate erythritol secretion [17].

1.4 Impact of Process Parameters

Aside from overexpressed genes and transporters, process parameters are also known to have an impact on the PPP [23]. Together with carbon and nitrogen source (*vide infra*), transcription factors and oxidative stress are the most likely influences on the regulation of the PPP [23].

1.4.1 Carbon sources

Xylose

As the second most prosperous sugar in nature, Xylose has previously been reported to facilitate erythritol production in yeasts and yeast-like fungi [24]. Xylose is the leading sugar present in wheat straw hydrolysate [25]. While no extracellular erythritol could be observed in *Trichoderma reesei* using wheat straw hydrolysate as carbon source, mycelial production of about 210 µg per g mycelium is still promising [17]. Xylose enters the PPP *via* the intermediate xylulose as xylulose-5-phosphate (see figure 1.3). Both xylose and xylulose can form xylitol in one step.

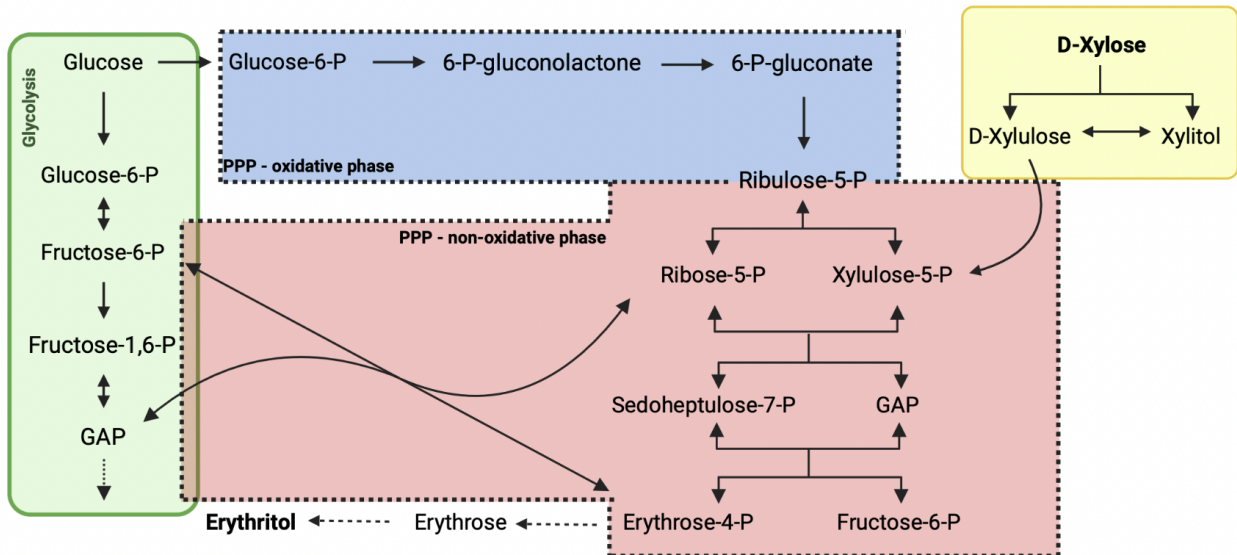


Figure 1.3: Xylose entry into PPP

Lactose

Lactose is among the main compounds in bovine milk and is synthesized from glucose and UDP-galactose [26]. It is the primary sugar in by-products of the dairy industry [27]. Contrary to the previously discussed sugars, it is a disaccharide and cannot directly enter the PPP. In filamentous fungi, it first has to be split into glucose and galactose before entering glycolysis and the PPP (see figure 1.4). The exact lactose metabolism and its regulation in *Trichoderma reesei* are not yet fully understood [28].

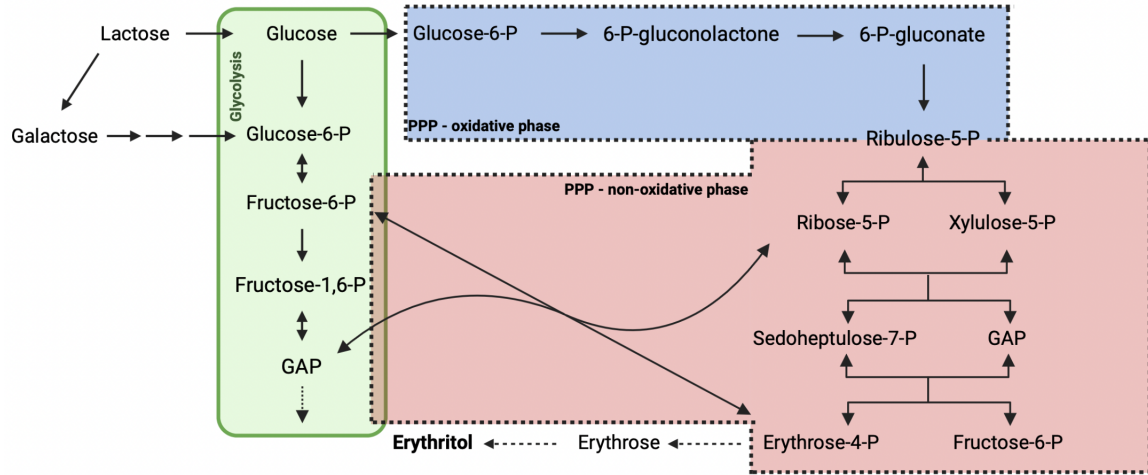


Figure 1.4: Lactose entry into PPP

Glycerol

Glycerol is a 3-carbon polyol, which can be used as a primary carbon source, directly going into gluconeogenesis (see figure 1.5) [29]. As already mentioned in section 1.2, glycerol is an interesting waste-product, which has been shown to facilitate erythritol production of up to 220 g/l in *Y. lipolytica* [14].

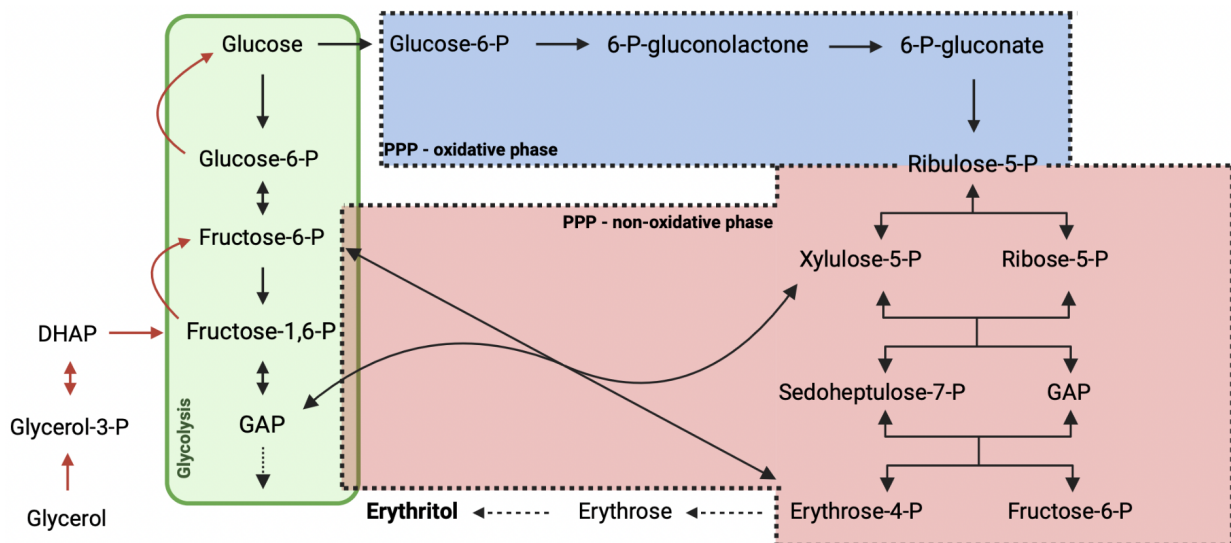


Figure 1.5: Glycerol entry into PPP

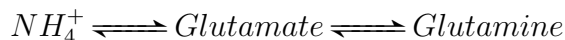
1.4.2 Nitrogen sources

The source of nitrogen is known to have an impact on growth of *Trichoderma* species [30]. Besides direct supplementation with amino acids, a variety of nitrogen sources can facilitate growth. Common sources are ammonium sulfate, nitrates of sodium, calcium or ammonium, and urea [31]. Also, undefined nitrogen sources are widely used, among which yeast extract, peptone and casamino acids proved to be beneficial for growth [30, 31].

The easiest form of nitrogen assimilation is the incorporation of ammonium into other molecules. If the nitrogen source is not directly ammonium, most will be converted to ammonium. After forming ammonium, most fungi fixate it using the NADP-dependent glutamate dehydrogenase to form glutamate [32]. Glutamate, in turn, can be used to form amino acids from α -keto acids using a transaminase. Glutamine, for example, can be directly formed from glutamate and is involved in the biosynthesis of a plethora of compounds, including inosinmonophosphate and uridinmonophosphate, which are building block for DNA.

Ammonium

Ammonium can be incorporated according to the following reaction equation.



There are many more options to incorporate ammonium into other molecules. Besides transamination of α -ketoglutarate and glutamate, ammonia can also be incorporated into pyruvate to form α -Alanine, into aspartate to form asparagine, fumarate to form aspartate or directly combined with water and carbon-dioxide to form carbamyl phosphate.

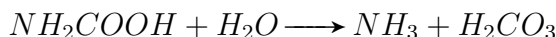
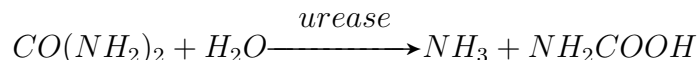
Nitrate

Nitrate is among the most abundant sources of nitrogen on the planet [33]. In fungi, nitrate has to undergo denitrification to the preferred ammonium [34]. In turn, the enzymes nitrate reductase and nitrite reductase have to be synthesized when using nitrate as sole nitrogen source [34]. The following reaction equation shows the suggested process of denitrification for generation of ammonium [23].

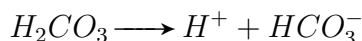


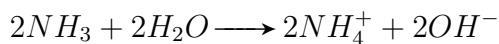
Urea

Urea is an organic compound used to excrete ammonia in mammals. In contrast, filamentous fungi can use urea as source of nitrogen, gaining two moles of NH_4^+ per mol of urea. The mechanism for generation of ammonium from urea is shown below [35].



At neutral pH, ammonia is protonated and the carbonic acid dissociates according to the following reaction equation, resulting in an increase of pH.





1.5 Research Goals

While many factors are known to have an impact on growth and polyol production of *T. reesei*, the main emphasis in the following experimental part will be laid on different carbon and nitrogen sources as well as the pH during cultivation. These were expected to have the biggest impact on the PPP. Other potentially interesting parameters, which will not be discussed in detail, include osmotic pressure, stirring and oxygenation, temperature and concentrations of essential elements/ions. Glucose, xylose and lactose are the compared carbon sources. Glucose is the main source of energy of the previously developed process. Lactose will be investigated due to its presence in whey, a by-product of the dairy industry [27]. Xylose is the most abundant sugar in wheat straw hydrolysate and is known to facilitate erythritol production in *T. reesei* [25]. Ammonium sulfate, sodium nitrate, yeast extract and urea are the compared nitrogen sources. Ammonium sulfate is the reference N-source of the previously developed process. Nitrate, yeast extract and urea are chosen because they are commonly used nitrogen sources that were available in-house.

Chapter 2

Materials and Methods

2.1 Strains

Two different strains of *Trichoderma reesei* were used to investigate the impact of process parameters. A patented strain, generated by Katharina Regnat [36], was compared to a single deletion strain generated by Matthias Steiger [22]. The single deletion strain comprises an inactivation of the gene *tmus53*, which is responsible for non-homologous end-joining. The patented strain, constructed using the single deletion strain, entails several modifications aiming at a higher erythritol production. Overexpressed genes are erythritol reductase *err1* and alditol transporter gene *fps 1*. The gene encoding mannitol 1-phosphate-5-dehydrogenase *mpdh* was inactivated.

2.2 Media composition

The concentrations of rare elements and ions for one cultivation are displayed in table 2.1. Glucose and nitrogen source are added separately. For better handling, trace element stock solutions were used. The exact composition of these stock solutions are mentioned in table 2.2.

Table 2.1: Fermentation Media Recipe

Compound	Concentration (g/l)
C-source	varies
N-source	varies
$MgSO_4 * 7H_2O$	1
KH_2PO_4	4
$NaCl$	0.5
Tween80	0.5
Pepton	0.1
$FeSO_4 * 7H_2O$	0.005
$MnSO_4 * H_2O$	0.0017
$ZnSO_4 * 7H_2O$	0.0014
$CaCl_2 * 2H_2O$	0.002

The stock solution labeled 'F-media' was 10X concentrated in tap water. For a reaction

volume of 100 ml, 10 ml of 'F-media' had to be added. The stock solution labeled 'Tracer' was 100X concentrated in distilled water (dH_2O). For a reaction volume of 100 ml, 1 ml of 'Tracer' had to be added. Both solutions were autoclaved at 120 °C for 20 min before usage.

Table 2.2: Concentrations of Stock Solutions

F-media Stock Solution (10X)		Tracer Stock Solution (100X)	
Compound	Concentration (g/l)	Compound	Concentration (g/l)
$MgSO_4 * 7H_2O$	10	$FeSO_4 * 7H_2O$	0.5
KH_2PO_4	40	$MnSO_4 * H_2O$	0.17
$NaCl$	5	$ZnSO_4 * 7H_2O$	0.14
<i>Tween</i> 80	5	$CaCl_2 * 2H_2O$	0.2
<i>Pepton</i>	1		

While the concentrations of 'F-media' and 'Tracer' remained constant across all reactions, the rest of the mix (C/N-source) was frequently adjusted according to recent findings. Unless otherwise mentioned, all solutions have been autoclaved at 120 °C for 20 min before usage. To avoid unwanted reactions, carbon sources, nitrogen sources, and both stock solutions were autoclaved separately.

2.2.1 Carbon Sources

The tested carbon sources include xylose, lactose and glucose. Concentrations were used as displayed in table 2.3. Glucose was solved in dH_2O , lactose and xylose in tap water.

Table 2.3: Concentration Carbon Sources

Sugar	Concentration (mM)	Concentration (g/l)
Glucose	55.5	10
	277.5	50
Lactose	55.5	19
	277.5	95
Xylose	55.5	8.3
	277.5	41.6

2.2.2 Nitrogen Sources

The tested sources of nitrogen include ammonium sulfate ($(NH_4)_2SO_4$), sodium nitrate ($NaNO_3$), yeast extract (YE) and urea ($CO(NH_2)_2$). Concentrations have been chosen based on the previous standard media of 2.8 g/l ammonium sulfate and are displayed in table 2.4. The initial concentration was changed to 20 mM or 2.64 g/l for better comparison between nitrogen sources and easier handling.

All chemicals have been dissolved accordingly in tap water. Urea can slightly degenerate at high temperatures, and was sterile filtered in later reactions. The point at which autoclaving was replaced with sterile filtering will be discussed in the following chapter.

Table 2.4: Concentrations Nitrogen Sources

N-source	Concentration (mM)	Concentration (g/l)
Ammonium Sulfate	20	2.64
	80	10.57
Nitrate	20	1.70
	80	6.80
YE	-	2
	-	8
Urea	20	1.20
	80	4.80

2.3 Cultivation Parameters

2.3.1 General Parameters

All shake flask cultivations have been carried out in 250 ml erlenmeyer flasks with a reaction volume of 100 ml unless otherwise mentioned. Temperature was set to 30 °C and incubation times varied from 96 to 192 hours. The pH of cultivations was adjusted to 5 using 1M citric acid before incubation or 1M hydrochloric acid during cultivation.

For inoculation, a photometrically measured starting OD_{700} of 0.05 was used. All experiments were started from spores.

Spore Suspension

After plating the strains from a -80 °C stock suspension (50 % v/v glycerol/spore suspension) and incubation in the dark for 2 days at 30 °C, a 0.5 cm² piece with mycelium was transferred to a new plate. The new plate was subsequently incubated at 30 °C in the dark for 3 days and finally at room temperature and light for two days to allow sporulation. Spores were then scraped off the plate using a sterile spatula and transferred into 1 ml of a 0.8 % NaCl and 0.05 % Tween80 solution. Finally, the spores were separated from the mycelia by filtering through sterile glasswool. To calculate the necessary volume of spore suspension, equation 2.1 was used.

$$\frac{0.05 * 100 \text{ ml}}{OD_{700}} = x \text{ ml} \quad (2.1)$$

2.4 Evaluation Parameters

2.4.1 Biomass

The biomass generation was measured at the end of culture, filtering the whole culture broth through a pre-weighted Whatman filterpaper and drying at 80 °C until dry. For faster filtration times, vacuum was applied until a minimum of 400 mbar.

2.4.2 Sugar consumption and Polyol Production

Measurements of carbon source utilization and polyol production were carried out using a Shimadzu HPLC system with Real Time analysis, Postrun analysis and Lab solution as

programs for computation and visualization. Components of the Shimadzu system are a DGU-ZOA 3R degassing unit, Nexera XR LC-20AD 2XLiquid Chromatograph, SIL20AC Autosampler, CTO-20A column oven and RID 20A Refractive index detector. For quantitation, a calibration curve entailing glucose, lactose, galactose, mannitol, erythritol, xylitol and glycerol has been established using 7 different dilutions. The results were quantified in g/l. For sampling, 1 ml of culture broth was filtered through 0.2 μ m filters into 1.5 ml HPLC-tubes. The mobile phase consisted of 5 mM sulfuric acid, diluted from concentrated sulfuric acid using deionized water. Pressure through the column was 16-17 bar with a flow rate of 0.6 ml per minute. The temperature of the column was 50 °C. Automatic or manual integration of the results gave quantitative results of any given calibrated compound.

Chapter 3

Results and Discussion

3.1 Carbon Source

3.1.1 Comparison of Xylose, Lactose and Glucose

Xylose, Lactose and Glucose were tested in the first set of experiments, using two concentrations on the patented and wildtype strain. The concentrations of carbon sources is based on 55.5 mM and 277.5 mM glucose (see table 2.3). Each condition was tested as duplicate. The rest of the media entailed the fermentation media as shown in table 2.1 as well as 21 mM Ammonium sulfate as nitrogen source. The cultivation time was set to 96 hours. No external osmotic pressure was applied. All conditions were tested in biological duplicates to increase the number of different conditions. Results of carbon source utilization are depicted in figures 3.1-3.3. Abbreviation 'wt', 'fs', 'wt hc' and 'fs hc' correspond to 'wildtype', 'final strain', 'wildtype high C-source', 'final strain high C-source'.

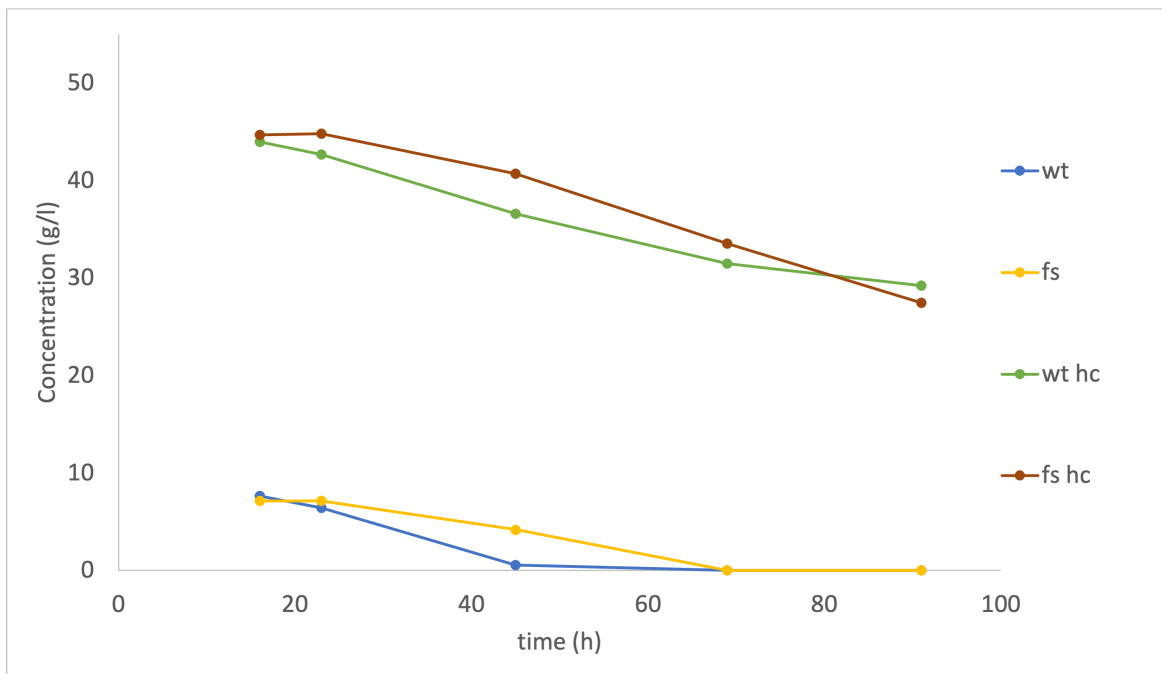


Figure 3.1: Comparison of different C-sources using different strains: Glucose consumption within 96 hours

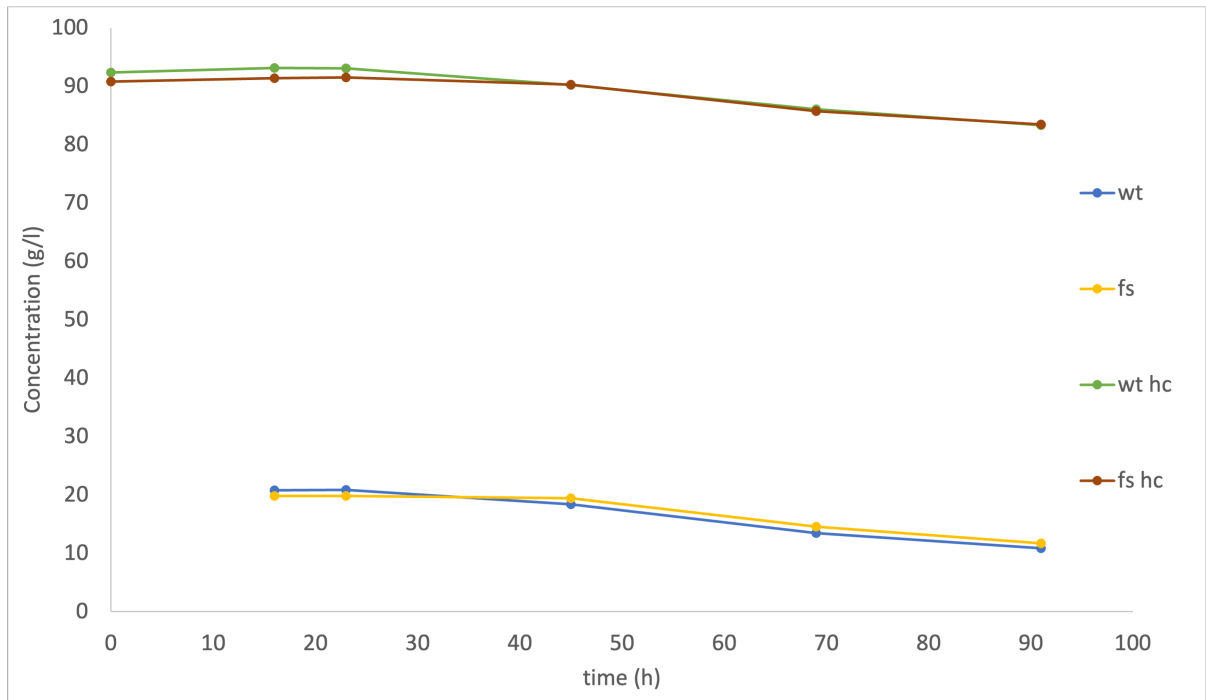


Figure 3.2: Comparison of different C-sources using different strains: Lactose consumption within 96 hours

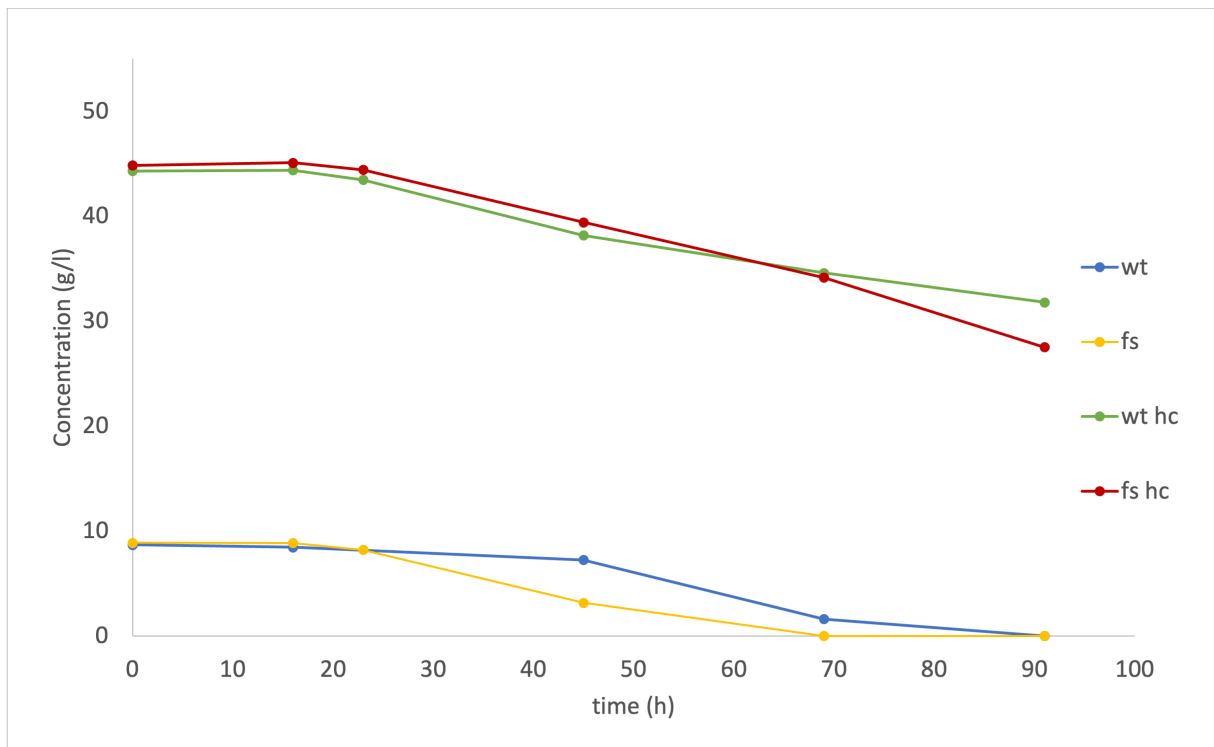


Figure 3.3: Comparison of different C-sources using different strains: Xylose consumption within 96 hours

The results concerning polyol production and biomass are given in table 3.1.

Table 3.1: Comparison of different C-sources and Strains - Influence on DCW and Polyols

	g/l				g
	Mannitol	Xylitol	Erythritol	Glycerol	DCW
WT low Glucose	-	-	-	-	0.252
PS low Glucose	-	-	-	-	0.339
WT high Glucose	0.732	-	0.008	1.196	0.375
PS high Glucose	0.091	-	0.016	1.935	0.501
WT low Lactose	-	-	-	-	0.347
PS low Lactose	-	-	-	-	0.383
WT high Lactose	-	-	-	-	0.387
PS high Lactose	-	-	-	-	0.384
WT low Xylose	-	-	-	-	0.299
PS low Xylose	-	-	-	-	0.329
WT high Xylose	-	0.162	-	0.560	0.294
PS high Xylose	-	0.090	-	0.752	0.457

This set of experiments indicated differences between different carbon sources and strains. Growth was comparable between xylose and glucose while consuming less carbon source when using lactose. In low sugar conditions, no polyols were measured in the supernatant. The final strain produced more biomass. Conditions using high glucose concentrations formed the highest concentrations of polyols. When using xylose, the total polyol concentration was lower. Only glycerol and xylitol concentrations were above the detection limit of the HPLC. As discussed in section 1.4.1, figure 1.3, the pathway of xylitol into the PPP indicates a favorable production of xylitol over erythritol and mannitol. The condition using high xylose could prove this. Glycerol is the only product formed in both glucose and xylose conditions. The main observed differences between strains in high glucose conditions concern polyol formation. The wildtype produced more mannitol but less glycerol. The patented strain produced slightly more erythritol and glycerol with a decreased mannitol concentration due to the deleted enzyme catalyzing the reduction of fructose-6-phosphate to mannitol-1-phosphate. The decreasing amount of mannitol seemed to increase glycerol production rather than erythritol production. The yield of erythritol per gram carbon-source was maximum at 50 g/l glucose in the patented strain with a value of 0.3 mg/l; the wildtype resulted in 0.16 mg/l. As a result, glucose is the best energy source for erythritol formation between the tested carbon sources. To increase the number of factors potentially tested in the same set of experiments, the wildtype strain was exclusively used for future experiments.

3.2 Nitrogen Source

3.2.1 Comparison of Ammonium, Nitrate, Yeast Extract and Urea on Biomass

After deciding upon carbon source and strain, the next step was to evaluate the influence of different nitrogen sources. The chosen substances were ammonium sulfate, sodium nitrate, yeast extract, and urea. Besides being known nitrogen sources, the choice was because of availability in the general stock. Due to a shortage of flasks, this set of experiments used

Table 3.2: DCW using different N-sources

	DCW (g)
40 mM Ammonium	0.251
50 mM Ammonium	0.257
40 mM Nitrate	0.156
50 mM Nitrate	0.152
4 g/l YE	0.337
5 g/l YE	0.343
40 mM Urea	0.309
50 mM Urea	0.311

50 ml reaction volumes in 100 ml Erlenmeyer flasks. Thus, only a comparison of biomass seemed interesting. Cultivation time was 96 hours in 50 g/l glucose. Biological duplicates are used to increase the number of different conditions. Results are shown in Table 3.2.

Despite the variations compared to previous assays, usage of different nitrogen sources led to differing behavior concerning biomass production. Sodium nitrate yielded the least biomass, with slightly more growth when using ammonium sulfate. YE and urea appeared more beneficial for biomass formation. Reasons for this difference are not clear. YE could contain sugars. Additional sugar might lead to higher biomass. Urea contains one carbon atom, which might be used as an additional C-source. The concentrations did not seem to have a significant impact on either N-source. Following these results, the comparison of N-sources was repeated using broader concentration gaps and cultivation using 250 ml flasks.

3.2.2 Comparison of Ammonium, Nitrate, Yeast Extract and Urea on Biomass and Polyols

This further comparison of nitrogen sources aimed to find the right N-source and the impact of concentrations on the cultivation. Cultivation time was 96 hours using 100 ml broth in 250 ml flasks with biological duplicates. Glucose concentration was 50 g/l. The results are shown in Table 3.3. Biological duplicates are used to increase the number of different conditions.

Table 3.3: Polyols and DCW using different N-sources

	g/l				g
	Mannitol	Xylitol	Erythritol	Glycerol	DCW
20 mM Ammonium	-	-	0.018	1.239	0.384
80 mM Ammonium	-	-	0.028	2.419	0.417
20 mM Nitrate	-	-	-	0.055	0.107
80 mM Nitrate	-	-	-	0.139	0.117
2 g/l YE	-	-	0.028	0.259	0.693
8 g/l YE	-	-	0.521	2.577	0.446
20 mM Urea	-	-	0.030	1.822	0.557
80 mM Urea	-	-	0.301	3.158	1.01

Similar to the previous experiment, the source of nitrogen had a significant impact on the process. Biomass behaved similarly, with YE and urea producing more biomass than ammonium or nitrate. The results on polyol production coincide with biomass production.

The standard condition of 20 mM ammonium sulfate produced the expected amount of 15-30 mg/l erythritol. In higher concentrations of ammonium sulfate, the amount of polyols in the supernatant was twice as high. Nitrate behaved as expected with negligible amounts of polyols. YE and urea proved to be very effective for improving polyol production. In 2 g/l YE conditions, polyol production was limited. At 8 g/l, 0.521 g/l erythritol formed. Urea also seemed to be very beneficial for erythritol production. While 20 mM urea yielded comparable amounts to ammonium sulfate, 80 mM urea increased the volumetric production of glycerol and erythritol. Nitrogen sources can have a significant impact on the PPP, but the exact mechanism is unknown and only suggested [23]. YE and urea appear to increase the activities of enzymes helping in the formation or secretion of erythritol and other polyols. The yield of erythritol per g substrate was 10.42 mg/g in 8 g/l YE and 6.02 mg/g in 80 mM urea. Despite the higher yield when using YE as the nitrogen source, urea appeared more compelling as a nitrogen source. Urea has a known composition and is a waste product. Furthermore, YE already entails erythritol in measurable amounts. A 10 g/l solution of YE contains 30-40 mg/l erythritol.

3.3 DoE

3.3.1 DoE-1 Urea/Glucose

Design of Experiments - based on a Doehlert design matrix - was the next step to optimize conditions following the screening of parameters. The design (see Figure 3.4) used five different concentrations of urea and three different concentrations of glucose. The seven different conditions of a two-dimensional DoE are referred to as treatments, as *T. reesei* is treated in seven different ways. The values of the respective treatments are in table 3.4. Biological duplicates were used to reduce number of flasks.

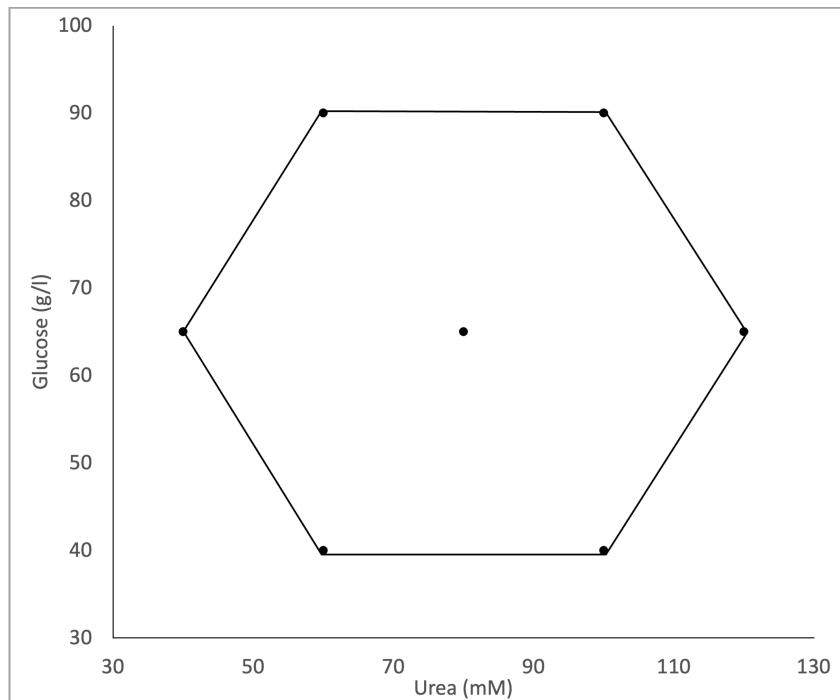


Figure 3.4: Design Space DoE-1

Table 3.4: Numeric Design Space DoE-1

Treatment	Experimental variables			
	Glucose	Urea	Glucose (g/l)	Urea (mM)
1	0	-1	65	40
2	0.866	-0.5	90	60
3	-0.866	-0.5	40	60
4	0	0	65	80
5	0.866	0.5	90	100
6	-0.866	0.5	40	100
7	0	1	65	120

The cultivation time increased to 120 h to understand behavior on longer cultivation times. The results of the DoE-1 are in table 3.5.

Table 3.5: Results DoE-1 - Glucose/Urea

Treatment	g/l				g
	Mannitol	Xylitol	Erythritol	Glycerol	
1	0.12	-	0.19	2.77	0.761
2	0.02	-	0.28	3.92	0.814
3	0.13	-	0.57	2.78	0.838
4	-	-	0.38	4.07	0.957
5	0.22	0.09	0.04	4.17	0.689
6	0.39	0.17	0.16	4.13	0.773
7	0.46	0.18	0.14	3.24	0.535

Contrary to previous experiments, mannitol and xylitol were present in measurable amounts. Especially high urea concentrations appear to facilitate xylitol excretion. Even before determining optimal conditions using a DoE software, erythritol production seemed to be highest between 60-80 mM urea.

The contour plot, as suggested by the software based on the generated data (see figure 3.8), shows a urea concentration of 72-100 mM with a glucose concentration of 40-50 g/l as the best condition. For a good design, the red area (representing maximum response) should be entirely within the chosen design space. Unfortunately, this was not the case in this set of experiments. The suggestion to reduce glucose concentrations below 40 g/l would also reduce potential erythritol recovery, as *T. reesei* can use erythritol as carbon source after glucose depletion. Thus, only the values for urea have been regarded as interesting for future experiments. Results of a DoE cannot be measured at more than one time point. Neither can it counterbalance human errors. Biggest drawback for a biological system is the number of different experiments needed for statistical significance. Regardless, this powerful tool helped understand the influence of glucose and urea better. The combination of 40 g/l glucose with 60 mM urea resulted in the highest measured erythritol formation. The condition using 65 g/l glucose and 80 mM urea gave 0.38 g/l erythritol. Following these results, the optimum is likely to be found close to these concentrations.

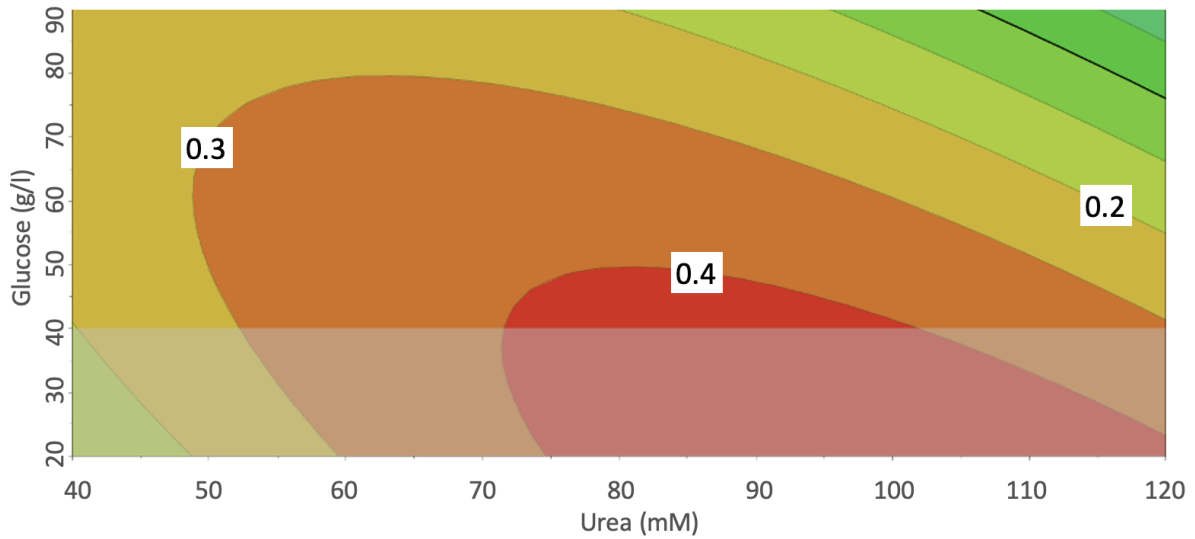


Figure 3.5: Contour Plot DoE-1

3.3.2 DoE-2 Urea/Glucose

The design space of the follow-up DoE used the values shown in table 3.6. The reaction time was 168 hours.

Table 3.6: Numeric Design Space DoE-2

Treatment	Experimental variables			
	Glucose	Urea	Glucose (g/l)	Urea (mM)
1	0	-1	60	20
2	0.866	-0.5	80	40
3	-0.866	-0.5	40	40
4	0	0	60	60
5	0.866	0.5	80	80
6	-0.866	0.5	40	80
7	0	1	60	100

The longer cultivation time had a significant impact on the process. Most conditions were depleted of glucose after 168 h, making it impossible to generate a useful analysis with the MODDE software. Also, a decrease in erythritol and glycerol concentration happened after glucose depletion, indicating their potential to be used as a carbon source when no preferred carbon source is present. There was no clear difference between erythritol and glycerol decrease. The maximum yield of erythritol per g glucose was determined to be 15.4 at 80 mM urea and 40 g/l glucose after 144 hours. The exact results of volumetric polyol production after 168 hours are in table 3.7.

The lower amount of polyols at the same urea concentrations is due to glucose depletion. Analysis of the response parameters indicates only a slight influence of glucose concentration on the process. Arguably, the impact is only measured because some treatments were void of glucose in the end. No influence of glucose concentration - so long as it is present - is assumed as a consequence. This statement is only reasonable within the tested range of 40 to 80 g/l glucose.

Table 3.7: Results DoE-2 - Glucose/Urea

Treatment	g/l				g
	Mannitol	Xylitol	Erythritol	Glycerol	DCW
1	0.215	-	0.061	2.07	0.721
2	0.102	-	0.322	3.71	0.958
3	0.113	-	0.174	3.03	0.927
4	0.638	-	0.537	3.31	1.599
5	-	-	0.856	5.32	1.330
6	-	-	0.617	4.22	1.136
7	0.281	-	0.734	8.11	1.215

3.3.3 DoE-3 Urea/Glucose

The need for better identification of outliers followed the previously discussed results. Using biological triplicates instead of duplicates promised to be sufficient. The range of glucose concentrations was 50 - 90 g/l while urea remained similar to the previous set of experiments (see table 3.8).

Table 3.8: Numeric Design Space DoE-3

Treatment	Experimental Variables			
	Glucose	Urea	Glucose (g/l)	Urea (mM)
1	0	-1	70	20
2	0.866	-0.5	90	40
3	-0.866	-0.5	50	40
4	0	0	70	60
5	0.866	0.5	90	80
6	-0.866	0.5	50	80
7	0	1	70	100

After discovering a potential heat reaction of urea during autoclaving, urea needed to be sterile filtered moving forward. Although glucose concentrations were adjusted to the previous findings, glucose was depleted in the treatment with 50 g/l glucose and 80 mM urea. The depletion already occurred after less than 144 h, invalidating the DoE once again. In contrast to previous experiments, there appeared to be an inhibition in conditions with 100 mM urea. As shown in figure 3.6, erythritol production slows down between 72-96 h, paired with a halt in glucose decrease.

While the measurement at 72 h still behaved close to expectations, the following samples all show only slight changes to the previous value. Three things are potentially causal. First, the decomposition of urea in aqueous solutions raises the pH of the system, thus possibly inhibiting growth and polyol production. The pH increases more when a higher amount of urea is used. Secondly, the decomposition of urea in aqueous solutions produces ammonia, which is toxic and harmful. Thirdly, no decomposition occurs when sterile filtering urea. As a result, the concentration is higher - and potentially inhibitory - than when autoclaving. All three are likely to be influential. Results of polyol production after 168 h are in table 3.9.

A condition of 80 mM urea and 90 g/l glucose resulted in the highest volumetric production of erythritol. When taking the yield of erythritol per g consumed glucose into account,

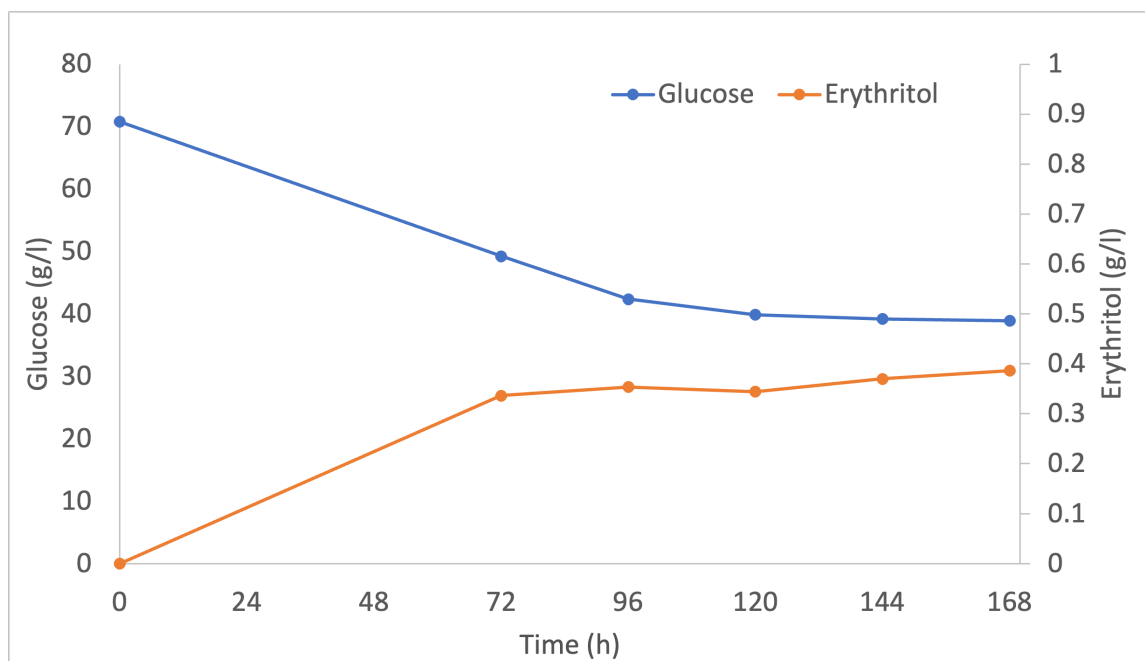


Figure 3.6: Inhibition of glucose consumption and erythritol formation in 100 mM urea - DoE-3

Table 3.9: Results DoE-3 - Glucose/Urea

Treatment	g/l				g
	Mannitol	Xylitol	Erythritol	Glycerol	
1	0.147	-	0.051	3.28	0.665
2	0.243	-	0.254	5.32	0.916
3	0.668	-	0.278	4.27	0.994
4	1.080	-	0.550	4.61	1.624
5	0.786	-	0.952	9.23	1.582
6	-	-	0.733	4.18	1.293
7	-	0.098	0.387	5.83	0.567

13.6 mg erythritol form per g glucose. At 80 mM urea and 50 g/l glucose, 14.7 mg erythritol formed per g substrate after 168 h. In this condition, the maximum obtainable yield was 19.1 ± 0.84 mg per g after 120 h. Depletion of glucose occurred in treatment six between 120 and 144 hours after inoculation. The results after 120 hours were subsequently used for analysis with the DoE software. Contrary to DoE-1, there seemed to be no statistical influence of glucose concentrations on the process from 50-90 g/l. Consequently, the corresponding contour plot (see figure 3.7) only gave estimations on ideal urea concentrations. The software suggested an ideal range of urea concentrations between 64 and 84 mM. A smaller area of urea concentrations and a clearer indication of influence of glucose would have been beneficial for future investigations. Still, DoE proved to be a helpful tool for estimating influences of urea and glucose concentrations and their respective interactions. Urea concentrations had a significant impact on erythritol concentration in the supernatant, while glucose concentrations were less significant. The initially found influence of glucose concentration is likely due to depletion of glucose in some flasks. There appeared to be no clear interaction between glucose and urea concentrations on the process.

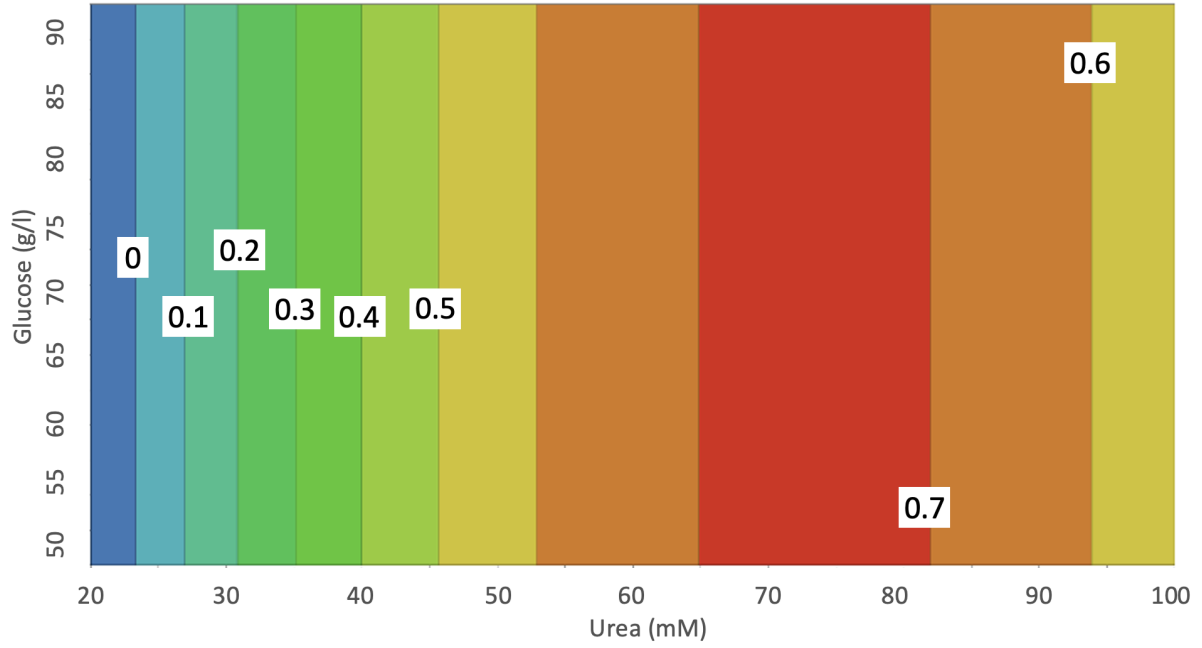


Figure 3.7: Contour Plot DoE-3

3.4 Further Optimization

3.4.1 Limit of Urea Concentration

With the new findings of glucose and urea, the following step was to understand the influence of more urea concentrations, using only one glucose concentration. Glucose concentration was 65 g/l and the range of urea to 60-120 mM in steps of 10 mM (experiments 1-7), including a control of 45 g/l glucose and 80 mM urea (experiment 8). The exact results are in table 3.10.

Table 3.10: Results Limit of Urea Concentration

Experiment	g/l				g
	Mannitol	Xylitol	Erythritol	Glycerol	DCW
1	0.369	0.132	0.152	6.770	1.178
2	0.718	0.231	0.837	6.799	0.801
3	0.519	0.137	0.561	8.066	0.792
4	0.426	0.255	0.429	6.208	0.547
5	0.125	0.086	0.198	3.906	0.483
6	1.178	0.247	0.169	4.581	0.493
7	0.641	0.046	0.105	3.798	0.436
8	-	0.072	0.695	6.002	0.929

In control conditions, glucose depleted after 120 h, thus stopping polyol production. Results still show a decrease in polyol production in urea concentrations beyond 90 mM, with potential reasons mentioned in Chapter 3.3.3. The maximum yield of erythritol per g glucose was at 70 mM urea with 18.6 mg/g. The yield of standard conditions was 15.4 mg/g.

3.4.2 Impact of pH

In previous experiments, outliers in erythritol production usually entailed a different pH compared to the other replicates. Lower pH seemed to promote erythritol production. Higher pH corresponded to lower erythritol formation. Thus, understanding the influence of pH on the process was a logical step for further optimization. Parameters were 70 g/l glucose and 80 mM urea, the standard parameters as decided before. To measure the influence of pH, 1M HCl was added to the flasks until reaching a pH of 5 or lower. Mistakenly, the control condition also used an adjusted pH value, making results lack comparability. Still, the cultivation - carried out in six biological replicates - gave insights into the robustness of the process. The yield was comparable to the standard condition with 15.2 mg erythritol per g glucose. The volumetric production of erythritol after 168 hours was 0.978 ± 0.066 g/l, marking the highest obtained value.

Chapter 4

Conclusion and Outlook

Throughout the thesis, the process could be improved in a significant manner. The initial research goals were met. The knowledge of impact of C and N source on erythritol formation led to a highly improved process for erythritol production in *T. reesei*. Among the compared carbon sources, glucose gave the highest amount of erythritol. Erythritol formation was reduced in any other carbon source. As discussed in Chapter 1, xylose is very unlikely to produce erythritol. This appears to be linked to the formation of xylitol. Lactose on the other hand appears to be strongly reducing growth with no measurable polyol production. The wildtype seemed more interesting to investigate, as there were no big differences between the patented strain and the wildtype. The possibility to produce erythritol with a wildtype strain appeared of greater interest than the slightly increased volumetric production of the patented strain.

Examining the impact of different nitrogen sources gave interesting results. While the previously used standard of ammonium sulfate gave the expected results, using nitrate inhibited both growth and polyol production. YE and urea seemed to be promoting polyol formation and secretion, making them better sources of nitrogen. Here, the yield of erythritol per g glucose was increased from 0.3 mg/g in original conditions to 6.02 mg/g (+ 3373 %). The yield per g mycelium increased from 3 to 29.9 mg/g (+897 %).

The combined impact of glucose and urea on the process was investigated using DoE. Results indicated no influence of glucose concentrations in the range of 40 - 90 g/l. The predicted influence is likely based on glucose depletion in some flasks, as erythritol and other polyols can be used as carbon source after glucose depletion. Still, with many different conditions tested, the yield of erythritol per g glucose increased to 14.7 mg/g (+ 4667 % of original yield), the yield per g mycelium increased to 60.2 mg/g (+ 1906 % of original yield). The best condition was an 80 mM urea solution with 50 - 90 g/l glucose. To avoid glucose depletion, the standard glucose concentration was 70 g/l. Thus, the fermentation media presented in table 2.1 with 80 mM sterile filtered urea and 70 g/l glucose was the best condition. After being able to better understand the impact of high urea concentrations and pH, the yield of erythritol per g glucose finally arrived at 15.27 mg/g (+ 4990 % of original yield), the yield per g mycelium was 80.06 (+ 2569 % of original yield).

With the success achieved during this thesis, the research group managed to scale the cultivation up to bioreactors, where the optimized process will undergo further investigations. Also, more parameters are expected to have an impact, (*i.e.*, the temperature of incubators, starting pH and pH during culture, rotations per minute, ...) and will be tested in the future. Ideally, the scale-up will be accompanied by further shake flask experiments for optimization to maximize progress. Furthermore, the impact of the process on the PPP

should be tested *via* a real-time quantitative PCR. This could lead to a better understanding of changes in specific enzymatic activities when using different sources of carbon or nitrogen. A better understanding of the regulation of the PPP in response to nutrients could lead to the development of new strains with much higher productivities, which could lead to an industrially viable process.

Abbreviations

Polyol	Polyalcohol
PPP	Pentose Phosphate Pathway
DoE	Design of Experiments
F-media	Fermentation Media
Tracer	Trace Elements Solution
OD	Optical Density
HPLC	High-Performance Liquid Chromatography
WT	<i>T. reesei</i> QM6a Δ <i>tmus53</i>
PS	Patented Strain (see Chapter 2.1)
DCW	Dry Cell Weight

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List of Figures

1.1	Chemical structure of meso-erythritol	5
1.2	Erythritol synthesis in fungi	6
1.3	Xylose entry into PPP	7
1.4	Lactose entry into PPP	8
1.5	Glycerol entry into PPP	8
3.1	Comparison of different C-sources using different strains: Glucose consumption within 96 hours	15
3.2	Comparison of different C-sources using different strains: Lactose consumption within 96 hours	16
3.3	Comparison of different C-sources using different strains: Xylose consumption within 96 hours	16
3.4	Design Space DoE-1	19
3.5	Contour Plot DoE-1	21
3.6	Inhibition of glucose consumption and erythritol formation in 100 mM urea - DoE-3	23
3.7	Contour Plot DoE-3	24

List of Tables

2.1	Fermentation Media Recipe	11
2.2	Concentrations of Stock Solutions	12
2.3	Concentration Carbon Sources	12
2.4	Concentrations Nitrogen Sources	13
3.1	Comparison of different C-sources and Strains - Influence on DCW and Polyols	17
3.2	DCW using different N-sources	18
3.3	Polyols and DCW using different N-sources	18
3.4	Numeric Design Space DoE-1	20
3.5	Results DoE-1 - Glucose/Urea	20
3.6	Numeric Design Space DoE-2	21
3.7	Results DoE-2 - Glucose/Urea	22
3.8	Numeric Design Space DoE-3	22
3.9	Results DoE-3 - Glucose/Urea	23
3.10	Results Limit of Urea Concentration	24