TECHNISCHE UNIVERSITÄT WIEN

DIPLOMA THESIS

Development of a filamentous fungi cultivation process in bioreactor for erythritol production

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[redacted]

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Abstract

Erythritol is a sugar alcohol that serves as a metabolite or storage substance in nature and is found in various fruits, mushrooms or seaweed. With increasing sugar consumption, erythritol is becoming more attractive, especially in its ability as a sweetener. Its near-zero calories and the fact that erythritol, unlike other sweeteners, does not cause changes in blood glucose levels or gastrointestinal problems make erythritol one of the most promising and safest candidates for sugar replacement.

Currently, erythritol is produced mainly in genetically modified osmophilic yeasts, such as *Torula sp.* or *Candida magnoliae*. However, due to widespread skepticism and strict European regulations towards GMOs, a promising approach would be to produce in wild types. A potential production organism is the ascomycete *Trichoderma reesei*. In the course of the whole project, the production of erythritol by a wild type of *T. reesei* will be investigated and a process for this should be developed. This thesis follows up on experiments performed in shake flasks. The aim is to establish a process for erythritol production by *T. reesei* in bench-scale bioreactors. For this purpose, the influence of stirring and aeration, fermentation time, batch and discontinuous fed-batch operation modes, and reactor volume on erythritol production in bioreactors was investigated. In addition, further studies were carried out in shake flasks to assess the influence of media components such as Tween80, peptone or antifoam on product formation. A Design of Experiments (DoE) was also conducted to determine the effect of temperature and initial pH on the process. However, foam formation and uncontrolled growth in bioreactors led to a premature end of the fermentation after 96 hours of cultivation. This shortened cultivation time was the faced challenge with a significant impact on erythritol formation. Foam formation was reduced/controlled by the addition of antifoam. Nevertheless, only yields of a maximum of 17,29 mg erythritol per gram of biomass formed and 11,02 mg erythritol per gram of glucose consumed could be achieved, which was significantly below the yields obtained in shake flasks. For comparison, in shake flasks, yields of 15,27 mg of erythritol per gram of glucose and 80,06 mg of erythritol per gram of biomass could be realized. However, insights were gained on the impact of certain parameters and it opens the path to ideas to test for an improvement of the process. A Design of Experiments showed that initial pH and temperature correlate with each other and significantly influence erythritol production, indicating that it has not yet been performed at the optimum of these two parameters. Moreover, knowledge was gained about the influence of stirring and aeration and the operation mode. Based on these experiences, further process development should be carried out.

Kurzfassung

Erythritol ist ein Zuckeralkohol, der in der Natur als Stoffwechselprodukt oder Speicherstoff dient und in verschiedenen Früchten, Pilzen oder Algen vorkommt. Mit zunehmendem Zuckerkonsum gewinnt Erythritol an Attraktivität, insbesondere als Süßungsmittel. Die Tatsache, dass Erythritol nahezu keine Kalorien enthält und im Gegensatz zu anderen Süßungsmitteln keine Veränderungen des Blutzuckerspiegels oder Magen-Darm-Probleme verursacht, macht Erythritol zu einem der vielversprechendsten und sichersten Kandidaten für Zuckerersatz.

Derzeit wird Erythritol hauptsächlich in gentechnisch veränderten osmophilen Hefen wie *Torula sp.* oder *Candida magnoliae* hergestellt. Aufgrund der weit verbreiteten Skepsis und der strengen europäischen Vorschriften gegenüber GVOs wäre ein vielversprechender Ansatz die Produktion in Wildtypen. Ein möglicher Produktionsorganismus ist der Ascomycet *Trichoderma reesei*. Im Verlauf des gesamten Projekts soll die Produktion von Erythritol durch einen Wildtyp von *T. reesei* untersucht und ein Verfahren dafür entwickelt werden. Diese Arbeit knüpft an Experimente an, die in Schüttelkolben durchgeführt wurden. Ziel ist die Etablierung eines Verfahrens zur Erythritolproduktion durch *T. reesei* in Bioreaktoren im Labormaßstab. Zu diesem Zweck wurde der Einfluss von Rühren und Belüften, Fermentationszeit, den Fermentationsmodi Batch- und diskontinuierlicher Fed-Batch sowie des Reaktorvolumens auf die Erythritolproduktion in Bioreaktoren untersucht. Darüber hinaus wurden weitere Untersuchungen in Schüttelkolben durchgeführt, um den Einfluss von Medienkomponenten wie Tween80, Pepton oder Antischaummittel auf die Produktbildung zu bewerten. Außerdem wurde ein Design of Experiments (DoE) ausgeführt, um die Auswirkungen von Temperatur und initialen pH-Wert auf den Prozess zu ermitteln. Schaumbildung und unkontrolliertes Wachstum in den Bioreaktoren führten jedoch zu einem vorzeitigen Ende der Fermentation nach 96 Stunden Kultivierungszeit. Diese verkürzte Kultivierungszeit war eine Herausforderung mit großem Einfluss auf die Erythritolbildung. Die Schaumbildung konnte durch Zugabe von Antischaum reduziert/kontrolliert werden. Dennoch konnten nur Erträge von maximal 17,29 mg Erythritol pro Gramm gebildeter Biomasse und 11,02 mg Erythritol pro Gramm verbrauchter Glukose erzielt werden, was deutlich unter den in Schüttelkolben erzielten Erträgen lag. Zum Vergleich: In Schüttelkolben konnten Ausbeuten von 15,27 mg Erythritol pro Gramm Glukose und 80,06 mg Erythritol pro Gramm Biomasse erzielt werden. Es wurden jedoch Erkenntnisse über die Auswirkungen bestimmter Parameter gewonnen, die neue Wege für eine Prozessverbesserung eröffnen. Das Design of Experiments zeigte, dass der initiale pH-Wert und die Temperatur miteinander korrelieren und die Erythritolproduktion erheblich beeinflussen und deutet darauf hin, dass das Optimum dieser beiden Parameter noch nicht erreicht wurde. Darüber hinaus wurden Erkenntnisse über den Einfluss von Rühren und Belüften sowie der Fermentationsmodi gewonnen. Auf der Grundlage dieser Erfahrungen sollte weitere Prozessentwicklung durchgeführt werden.

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1 Introduction

Sweetness is one of the most popular perceptions we have. The delight we feel when tasting something sweet is innate and already discovered before birth [1]. However, nowadays, sugar is consumed excessively. High sugar consumption results in excessive energy intake, leading to obesity, a risk factor for, among others, type 2 diabetes mellitus and hypertension [2]. Additionally, the increasing consumption of sugary foods means that nearly 100 % of adults have already been affected by tooth decay [3]. Reducing sugar in our diet can therefore be beneficial. One way to achieve this could be through sweeteners. Generally, sweeteners can be divided into noncaloric, high-intensity- and nutritive sweeteners known as sugar alcohols [4]. These sugar substitutes mimic the sweetness of sucrose but have few to no calories and a little glycemic response [5]. Moreover, they are usually non-cariogenic as oral bacteria cannot metabolize them [6].

1.1 Polyols

Sugar alcohols, also known as polyols, are sugar derivatives with the general formula $HOCH_2(CHOH)_nCH_2OH$ [7]. In nature, polyols occur in small amounts in fruits, mushrooms, and vegetables. They are produced by reducing the aldehyde or ketone functions in carbohydrates and most of them have GRAS (generally recognized as safe) status [8, 9]. Besides being low in calories and non-cariogenic, polyols have other advantages over sucrose: they are good antioxidants, protect blood vessels, and have no effect on bone density [10]. Unlike artificial sweeteners, polyols can be added in amounts similar to sucrose. Therefore, they also can be used for bakery [5]. Polyols are usually absorbed in the small intestine. However, they are only incompletely absorbed, causing the glucose level in the blood to rise less than sucrose. This property makes sugar alcohols popular for low-sugar diets but leads to digestive problems such as diarrhea or flatulence when consumed in excess. Usually, people develop tolerance to these symptoms with regular consumption [7].

Sugar alcohols include sorbitol, xylitol, mannitol, maltitol, lactitol, isomalt, and erythritol. Polyols have a wide range of applications: besides their function as sweeteners, they can also be used as thickeners, humectants, stabilizers, or emulsifiers [8]. A commonly used method for obtaining most of these polyols is the catalytic hydrogenation of sugars. However, this happens under high temperatures and pressure and requires the obtained product's complex and expensive purification processes. Therefore, biotechnological production has been pushed recently [11]. Unlike the other polyols, erythritol was neither chemically produced in the past. Although a process was found in 1960 to produce erythritol from starch, using high temperatures and a nickel catalyst, it has been produced by fermentation since the 1950s. This is due to the high energy requirement and relatively low yield [12, 13].

1.1.1 Erythritol

Within all polyols, erythritol plays a unique role. In contrast to the other sugar alcohols, erythritol consists of only four carbon atoms. Consequently, it has different physical and chemical properties. For example, erythritol produces higher osmotic pressure or, when dissolved, lower water activity and lower viscosity than other polyols [10]. In nature, erythritol is a metabolite or storage substance in seaweed or fungi but is also present in various fruits, such as melons or grapes. Erythritol is symmetrical, so it exists only in the meso-form (Figure 1). Like the other polyols, it has no reducing end-groups, which gives it excellent heat and acid stability. It forms anhydrous crystals with a moderate sweetness of 60-80 % compared to sucrose. Erythritol also acts as a free radical scavenger with an antioxidant effect [14].

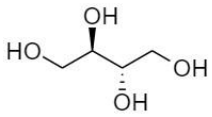


Figure 1: Chemical structure of erythritol

Although erythritol has about two-thirds the sweetness of sucrose, it has only 0 to 0,2 calories per gram, while sucrose has four calories per gram. Like the other polyols, this property makes erythritol a common sweetener in beverages or low-calorie foods [15]. However, while most polyols can cause side effects such as flatulence, abdominal cramps, or diarrhea when consumed excessively, erythritol has no laxative effect and has the highest digestive tolerance among all sugar alcohols [8, 16]. All the advantages of erythritol mentioned above make it a widely applicable sugar substitute with a growing market. Forecasts indicate a 22 % increase in global market size to $2748 million within the next six years [17]. Therefore, it is reasonable to optimize production processes to make them more cost- and resource-friendly.

1.2 Pentose phosphate pathway

Erythritol is produced in yeasts and fungi via the pentose phosphate pathway (PPP). This pathway is essential for maintaining carbon balance, producing precursors for nucleotides- and amino acids biosynthesis [18]. Moreover, it provides NADPH as a reducing agent for anabolism and maintains the redox potential necessary for protection against oxidative stress [19].

The oxidative Pentose phosphate pathway (OPPP) is unidirectional as three irreversible reactions occur. At the end of this phase, glucose-6-phosphate has been converted to ribulose-5-phosphate with the formation of 2 NADPH. The OPPP is followed by the non-oxidative pentose phosphate pathway (NOPPP), which links to glycolysis and other metabolic pathways. This phase involves only reversible reactions. Thus, the cell can control the reaction directions based on its need [20]. Depending on the requirements, ribose-5-phosphate can be used directly to synthesize nucleotides. However, it can also be further converted in NOPPP to erythrose-4-phosphate, a building block for the biosynthesis of aromatic amino acids. Due to its flexibility, the PPP is a popular target for metabolic engineering and biotechnology [15, 18].

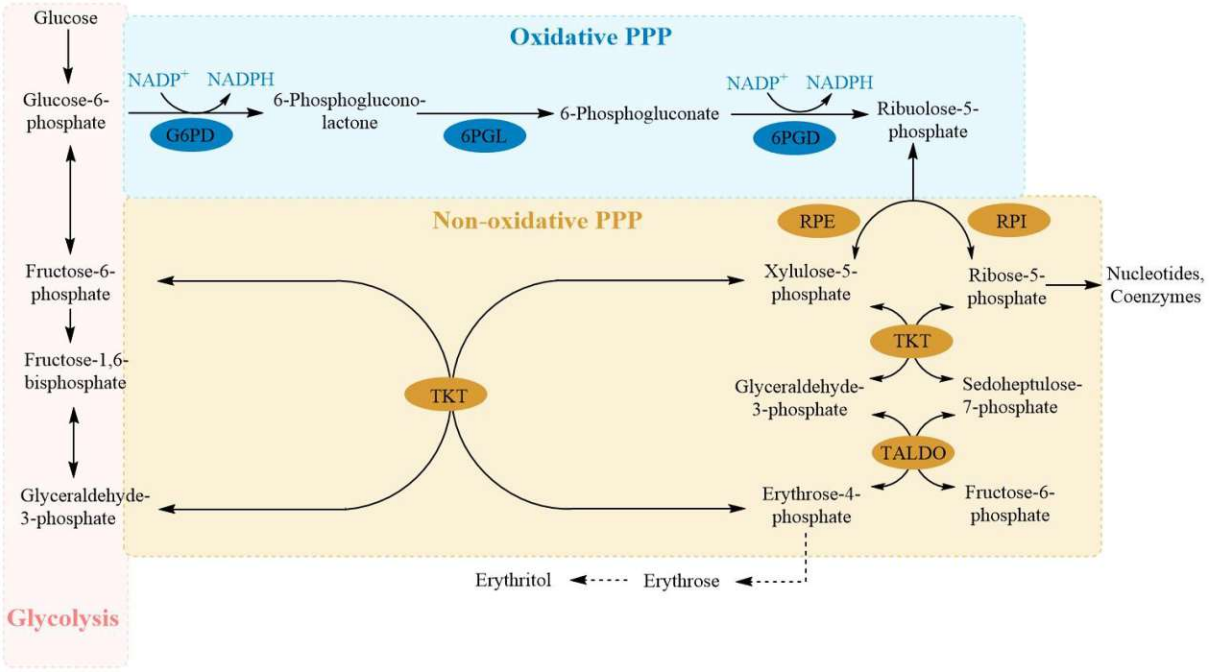


Figure 2: Pentose Phosphate Pathway (PPP) and its crossover to glycolysis. The main enzymes catalyzing reactions in the PPP are Glucose-6-phosphate-dehydrogenase (G6PD), 6-Phosphogluconolactonase (6PGL), 6-Phosphogluconate-dehydrogenase (6PGD), Ribose-5-phosphate-isomerase (RPI), Ribulose-5-phosphate-epimerase (RPE), Transketolase (TKT), and Transaldolase (TALDO) [21]

As shown in Figure 2, erythrose-4-phosphate is formed in NOPPP. In the presence of ADP, this can be further converted to erythrose by the enzyme phosphatase. Finally, it can be reduced to erythritol under NADPH oxidation with the help of erythrose reductase. Hence, the higher the key enzyme activity, the higher the erythritol yields. Therefore, increasing their activity should benefit erythritol production [15].

1.3 Production approaches

Yeasts and yeast-like species can grow even at high osmolarity and are considered osmophilic. These osmotolerant species produce polyols, like erythritol, in response to osmotic stress [22, 23]. For higher production rates, mutations were generated by UV irradiation or chemicals. Afterward, they were scanned for increased productivity. This procedure resulted in some yeasts, such as *Aureobasidium sp.*, *Trichosporonoides sp.*, *Torula sp.*, or *Candida magnoliae*, which can achieve erythritol-yields of around 40 % (w/w) at high initial glucose concentrations [23, 24]. Moreover, reducing byproducts, mainly glycerol, has been focused on lately as they are often hard to remove in the downstream process [25].

As already mentioned, osmotic stress is required to produce erythritol. This effect can be generated by high sugar- or salt concentrations. In most cases, glucose is used for this purpose. In erythritol production, for example, concentrated glucose syrup (16-40 % (w/v)) is widely used both as a carbon source and as a trigger for osmotic stress. This is done because of its ease of use, safety, purity, and selectivity. However, the use of glucose also has a disadvantage: it increases the production costs of erythritol. The price of erythritol is 75 % determined by the raw material, which is usually glucose [26]. Even nowadays, erythritol is still expensive to

produce. Therefore, there are three approaches to reduce this price problem: 1) Increasing productivity; 2) Reducing byproduct formation; 3) Using cheaper substrates. Points 1 and 2 can be achieved by strain-engineering on the one hand and by adjusting the production parameters on the other hand (see section 1.3.2). An approach to cheaper substrates is using production organisms that can use lignocellulosic biomass as a substrate for erythritol production. Some filamentous fungi produce large amounts of cellulases and hemicellulose enzymes, making them potent biomass degraders. They can also degrade xylans and metabolize them in the PPP into polyols, such as erythritol [24, 27].

1.3.1 Production organism – *Trichoderma reesei*

The ascomycete *Trichoderma reesei* can use non-food biomass as a substrate by producing cellulases and hemicellulases to degrade lignocellulosic material into hexose and pentose monosaccharides. Therefore, this fungus can grow on cheap substrates containing lignocellulose, such as wheat straw. Although *T. reesei* has been used intensively in the industry for a long time, mainly for cellulases production, its use as an erythritol producer is just starting [15, 28, 29]. Jovanovic et al. characterized the key enzyme erythrose reductase from *T. reesei* in 2013 [30], confirming the ability of this fungus to produce erythritol. Based on the properties mentioned above, *T. reesei* as a production organism offers a promising and less expensive approach for erythritol production. However, its productivity for this purpose is still significantly below the yeasts' mentioned in section 1.3 [24]. Therefore, further strain improvements and media and process optimization are essential to finding a favorable production method for erythritol using *T. reesei* [12].

1.3.2 Fermentation in bioreactors

An approach to industrial production of erythritol by *T. reesei* is fermentation in bioreactors. Submerged fermentation is the aerobic cultivation of microorganisms in nutrient-rich media [31]. Submerged cultivation is preferable for the fermentation of filamentous fungi, usually done in stirred tank reactors (STRs). However, in these submerged cultivation processes, there is a dynamic interplay between environmental conditions and the growth patterns of the organisms to be cultivated [32, 33]. Mycelium in submerged cultures often grows in an uncontrolled manner. Thus, mycelium often winds around impellers or in and around ports and probes, causing blockages and limitations in oxygen transfer [31]. Therefore, it is essential to define the parameters so the process leads to the desired biomass and product formation. Cultivating filamentous fungi in submerged cultures is a complex multiphase- and multicomponent process. Cell growth and resulting product formation are influenced by many parameters: temperature, pH, shear stress, inoculum, oxygen supply, rheology, and morphology. However, it is essential to consider these parameters individually and as an overall construct [34].

1.3.2.1 Reactor Design and Scale-Up

Shake flask experiments are usually performed for the first steps in bioprocess development. Shake flasks have many advantages, particularly beneficial at the beginning of process development. Many "reactors" can be used simultaneously, making it especially interesting for mass screening. Screening processes are beneficial, for example, to determine the influence of different media compositions, total cultivation time, pH and temperature. Screening in shake flasks can also be used to evaluate fundamental kinetic data. There is a wide range of different vessel sizes and they are considered the cheapest and simplest type of bioreactors. In addition, shake flasks have an advantage over, for example, STRs, which is especially important for initial screening processes: they have a well-defined gas/liquid mass transfer area, which remains relatively constant during all experiments. Thus, screening for other parameters is possible without this influence distorting the results [35].

However, shake flasks also have disadvantages that make it necessary to switch to other reactors sooner or later. Particularly critical is that oxygen limitation often occurs during fermentation in shake flasks. This can lead, for example, to the entire metabolism of the microorganism being slowed down or only the product formation being unknowingly influenced by this. It is also difficult to constantly monitor parameters such as pH value. In addition, some effects, such as foam formation or stirring, which are important parameters in later large-scale production, do not play a role in shake flask fermentations [35]. All these aspects make further scale-up on bench-scale reactors necessary.

An STR is generally the reactor of choice for viscous fermentation broths, such as filamentous fungi fermentation broths. An important parameter is the aspect ratio between height and diameter. It can be calculated using Equation 1. When cultivating filamentous fungi, the aspect ratio should be 2-3 [36, 37].

Equation 1

$$Aspect\ ratio = \frac{height_{Liquid}}{diameter_{Reactor}}$$

STRs are an excellent choice for viscous broths but are not practical for volumes above 500m³, as the energy consumption becomes too high [33]. Therefore, for example, airlift reactors should be considered as an alternative. Airlift reactors do not require mechanical action. This property helps on the one hand to overcome shear-related problems. On the other hand, it enables easier scale-up. However, it lacks flexibility compared to STRs [38]. Schematic drawings of both reactors can be seen in Figure 3.

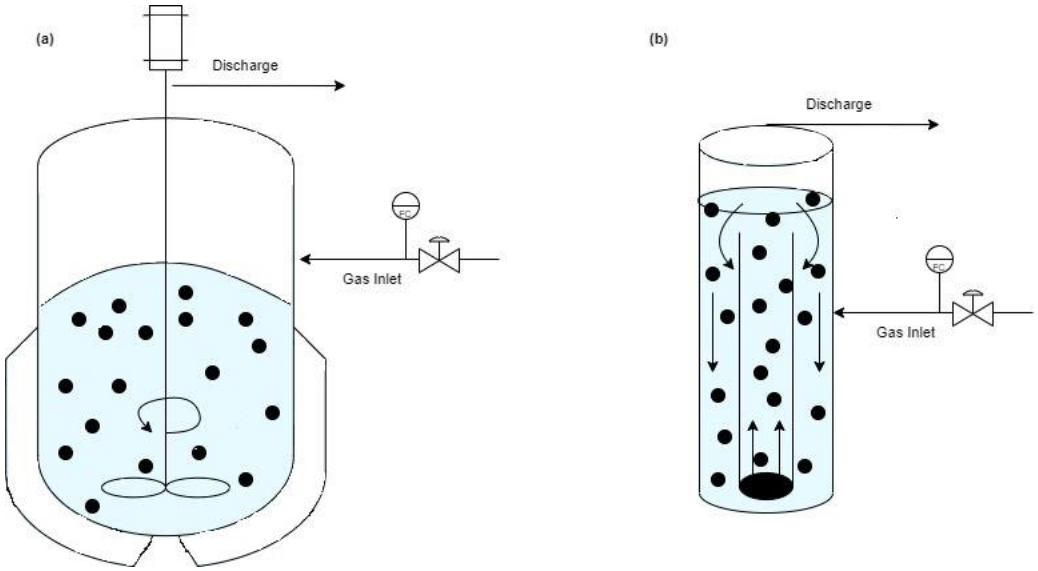


Figure 3: Schematic figure of (a) stirred tank reactor and (b) airlift reactor [39]

As initial experiments and optimizations for erythritol production in *T. reesei* in shake flasks have already taken place [40], it is time to go through further process development steps in bench-scale STRs. For the reasons mentioned above, using shake flasks in developing a large-scale process is practical but only helpful to a certain point. In order to be able to optimize parameters such as agitation, rheology, foaming and energy requirements, the next reasonable step is cultivation in STRs.

Once a sufficiently satisfactory process has been found in bench-scale reactors, further up-scaling of the reactors can take place. There are general problems in scaling up the fermentation of filamentous fungi due to the complex relationship between different process parameters. However, a reasonable prediction can often be made with the help of the maximum specific energy dissipation rate (EDCFεmax) [41]. EDCFε_{max} in W/m³ is shown in Equation 2.

Equation 2

$$EDCF\varepsilon_{max} = \frac{\varepsilon_{max}}{t_c}$$

The maximum local specific energy dissipation rate (ε_{max}) can be estimated from Equation 3, where ρ is the density of the broth (kg/m³), P_0 is the power number of the impeller, N is the rotational speed (s^{-1}), and D is the diameter of the impeller (m).

Equation 3

$$\varepsilon_{max} = 1{,}04 * \varrho * P_0^{\frac{3}{4}} * N^3 * D^2$$

The circulation time t_c can be calculated as shown in Equation 4, where V is the liquid volume in the bioreactor (m³), and Fl is the flow number of the impeller.

Equation 4

$$t_c = \frac{V}{Fl * N * D^3}$$

1.3.2.2 Stirring and Aeration

An essential aspect of bioreactor cultivation is the mixing of the broth. This prevents gradients in temperature, component concentrations, and pH. In addition, depending on the stirrer, the introduced oxygen bubbles are distributed into smaller oxygen bubbles, which ensures a uniform and sufficient oxygen supply [32]. Dissolved oxygen, and thus the oxygen available to the microorganism, is adjusted via the airflow and the stirrer speed. However, this often leads to high power consumption problems during scale-up [42].

Depending on the requirements, mainly two types of impellers are used in STRs. On the one hand, there are radial pumping impellers, for example, the Rushton disc turbine. This agitator type is associated with higher shear rates and better oxygen transfer. The second impeller type is the axial pumping impeller, such as the marine blade impeller. The advantage of these impellers is the reduced shear rate and the better homogenization of the broth. A high shear rate can cause damage to the mycelium of filamentous fungi, so it is important to find a balance between the shear rate needed for homogenization and oxygenation and the unfavorable effects of shear. [32, 33, 43]. The typical STR for the fermentation of filamentous fungi consists of a Rushton disc turbine below and one or more radial mixing impellers above [44].

1.3.2.3 Morphology and Rheology

The morphology of filamentous fungi mycelium varies in bioprocesses. The process parameters, such as stirring, influence the morphology and the morphology itself influences them [41]. Dispersed mycelium and high biomass lead to a highly viscous fermentation broth, increasing mixing time and energy consumption. In addition, submerged cultures of filamentous fungi often develop Non-Newtonian behavior. Therefore, viscosity changes during fermentation, making optimization and scale-up even more complex [36, 44].

Besides growing as free mycelium, filamentous fungi can also grow as pellets. Both growth forms are shown in Figure 4. In the case of pellets, the culture usually develops Newtonian behavior, making process design easier. However, pellet formation also leads to problems with the transport of substrates into the interior of the pellet. Whether pellet formation is beneficial or detrimental to the process depends on the product [33, 36]. There is no information about this yet regarding erythritol production in *T. reesei*.

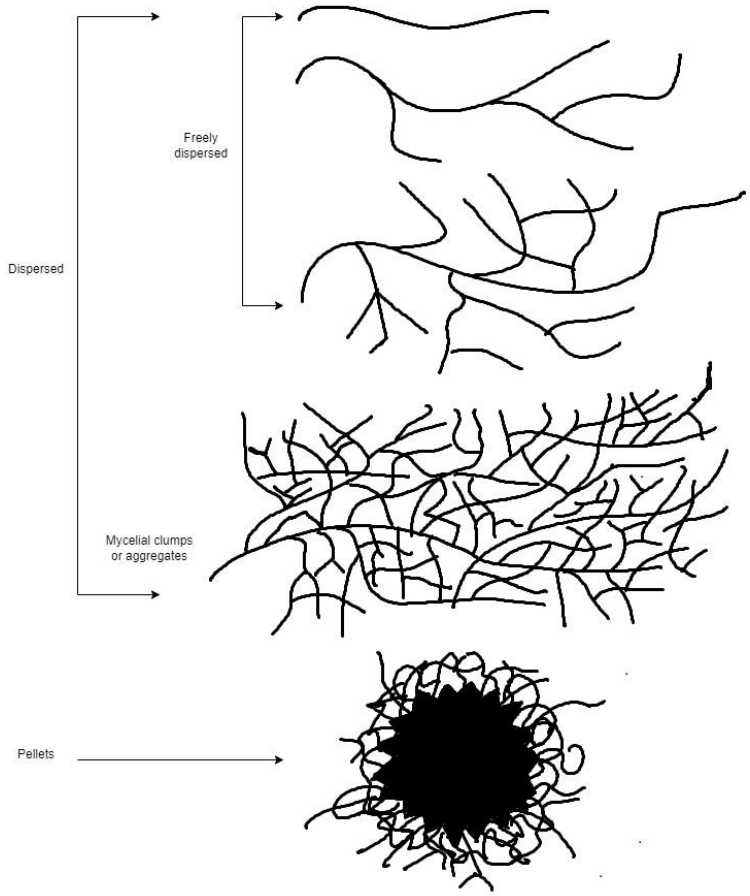


Figure 4: Overview of morphology forms in submerged cultures of filamentous fungi [45]

Numerous parameters influence the morphology of the culture: size and type of inoculum, stirring and aeration, media composition, temperature, and pH. However, the interrelationship of these parameters is highly complex. Therefore, identified co-dependencies are usually only applicable to specific processes with specific organisms and products. Due to these complex relationships, it is necessary to find the appropriate morphology and, thus, the associated process parameters for each process individually [46, 47].

1.3.2.4 pH and temperature

pH and temperature are important parameters when designing a bioprocess. Studies have shown that pH affects branching frequency and hyphal diameter in filamentous fungi and is thus related to morphology [43]. Furthermore, the pH of the microorganism influences its metabolism and is, therefore, an essential parameter in process design.

Filamentous fungi are currently used primarily for enzyme production, so most studies on the influence of temperature refer to this. However, they all concluded that temperature is essential for biomass and product formation [37, 48]. In 2003, Bai et al. found that increased oxidative stress also occurs at elevated temperatures, which must be considered in process design [49].

1.3.2.5 *Foaming*

Foam consists of liquid lamellas filled with gas and is stabilized by proteins. It is a result of the aeration of the culture medium in bioprocesses. The organism to be cultured can form the stabilizing proteins itself, or it is a component of the medium [50]. Problems with foaming are known in the cultivation of *T. reesei*. It impedes gas disengagement at the surface and thus affects oxygen transfer and erythritol yield [15, 32]. Therefore, foaming must be prevented as much as possible. A remedy for this problem is the addition of antifoam. Antifoaming agents induce bubble coalescence and thus prevent foam formation. However, adding antifoam also has disadvantages since it often interferes with downstream processing and can denature biological components. Moreover, it is also reported that antifoam influences biomass and metabolite production. Therefore, if possible, it should be prevented. [50, 51].

1.3.3 Metabolic considerations

Since erythritol is a metabolite of *T. reesei*, the metabolism must also be considered in process development. Particularly relevant for the process design are the operation mode on the one hand and the composition of the medium on the other.

1.3.3.1 *Operation Mode*

Generally, it can be distinguished between three operation modes. The different feed and volume profiles can be seen in Figure 5.

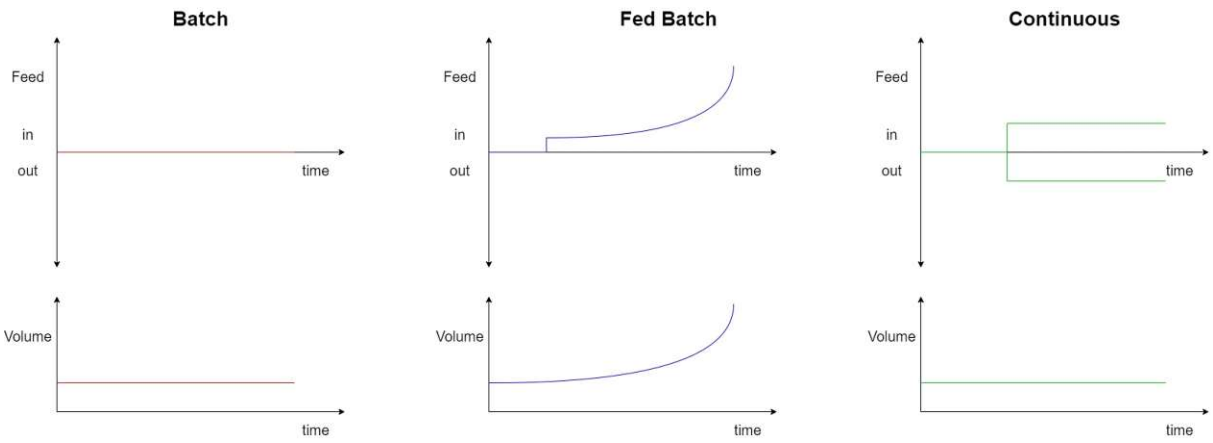


Figure 5: Feed and Volume profiles over the time of the three operation modes [52]

The first and simplest type of fermentation is batch fermentation. A closed system is used and the substrate is added in excess. The fermentation ends when all the substrate is used. The advantage of this variant is that there is a low risk of contamination. However, there are often relatively low substrate utilization rates and often overfeeding. Overfeeding often causes by-product formation and oxygen limitation in highly viscous processes and should be avoided [53, 54]. Moreover, substrate inhibition can occur [55].

In fed-batch fermentation, one or more nutrients are fed at least once during fermentation. A distinction can be made between a continuous and a discontinuous fed-batch. In a continuous

fed-batch, a feed rate is set, which can be linear or exponential, and as a result, the limiting component can be kept at a constant, low concentration. This allows the adjustment of the growth rate. This type of fed-batch is illustrated in Figure 5. In a discontinuous fed-batch, nutrients are supplied by individual discontinuous feeds. This can be a single feed, or it can be pulsed. Fed-batch processes are a way to prevent problems such as substrate inhibition, catabolite repression or overfeeding. By regulating the feeds, parameters such as biomass concentration, metabolic rates and oxygen availability can be better regulated [53, 54]. This fermentation method significantly improved productivity in previous biotechnological erythritol production approaches [15].

The inflow and outflow are constant in continuous fermentation, resulting in a constant reactor volume. This means that fresh medium is constantly added and degradation products and the desired product are removed. This is particularly beneficial for products that degrade quickly in the fermentation broth. The advantage over the fed-batch is that one does not have reactor-specific volume limits. In addition, continuous reactors need to be sterilized and re-inoculated less frequently [53]. However, the continuous fermentation method has not yet shown success in erythritol production [15].

Previous studies have shown that during erythritol production by *T. reesei*, erythritol already produced is degraded again if the glucose concentration is too low. Therefore, a batch process can only be carried out with a high initial glucose concentration [40]. However, fed-batch processes could also provide a remedy for production by *T. reesei.*

1.3.3.2 Media considerations

In addition to the process parameters mentioned in 1.3.2, the media composition is important for cultivating *T. reesei* and erythritol production. Former studies have shown that the type of carbon source is essential for erythritol yield. For example, when xylose is added, hardly any erythritol is produced, but xylitol is preferentially produced [40]. It has been shown that osmophilic microorganisms, such as *T. reesei,* favorably produce erythritol at high initial glucose concentrations [5, 40]. On the one hand, glucose serves as a carbon source. As shown in Figure 2, glucose can be metabolized in glycolysis and PPP. On the other hand, a high glucose concentration also leads to osmotic stress for the cell, which boosts the production of osmolytes such as erythritol [56]. High salt concentrations, such as NaCl, can also produce osmotic stress. However, salt-induced osmotic pressure can negatively affect cell growth, at least in yeasts, and should be avoided if possible [57].

The type of nitrogen source also influences erythritol production. While nitrate hindered biomass growth and thus product formation, yeast extract and urea generated significantly higher erythritol yields than the standard nitrogen source, ammonium sulfate [40].

1.4 Project goal

The production of sugar alcohols, especially erythritol, is often done biotechnologically. However, production is still expensive. This can be counteracted on the one hand by increasing the productivity of the production organisms and, on the other hand, by using cheap substrates. The latter can happen, for example, through filamentous fungi. Therefore, a project is underway at the Vienna University of Technology in which an attempt is being made to develop a process for the industrial production of erythritol using the filamentous fungus *T. reesei*. The aim is to develop a sustainable, cost-effective and resource-friendly process. The medium should be produced from industrial waste and *T. reesei* should be cultivated for erythritol production. This is to be done as efficiently as possible, which is why productivity should also be increased through process optimization in addition to strain optimization through genetic engineering. The former has achieved significant success on a shake flask scale. For example, Stark increased the yield of erythritol per g glucose by 4667 % to 14.7 mg/g by using high initial glucose concentrations (70 g/L) and 80 mM urea. He also found that extending the fermentation time from 96 h to 168 h and an acidic environment was beneficial for erythritol production. A yield of erythritol per g glucose of 15.27 mg/g could be obtained [40].

As mentioned in section 1.3.2.1, the knowledge acquired by shake flasks in process development is limited. Oxygen limitation, difficult constant monitoring, and the lack of information on the influence of other process parameters such as shear rate and foaming make upscaling to bench-scale bioreactors necessary. Therefore, this work will address the upscaling of the process to bench-scale bioreactors. It is based on Stark's previous results from shake flask experiments. The goal is to transfer the process developed in shake flasks into a bench-scale bioreactor. Ideally, this should be done while maintaining or improving the obtained yields. However, the behavior of this process in bioreactors was unknown. Therefore, the focus should also be on parameters that either had no influence on the process in shake flasks or could not be investigated in such a setting. For this purpose, this thesis will deal with the yet unknown process factors, such as agitation, aeration, foam formation, or the operation mode.

2 Methods and Materials

2.1 Media compositions

As a medium for cultivation on plates, MEX medium was used. MEX medium was made using tap water and sterilized through autoclaving.

Table 1: Composition of MEX medium

Compound	Concentration (g/L)
Malt extract	30
Peptone	1
Agar	15

As a carbon source, a 400 g/L glucose stock solution was used and diluted with dH_2O to the desired final concentration. Urea was also prepared separately as a 4 M stock solution, sterilized through a 0,22 µm sterile filter. The final fermentation medium composition is shown in Table 2.

Table 2: Fermentation medium composition

Compound	Concentration (g/L)
$MgSO_4*7H_2O$	1,0
KH_2PO_4	4,0
NaCl	0,5
Tween80[1]	0,5
Peptone[1]	0,1
$FeSO_4*7H_2O$	0,005
$MnSO_4*7H_2O$	0,0017
$ZnSO_4*7H_2O$	0,0014
$CaCl_2*2H_2O$	0,002
Glucose	Varies
Urea	80 mM

For easier handling, a stock solution of the remaining components was made. A 100x concentrated stock solution in tap water was prepared from the trace elements and autoclaved. This solution will be called Tracer from now on. The remaining components were combined into a 10x concentrated solution in tap water, now called F-medium. The concentrations of the stock solutions can be seen in Table 3.

[1] Only used in shake flasks

Table 3: Composition of the stock solutions

F-medium (10x)		Tracer (100x)	
Compound	**Concentration (g/L)**	**Compound**	**Concentration (g/L)**
$MgSO_4*7H_2O$	10	$FeSO_4*7H_2O$	0,50
KH_2PO_4	40	$MnSO_4*7H_2O$	0,17
NaCl	5	$ZnSO_4*7H_2O$	0,14
Tween80[1]	5	$CaCl_2*2H_2O$	0,20
Peptone[1]	1		

2.2 Preculture and Shake flasks

2.2.1 Preparing spore suspension

To prepare spore suspension, *Trichoderma reesei Qm6a Δtmus53* [58] was plated from a glycerol stock (50 % v/v spore suspension/glycerol), stored at -80 °C, onto MEX plates. These plates were then incubated for three days at 30 °C in the dark. A small piece of this plate was cut out and incubated on another MEX plate for three to four days at 30 °C in the dark. The plates were incubated at room temperature under light for 1-2 more days to allow them to sporulate.

Once the fungus sporulated well, the spores were scraped off using a sterile spatula and transferred to a reaction tube containing 1 ml NaCl/Tween80 solution. NaCl/Tween80 solution consisted of 0,8 % NaCl and 0,05 % Tween80. The suspension was vortexed and filtered through glass wool to separate the mycelium. To calculate the amount needed to prepare the shake flasks, Equation 5 was used. The OD700 of the spore suspension was measured on Nanodrop. The starting OD700 for the experiments should be 0,05.

Equation 5

$$Volume_{Spore\ suspension}(mL) = \frac{0{,}05 * Volume_{Shake\ flasks}(mL)}{OD700}$$

2.2.2 Shake Flask Experiments

Experiments performed only in shake flasks were prepared according to Table 4 under sterile conditions. The spore suspension was prepared as described in section 2.2.1, and the shake flasks were inoculated with the volume calculated in Equation 5. The total volume was 100 mL. If pH was adjusted before cultivation, this step was done before filling the shake flasks up to 100 mL.

Table 4: Recipe for Shake flask experiments

Compound	Amount (mL)
F-Medium (10x)	10
Tracer (100x)	1
4 M Urea	2
400 g/L Glucose	17,5
Tap water	to 100

After inoculation, shake flasks were incubated in shaking incubators at 180 rpm. Time and temperature varied depending on the experiment.

2.2.3 Preculture

Precultures were prepared very similar to the regular shake flask experiments. The volume of the preculture was 10 % of the reactor volume. The ratio between the individual components is identical (see Table 5), but the volume was adapted to the subsequent reactor.

Table 5: Composition of the precultures

Compound	Proportion (v%)
F-Medium (10x)	10
Tracer (100x)	1
4 M Urea	2
400 g/L Glucose	Varies
Tap water	to final volume

After inoculation, the precultures were incubated at 30 °C and 180 rpm in shaker incubators for about 18 h.

2.2.4 Design of Experiments

A design of experiments was carried out once. This was used to determine the influence of initial pH and temperature on erythritol production. It was performed as a screening process with a full factorial design at two levels with an interaction model and included two factors with four responses. This resulted in a total of 15 runs. Since resources were limited, the DoE was performed in shake flasks. The model created was fitted with multiple linear regression.

The medium recipe is identical to the other shake flask experiments, as shown in Table 4. The pH was adjusted with 1 M HCl and 1 M NaOH. Subsequently, incubation was performed at 180 rpm and various temperatures in shaker incubators for 96 h. A sample was drawn every 24 h, pH was measured, and the supernatant was frozen for HPLC.

2.3 Fermentation in Bioreactors

2.3.1 Batch Fermentation

2-L-bench top bioreactors (Bioengineering AG, Wald, Swiss) were used for cultivation in bioreactors. A plan of the setup is shown in Figure 6. The volume of the fermentation medium varied between 0,5 and 1,25 L. F-medium reactor, tracer and water were autoclaved with the reactor. The F-medium reactor has the same composition as in Table 4 but without Tween80 and peptone. If antifoam was added, Antifoam 204 from Sigma-Aldrich was used at a concentration of 0,01 %. This was also autoclaved with the reactor. After autoclaving, a 100 mL bottle of 1 M HCl was connected to the reactor via tubing under sterile conditions. Inoculation was done with a preculture whose volume was one-tenth of the total volume. This was mixed with the desired amount of 400 g/L glucose solution and pumped into the reactor for inoculation.

Sample one was always taken immediately after inoculation. Additional samples were taken at regular intervals and analyzed immediately (see section 2.4.2). As soon as the pH probe indicated a pH value above 7, the pH was manually adjusted to 3-4 with 1 M HCl. Automatic pH adjustment was not used in this case, as the probe was sometimes unreliable due to fungal growth on probes.

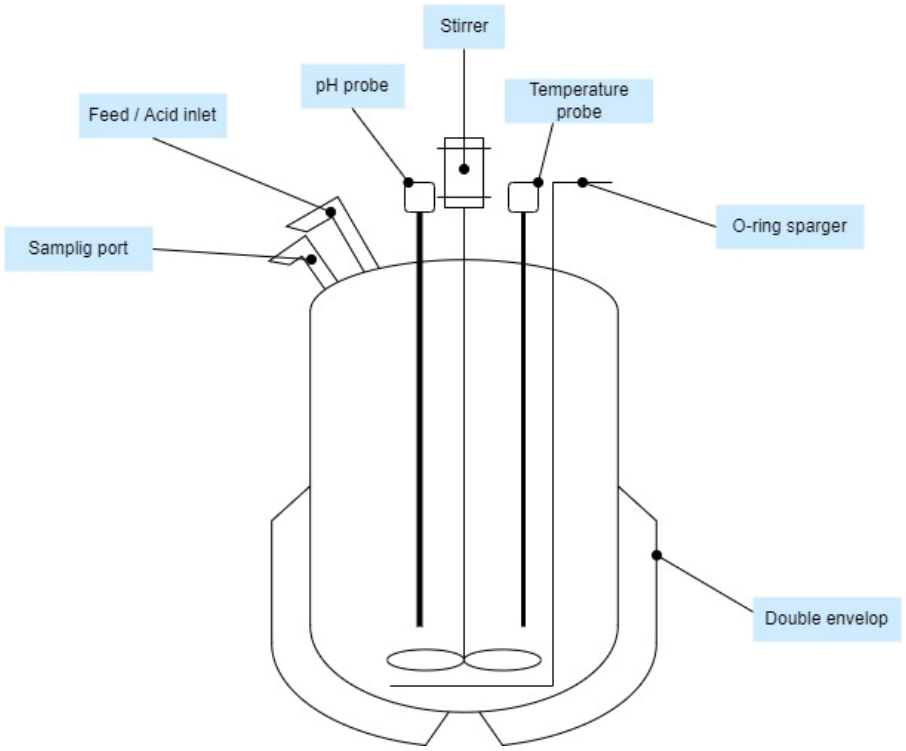


Figure 6: Schematic plan of the used bioreactor setup

2.3.2 Discontinuous Fed-Batch Fermentation

Discontinuous fed-batch cultivation also took place in 2-L-bench top bioreactors. The reactor volume was 0,5 L at the beginning. The medium preparation was identical to that for batch fermentations. In addition to the connection with the acid, a 400 g/L glucose feed was also

connected. Inoculation was also performed with a mixture of preculture and glucose solution. However, this mixture was no longer pumped in but injected via the sampling port using a syringe.

Sampling and pH adjustment was performed identically to that for batch fermentations. The glucose concentration in the samples was determined using a blood glucose meter (Medisana MediTouch® 2 mg/dL Blood glucose meter). As soon as the device indicated a glucose concentration of less than 25 g/L, glucose was fed until the initial concentration was restored.

2.4 Analysis

The subsequent analysis consisted of cell harvesting and determining dry cell weight. Moreover, samples were taken during the cultivations. These were analyzed by HPLC method.

2.4.1 Biomass determination

2.4.1.1 Shake flasks

At the end of cultivation, a final sample was taken. The culture broth was then filtered through a pre-weighed, dried Whatman filter paper. A minimum vacuum of 300 mbar was used for this purpose. The separated biomass was dried at 80 °C for one to three days and the dry cell weight was determined.

2.4.1.2 Bioreactor

After fermentation, the reactor was opened and large biomass agglomerates were transferred with a spoon onto a tared piece of aluminum foil. The remaining culture broth was then separated from the biomass via tared Miracloth. As in shake flasks, the biomass was dried at 80 °C for one to three days, and the dry cell weight was determined.

2.4.2 HPLC Analysis

For analysis via HPLC, samples were taken at regular intervals during the cultivations. 1 mL of these samples was filtered into HPLC vials using a 0,22 µm sterile filter. Vials were stored at - 20 °C until measurement.

A Shimadzu HPLC system with real-time and post-run analysis was used for the measurement. The system components were a DGU-ZOA 3R degassing unit, Nexera XR LC-20AD 2XLiquid Chromatograph, SIL20AC Autosampler, CTO-20A column oven and a RID 20A Refractive index detector. The mobile phase consisted of 5 mM sulfuric acid at a flow rate of 0,6 ml/min. The pressure across the column was 15-18 bar and the temperature was 50 °C.

A calibration curve was prepared for glucose, mannitol, xylitol, erythritol, and glycerol to allow quantitative analysis. For this purpose, a stock solution was set up from all these components. From this stock solution, eight dilutions were prepared and measured. For analysis, Lab solution was used as software.

3 Results

3.1 Volume reduction

3.1.1 Parameters

The first reactor was operated with conditions under which *T. reesei* could already be cultivated in bioreactors in the research group. The conditions are shown in Table 6. Since there were problems with overgrowth, the volume was later reduced to 0,5 L. Due to the lower volume, an impeller was also removed. This condition was tested twice. However, it should be noted that the reactor with a volume of 1 L was started with spore suspension, while the ones with reduced volume started with a preculture about 16 h old.

Table 6: Fermentation parameters for different volumes. The 0,5 L parameters will serve as standard conditions for the following chapters.

Parameter	**1 L volume**	**0,5 L volume**
Temperature	28 °C	28 °C
Aeration	13 PV	13 PV
Stirring speed	300 rpm	300 rpm
Cultivation time	72 h	72 h
Impeller type	2x marine blade	1x marine blade
Total volume	1 L	0,5 L
Spore suspension / Preculture	Spore suspension	Preculture

3.1.2 Results

Figure 7 and Figure 8 show the time course of glucose consumption and erythritol production at different volumes. It should be noted that measurements were only taken at individual points in time, marked here as triangles or dots. The interpolated straight lines in between are for better clarity and do not correspond to any measuring points. This will subsequently apply to all diagrams of this type. It should also be remarked that the 1 L reactor was started with a spore suspension, while the 0,5 L reactor was started with a preculture. The results are, therefore, only comparable to a limited extent.

Erythritol and glucose concentrations were measured via HPLC from the culture supernatant. The erythritol production corresponds to the measured erythritol concentration. Glucose consumption was calculated using the following equation:

Equation 6

$$c_{Glucose\ consumed}\left(\frac{g}{L}\right) = c_{Glucose}(0)\left(\frac{g}{L}\right) - c_{Glucose}(t)\left(\frac{g}{L}\right)$$

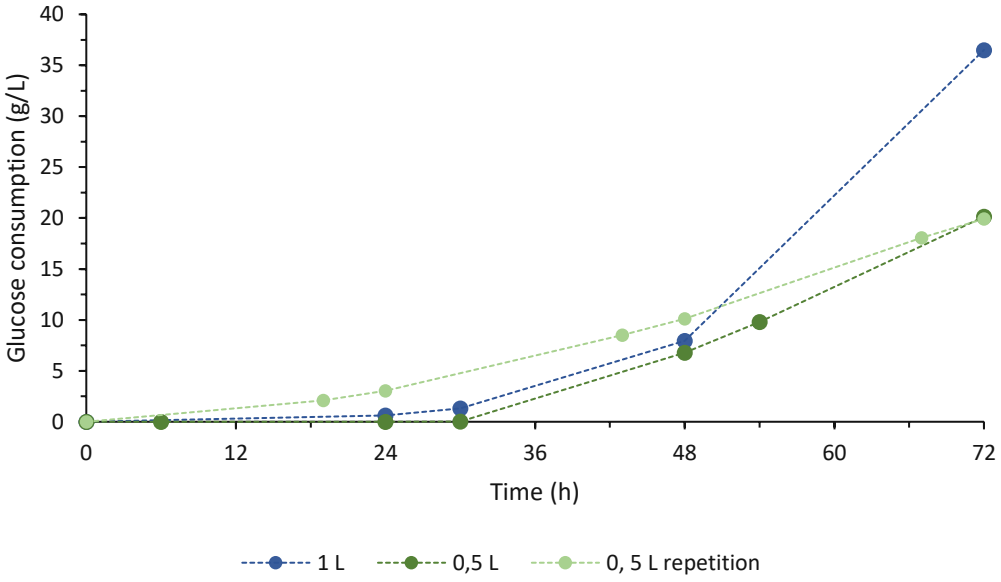


Figure 7: Glucose consumption over time at different volumes

It can be seen in Figure 7 and Figure 8 that glucose consumption and erythritol production are comparable for the 0,5 L reactors. The 1 L reactor shows a significant increase in both after 48 h.

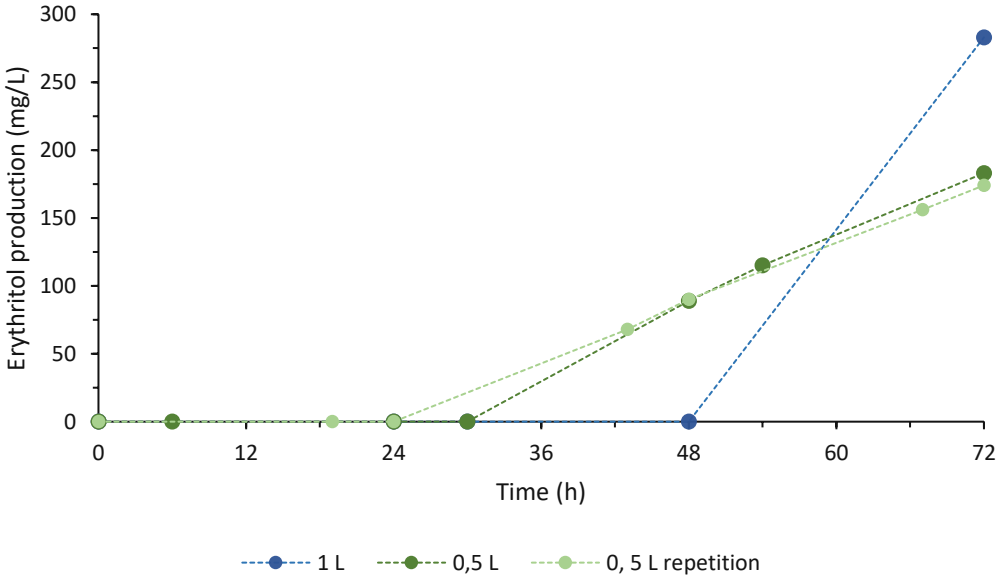
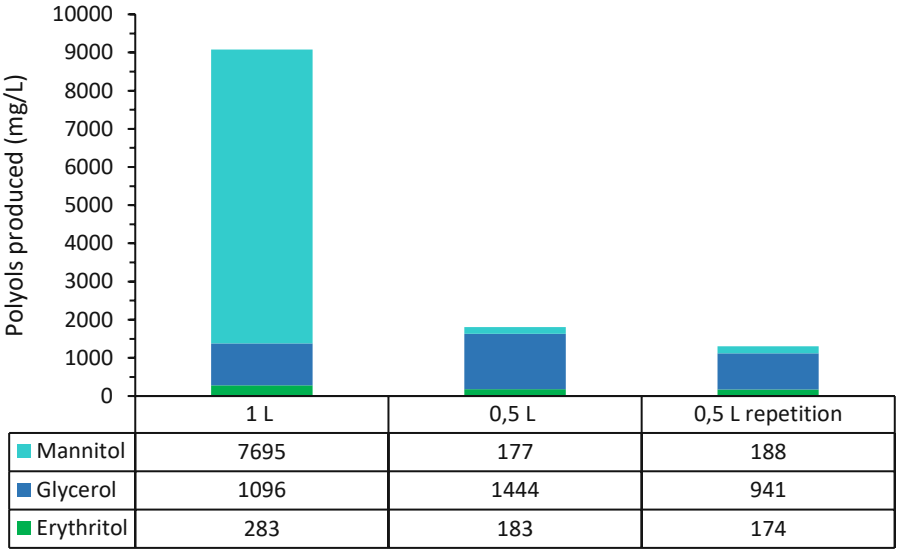


Figure 8: Erythritol production over time at different volumes

The polyol production of the two conditions can be seen in Figure 9. All polyol concentrations were measured via HPLC from the culture supernatant. It is visible that significantly more mannitol was produced in the reactor with a larger volume. In addition, about one-third less glycerol and more erythritol were produced. The repetition of the 0,5 L reactor showed less glycerol but a comparable amount of erythritol and mannitol.



	1 L	0,5 L	0,5 L repetition
Mannitol	7695	177	188
Glycerol	1096	1444	941
Erythritol	283	183	174

Figure 9: Polyols produced at different volumes

The yields and biomass are shown in Table 7. The different yields of the respective fermentations were calculated using Equation 7 to Equation 9. The meanings of the abbreviations are shown in chapter 9.

Equation 7

$$\frac{E}{Glu}\left(\frac{mg}{g}\right) = \frac{c_{Erythritol}(end)\left(\frac{g}{L}\right)}{c_{Glucose\ consumed}(end)\left(\frac{g}{L}\right)} * 1000$$

Equation 8

$$\frac{E}{X}\left(\frac{mg}{g}\right) = \frac{c_{Erythritol}(end)(\frac{g}{L})}{biomass\ (g) * Volume\ (L)} * 1000$$

Equation 9

$$\frac{X}{Glu}\left(\frac{g}{g}\right) = \frac{biomass\ (g) * Volume\ (L)}{c_{Glucose\ consumed}\ (end)(\frac{g}{L})}$$

Table 7: Biomass and Yields at different volumes

	1 L volume	**0,5 volume**	**0,5 L volume repetition**
Biomass (g)	17,600	6,838	6,494
E/Glu (mg/g)	7,754	8,046	8,736
E/X (mg/g)	16,080	13,381	13,397
X/Glu (g/g)	0,482	0,601	0,652

3.1.3 Determination of Standard conditions

For better comparison, standard parameters should be defined. These correspond to those parameters that have been practical for the fermentation of *T. reesei* in the past. In order to better understand the influence of individual parameters and parameter combinations on the process, the difference to these defined standard conditions will be presented in the other subchapters.

The 1 L volume reactor was started with spore suspension, while the ones with reduced volume started with a preculture about 16 h old. All subsequent reactors were also started with a preculture. Moreover, this was the only condition that was tested twice. Therefore, the 0,5 L reactor will be used as the standard condition for further comparison in the following chapters.

3.2 Medium Influence

As mentioned in section 1.3.3.2, media composition influences erythritol production. Stark already described the influence of carbon and nitrogen source on erythritol production by *T. reesei* [40]. However, other media components may also influence the process. Foam formation, for example, is promoted by some media components but proved to be one of the major problems during cultivation in bioreactors and regularly led to premature termination of fermentations. One way to prevent foaming is to discontinue using potential foaming agents, which in this case are Tween80 and peptone. Peptone is a mixture of peptides and amino acids that can have a foam-stabilizing effect. Tween80 is a non-ionic detergent that serves as an emulsifier. In this case, it can prevent the spores from sticking to each other, which is why it is added to the medium. It has surface-active properties and can therefore influence foam formation, depending on the other media ingredients [59]. In addition, there is a second possibility for preventing foam formation, which was investigated in this thesis: the addition of antifoam.

In this chapter, experiments in shake flasks are presented first, in which the influence of Tween80, Peptone and antifoam on erythritol production was investigated. These were carried out in shake flasks to obtain a certain degree of statistical validation. This would not have been possible in bioreactors due to a lack of resources. However, since the process often behaves differently in bioreactors, as shown in the following chapters, the influence of Tween80 was also tested in bioreactors.

3.2.1 Influence of foaming medium components and antifoam on erythritol production

3.2.1.1 Parameters

In order to be able to investigate the effects of the foam-influencing components, the fermentation parameters from Table 8 were selected. These correspond to the parameters tested by Stark for the production of erythritol in shake flasks [40]. The influence of Tween80 and Peptone were tested in a 168 h long experiment, while the influence of Tween80 and antifoam

were tested in a different experiment that took only 96 h. This has to be considered, especially when comparing polyols, biomass and yields.

Table 8: Parameters used for investigating the influence of foam-influencing media components in shake flasks

Parameter	Value
Temperature	30°C
Stirring speed	180 rpm
Cultivation time	96/168 h
Total volume	100 mL

The media composition from Table 4 was selected as standard shake flask conditions. The variation in the F-medium of the respective conditions compared to the standard shake flask conditions can be seen in Table 9.

Table 9: Overview of the foam-influencing media components in the tested conditions

Component	Standard shake flask conditions	Only Peptone	Only Tween80	No Peptone, no Tween80	Only Tween80, 96 h	Tween80 +Antifoam, 96 h	Antifoam, 96 h
Peptone	✓	✓	×	×	×	×	×
Tween80	✓	×	✓	×	✓	✓	×
Antifoam	×	×	×	×	×	✓	✓

3.2.1.2 Results

Figure 10 and Figure 11 show glucose consumption and erythritol production over time. It should be noted that measurements were only taken at individual points in time. An interpolated straight line was placed between these measurement points, which provided a better overview and did not correspond to the actual course. The glucose consumption was calculated according to Equation 6.

In Figure 10, it can be seen that Tween80 and peptone influence glucose consumption, but the trend of the interpolated curves remains similar. Here, the absence of Tween80 correlated with increased glucose consumption. The measurement point at 72 h, where in both experiments samples were taken, shows that the glucose consumption of “Only Tween80” in the 96 h series of measurements is also similar, although slightly higher. The addition of antifoam while retaining Tween80 did not yet cause a reduction in glucose consumption. Only when Tween80 was omitted a lower glucose consumption was observed in the 96 h experiment.

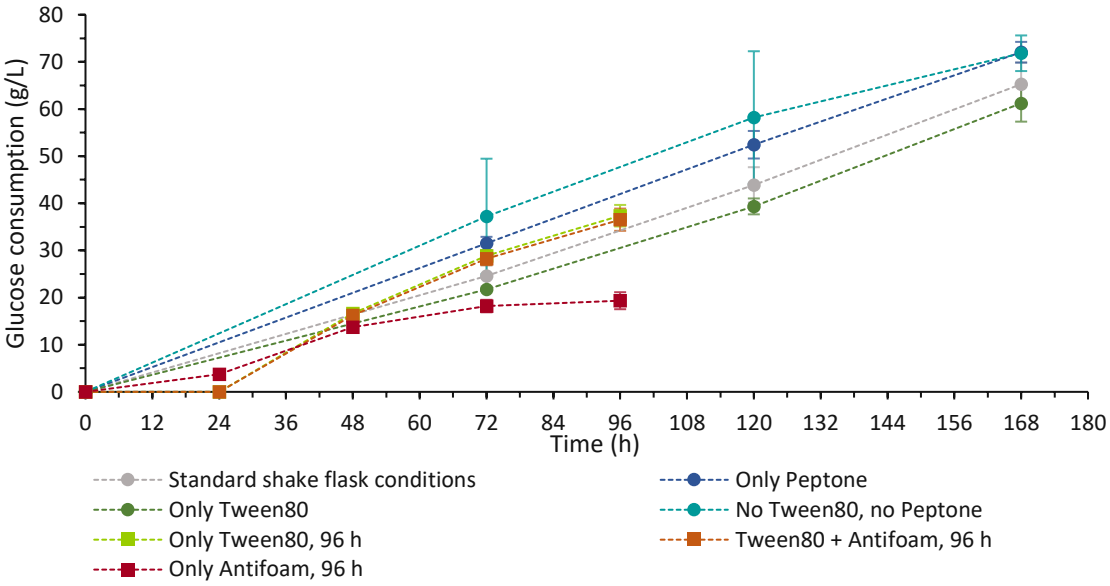


Figure 10: Glucose consumption over time with different (anti-)foaming media components

Figure 11 shows that the media composition influences erythritol production more than glucose consumption. Here it can be seen that from 72 h on, the omission of Tween80 correlates with a lower measured amount of erythritol. This is seen in both series of measurements but is particularly strong at longer fermentation times. The addition of peptone also seems to correlate with a higher amount of erythritol, but the effect is much less noticeable than with Tween80. The addition of antifoam appears to cause earlier erythritol production, but the slope of the interpolated straight line is weaker than under conditions without antifoam. The final erythritol production at 96 h does not seem to be influenced by the addition of antifoam. Moreover, in the two "Only Tween80" measurement series, a significant difference is seen at the measurement point at 72 h. The degree to which this can be attributed to measurement uncertainties or external circumstances, such as the condition of the inoculation culture, cannot be conclusively assessed and requires further testing for reliability.

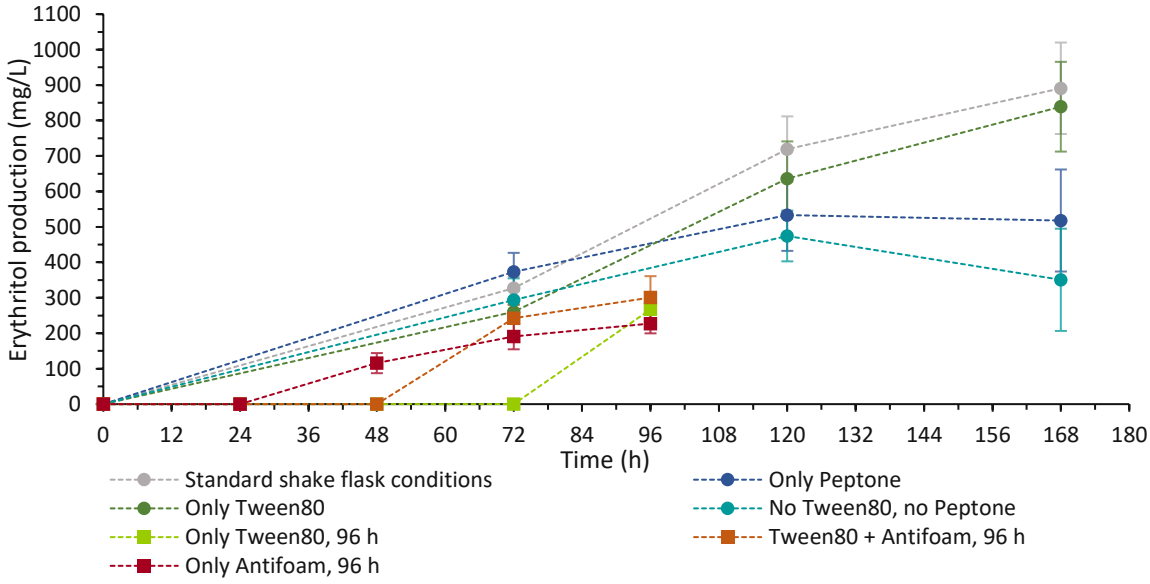
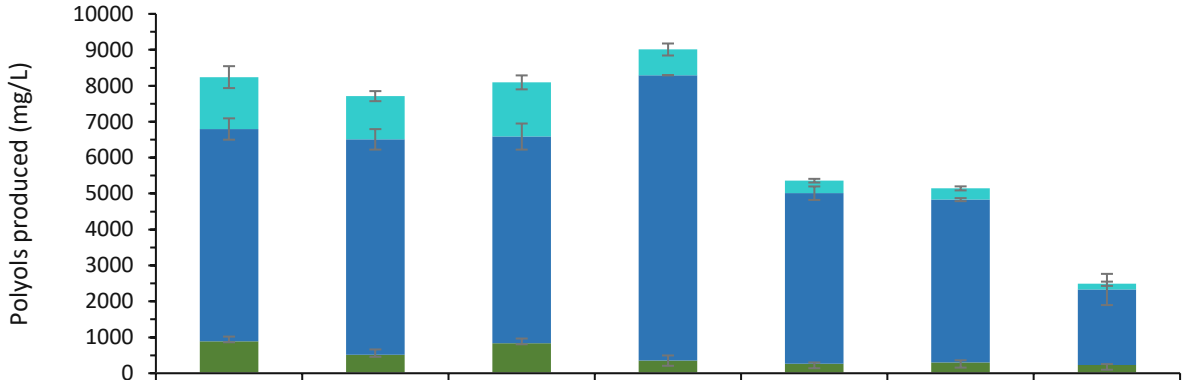


Figure 11: Erythritol production over time with different (anti-)foaming media components

The amount of polyols detected at the end of the fermentations can be seen in Figure 12. It should be noted again that these are two different fermentation durations. In that series of experiments, which went on for 168 h, it can be seen that Tween80 affects the amount of erythritol. This effect is not seen to the same extent with the other polyols. In contrast, the combination of the absence of Tween80 and peptone affected the production of the by-products. While the amount of glycerol increased by approximately 2000 mg/L, the amount of mannitol decreased significantly. The amount of erythritol also decreased more than the sum of the individual effects would suggest. However, in the series of measurements with 96 h fermentation time, hardly any effects on erythritol production can be seen. The addition of antifoam resulted in only slightly fewer by-products and only slightly more erythritol if Tween80 was present. However, omitting Tween80 here resulted in an approximate halving of glycerol and mannitol, with similar erythritol yields.



	Standard shake flask conditions	Only Peptone	Only Tween80	No Tween80, no Peptone	Only Tween80, 96h	Antifoam + Tween80 96h	Antifoam 96h
Mannitol	1444	1203	1508	715	348	313	159
Glycerol	5905	5990	5747	7943	4743	4530	2104
Erythritol	891	518	839	351	267	301	227

Figure 12: Polyols produced with different (anti-)foaming media components

Table 10 shows the biomass and the different yields at the different media compositions. The yields were calculated using Equation 7 to Equation 9. In the measurement series with 168 h, the omission of Tween80 correlates with higher biomass values. The opposite is true for the 96 h series of measurements. Similar results can be seen for the yields of erythritol per gram of glucose and yields of erythritol per gram of biomass. Tween80 appears to have a positive influence on the yields in the experiments with longer fermentation times, while the opposite is true for the shorter experiments. What can be seen, however, is that the addition of antifoam correlates with a biomass reduction and an increase in erythritol yields.

Table 10: Biomass and Yields at different media compositions. The standard deviation is given in grey. If no standard deviation was written, only two values were used for calculations because of an outlier.

	Standard shake flask conditions	Only Peptone	Only Tween80	No Tween80, no pep-tone	Only Tween80, 96h	Antifoam + Tween80, 96h	Antifoam, 96h
Biomass (g)	1,418	1,677	1,368	1,748	1,045	0,793	0,408
	±0,055	±0,034	±0,053	±0,235	±0,118	±0,082	±0,048
E/Glu (mg/g)	13,607	7,216	13,659	6,157	7,143	8,061	11,872
	±1,033	±2,161	±1,253		±0,596	±1,084	±2,213
E/X (mg/g)	62,667	31,016	61,204	26,511	26,017	37,654	56,416
	±6,628	±9,226	±7,422		±6,109	±3,907	±10,866
X/Glu (g/g)	0,218	0,233	0,224	0,243	0,281	0,214	0,213
	±0,008	±0,007	±0,006	±0,021	±0,041	±0,007	±0,039

3.2.2 Influence of Tween80 and antifoam in bioreactors

In order to investigate the influence of the medium components Tween80 and antifoam in bioreactors, standard fermentation conditions were used for setting up the reactor. They can be seen in Table 6. Figure 13 and Figure 14 show glucose consumption and erythritol production over time. Glucose consumption was calculated according to Equation 6. The lines between the measuring points are interpolated and serve only for clarity. They do not correspond to the actual course.

As shown in Figure 13, adding Tween80 seems to positively affect glucose consumption since the measurement points are almost all above the conditions without Tween80. Antifoam appears to increase glucose consumption slightly at the beginning of fermentation but results in very similar consumption at the end of fermentation.

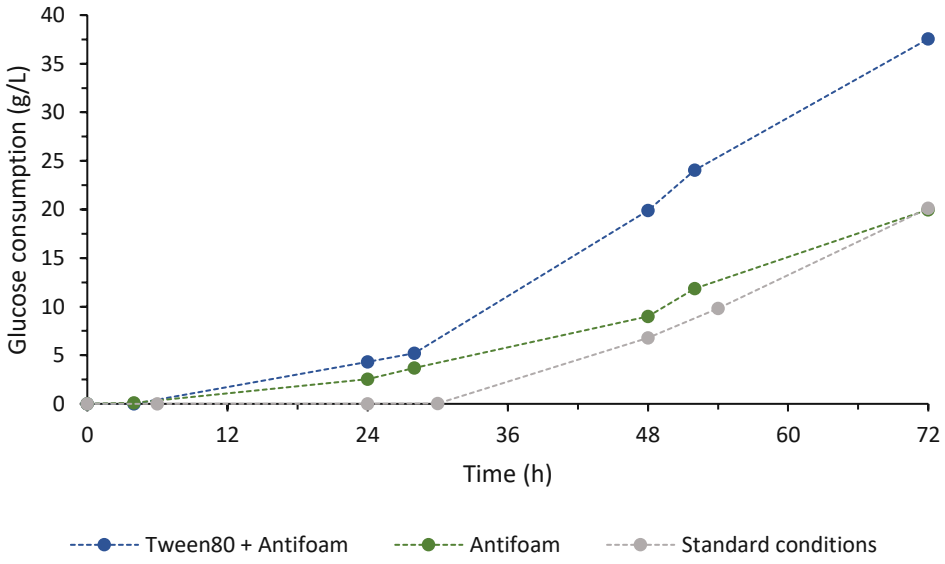


Figure 13: Glucose consumption over the time at testing the influence of Antifoam and Tween80 in bioreactors

The erythritol production, shown in Figure 14, is approximately synchronous for all three conditions. In the end, however, the amount of erythritol detected is slightly lower with the addition of antifoam than with standard conditions. The combination of antifoam and Tween80 has an even more negative effect on erythritol production.

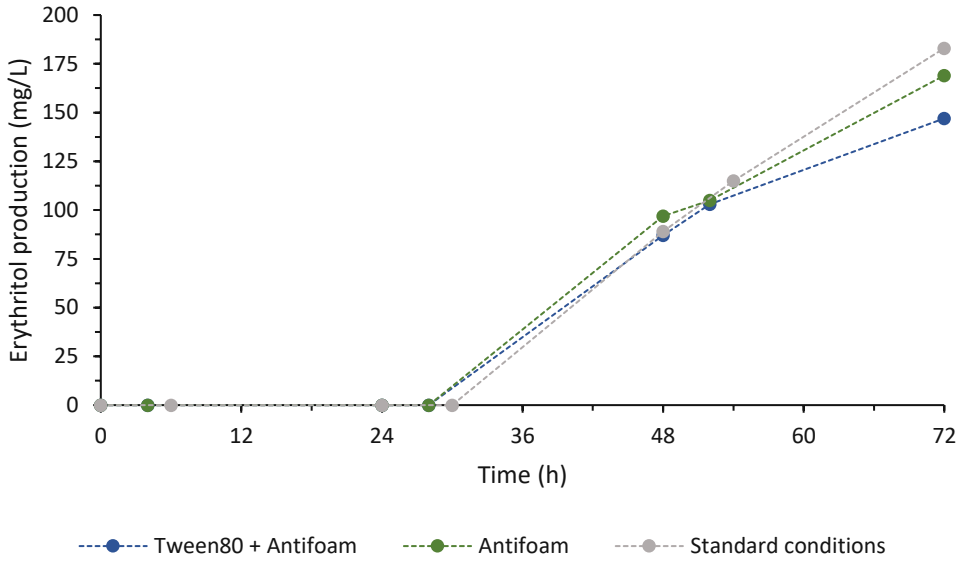
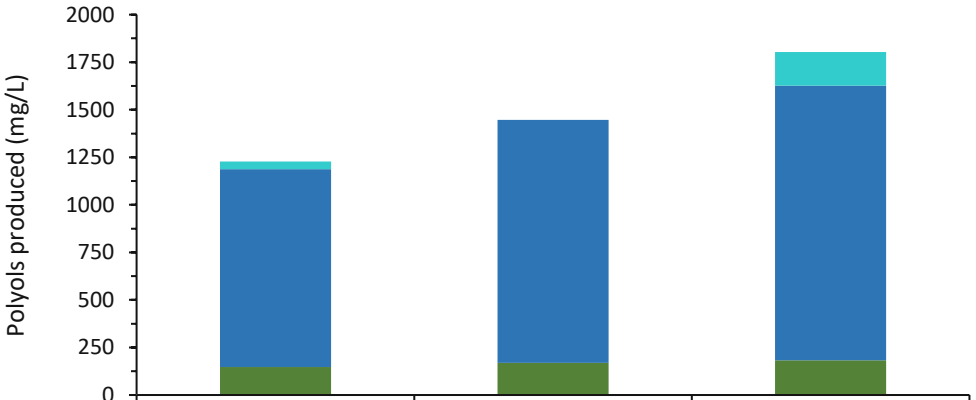


Figure 14: Erythritol production over the time at testing the influence of Tween80 and Antifoam in bioreactors

Figure 15 shows that the addition of antifoam reduced the amount of polyols. All polyols are present in lower amounts than under standard conditions. The combination of Tween80 and antifoam is also likely to harm polyol production. Especially mannitol production seems to be inhibited by antifoam, as shown by a reduction of approximately 80 % (when Tween80 is added) or 100 %. Erythritol production is least affected by this effect, but in the condition with Tween80, it is still around 20 % lower.



	Tween80 + Antifoam	Antifoam	Standard conditions
Mannitol	40	0	177
Glycerol	1040	1277	1444
Erythritol	147	169	183

Figure 15: Polyols produced at testing the influence of Tween80 and Antifoam in bioreactors

According to Equation 7 to Equation 9 calculated, the biomass yields are shown in Table 11. It can be seen that both Tween80 and antifoam increased biomass production. The increased biomass also affects the yields of erythritol per gram of biomass, which are decreased for both conditions compared to standard conditions. Especially, Tween80 is likely to have led to increased glucose consumption, as seen in Figure 13, which affects the yields of erythritol per gram of glucose and the yields of biomass per gram of glucose.

Table 11: Biomass and Yields at testing the influence of Tween80 and Antifoam in bioreactors

	Tween80 + Antifoam	**Antifoam**	**Standard conditions**
Biomass (g)	12,354	9,058	6,838
E/Glu (mg/g)	3,914	8,457	8,046
E/X (mg/g)	5,950	9,329	13,381
X/Glu (g/g)	0,658	0,907	0,601

3.3 Stirring and Aeration

3.3.1 Parameters

The following experiments in reactors should investigate whether higher stirring and aeration have a beneficial effect on erythritol production. For this purpose, these parameters have been alternated. The exact conditions can be seen in Table 12. It should be noted that the volume of the three reactors is different. This is because, as mentioned above, the volume had to be successively reduced due to overgrowth. According to this adaption, the fermentation time could be extended to 72 h at lower volumes. Of course, this affects the comparability of the results. Nevertheless, some trends can be read off.

Also, in contrast to the standard conditions defined in section 3.1.1, the temperature is at 30 °C. This was the initially desired fermentation temperature but could not be achieved in some experiments due to problems with the tower. As a recollection: under standard conditions fermentation was carried out at 300 rpm and 13 PV. The exact parameters for this condition are shown in Table 6. For better comparison, the results of the standard conditions will also be shown in section 3.3.2.

Table 12: Conditions for testing the influence of stirring and aeration

Parameter	High Stirring, High Aeration	Moderate Stirring, High Aeration	Moderate Stirring, High Aeration, Antifoam
Temperature	30 °C	30 °C	30 °C
Aeration	45 PV	45 PV	45 PV
Stirring speed	700 rpm	400 rpm	400 rpm
Cultivation time	48 h	48 h	72 h
Impeller type	Rushton turbine (bottom), marine blade (top)	Rushton turbine (bottom), marine blade (top)	Marine blade
Total volume	1,25 L	1 L	0,5 L

3.3.2 Results

Glucose consumption was calculated according to Equation 6 and is shown graphically in Figure 16. In gray is the standard condition for comparison. Again, as for Figure 17, it should be noted that the dashed lines are interpolated and do not necessarily reflect the actual trend. They are merely for clarity. It is visible that higher stirring and aeration correlates with higher glucose consumption. The addition of antifoam results in less glucose consumption.

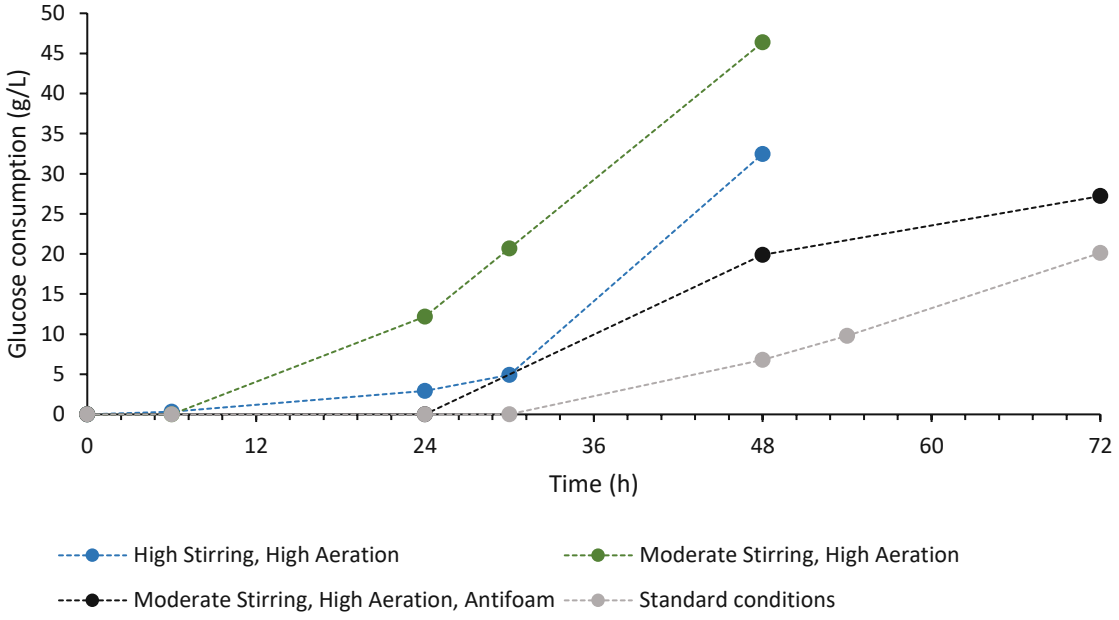


Figure 16: Glucose consumption over time at different stirring and aeration levels compared to standard conditions

Figure 17 shows the erythritol production under different conditions. It is visible that the amount of erythritol produced at elevated stirring and aeration levels is higher than that under standard conditions. Under moderate stirring and high aeration levels, erythritol could be detected earlier in the supernatant, after 30 h latest, compared to 48 h. The erythritol production was higher under moderate stirring and aeration levels than under standard conditions. Like glucose consumption, the green measurement series, meaning moderate stirring and

high aeration without antifoam, stands out remarkably. Here, the amount of erythritol produced is significantly above the level of the other conditions.

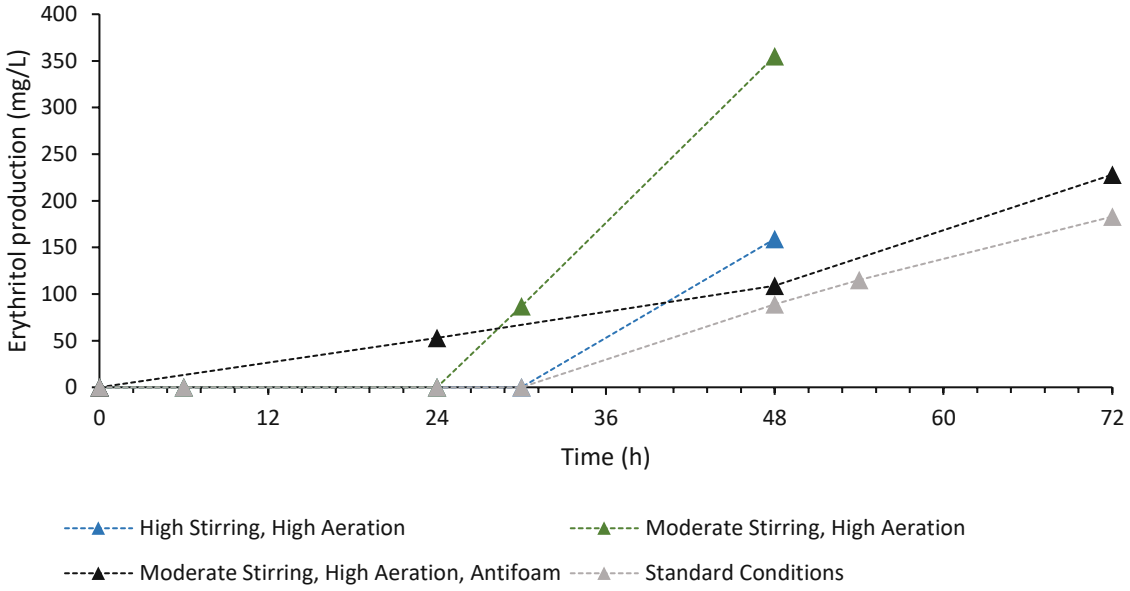
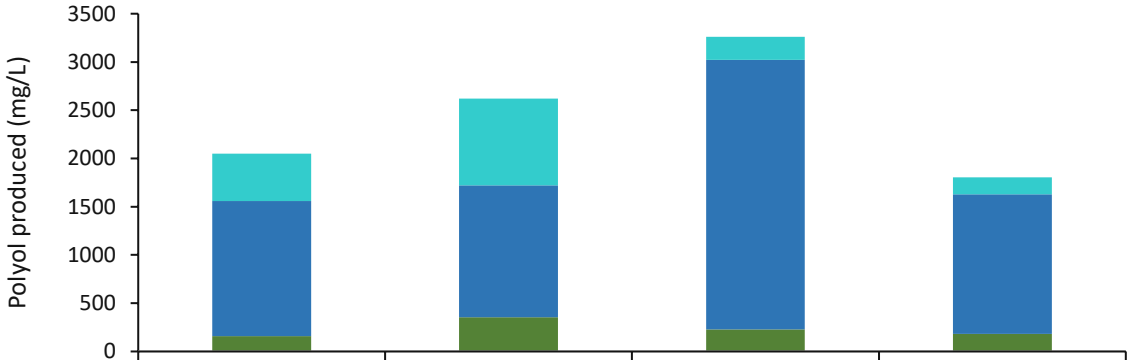


Figure 17: Erythritol production over time at different stirring and aeration levels compared to standard conditions

The polyols produced under various conditions are shown in Figure 18. It can be seen that higher stirring and aeration levels led to a higher total amount of polyols. While the glycerol concentration remained nearly constant without antifoam, it was nearly doubled by adding antifoam. The amount of mannitol was increased in all three conditions compared to the standard conditions, especially in moderate stirring and high aeration without antifoam. During strong stirring conditions, the absolute amount of erythritol at the end of fermentation is lower than under standard conditions. However, it should be noted that fermentation was 24 h longer under standard conditions. As can be seen in Figure 17, the amount of erythritol was still increased at 48 h.



	High Stirring, High Aeration	Moderate Stirring, High Aeration	Moderate Stirring, High Aeration, Antifoam	Standard conditions
Mannitol	490	897	239	177
Glycerol	1400	1367	2795	1444
Erythritol	159	355	228	183

Figure 18: Polyol production at different stirring and aeration levels compared to standard conditions

The yields were calculated according to Equation 7 to Equation 9 and are shown in Table 13. It can be seen that high stirring resulted in significantly lower erythritol yields than in standard conditions. However, biomass production appeared to have been significantly increased in this case, which was also consistent with visual impressions. In terms of yields of erythritol per gram of glucose consumption, the reactor with moderate stirring, high aeration and antifoam gave the best results. However, they are only slightly higher than under standard conditions. It also produced the most biomass per glucose consumed. In proportion to the biomass production, the most erythritol was produced with moderate stirring, high aeration and without antifoam.

Table 13: Comparison of Biomass and Yields at different stirring and aeration levels compared to standard conditions

	High stirring, high aeration	**Moderate stirring, high aeration**	**Moderate stirring, high aeration, antifoam**	**Standard conditions**
Biomass (g)	28,701	23,884	11,572	6,838
E/Glu (mg/g)	4,896	7,654	8,379	8,046
E/X (mg/g)	6,925	14,863	9,851	13,381
X/Glu (g/g)	0,707	0,515	0,851	0,601

3.4 DoE: Influence of pH and temperature

3.4.1 Parameters

A Design of Experiments (DoE) was conducted in shake flasks to determine if pH and temperature affect erythritol production. Different temperature and pH conditions were tested. The temperature range was chosen so that the previous standard fermentation conditions, namely 28 °C and a pH of 4 to 5, roughly corresponded to the center point. This resulted in temperature corner points of 20 and 35 °C and pH corner points of 3 and 6, corresponding to a center point of 27,5 °C and a pH of 4,5. The remaining fermentation parameters are shown in Table 14.

Table 14: Conditions used for testing the influence of pH and temperature

Parameter	**Value**
Temperature	varies
Stirring speed	180 rpm
Cultivation time	96 h
Total volume	100 mL

3.4.2 Results

Figure 19 and Figure 20 show glucose consumption and erythritol production over time. Glucose consumption was calculated using Equation 6. Measurements were only taken at individual points in time. The lines in between are interpolated and do not correspond to the actual course. They serve primarily for a better overview. In Figure 19, a clear difference in glucose consumption can be seen between temperatures. Most glucose was consumed at the

center point. While at 20 °C, significantly less glucose was consumed at the beginning than at the other temperatures, a clear increase can be seen in the interpolated lines from 48 h onwards. The curves of 35 °C behave almost in the opposite way. These still increase similarly to the center point in the first 48 h, but their increase is reduced after that. This indicates a slower glucose consumption in the second half of the fermentation. At 35 °C, a clear difference in glucose consumption at the different pH values can be seen at the end of the fermentation. However, the trends of the curves show apparent differences mainly between the different temperatures.

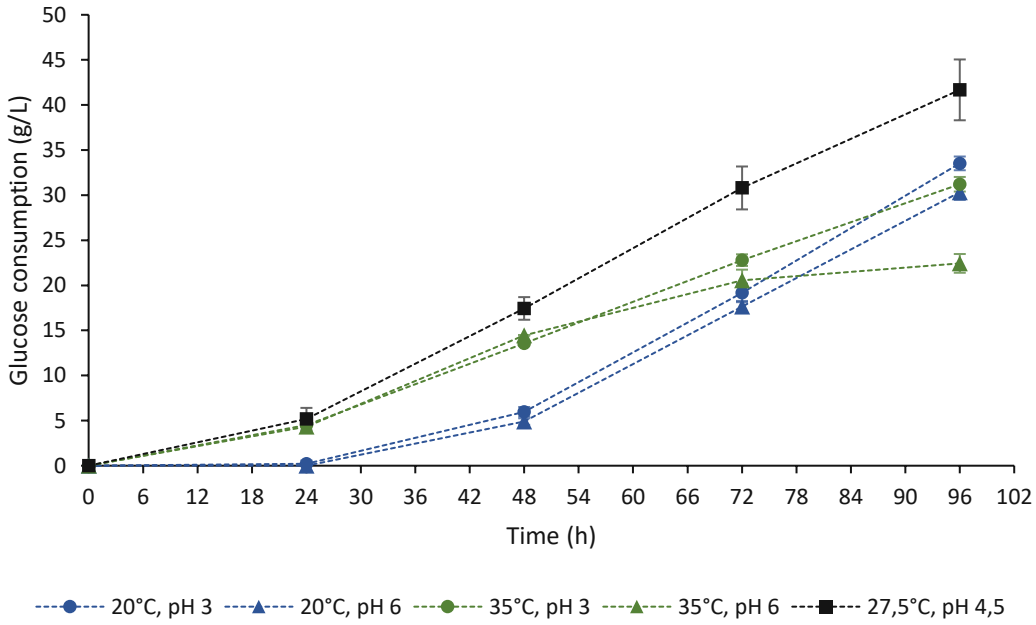


Figure 19: Glucose consumption over time at different pH and temperature combinations

The situation is different for erythritol production, as seen in Figure 20. Here, apparent differences can be seen even within one temperature. At 20 °C, the curves still show a similar trend but diverge in absolute values. In addition, the measurement points at the end of fermentation at these two conditions are loaded with a high standard deviation. The difference between the pH values at 35 °C is particularly significant. The curve of pH 3 is approximately the same as the center point, with slightly increased erythritol production. At pH 6, however, erythritol was hardly produced.

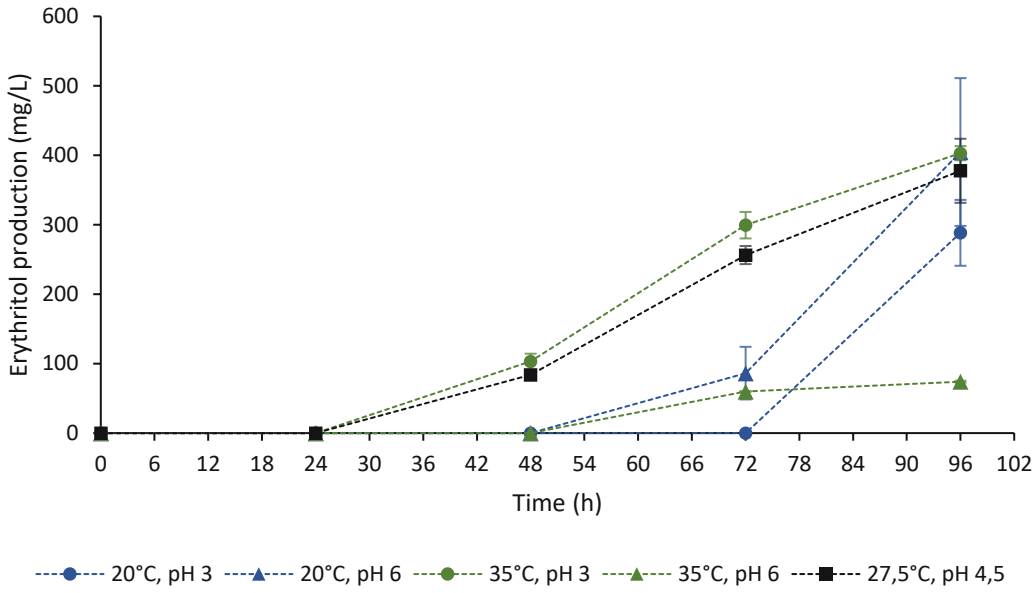
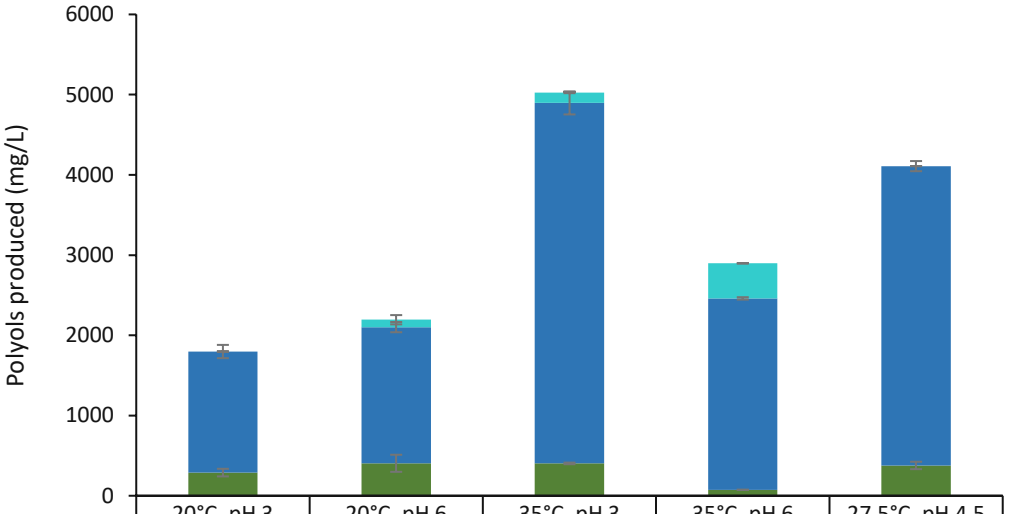


Figure 20: Erythritol production over time at different pH and temperature combinations

The amount of polyols produced is shown in Figure 21. It can be seen that the total polyol amount is lowest at 20 °C. The amount of glycerol was reduced by more than half compared to the center point, which was close to the standard conditions. For erythritol production, a clear difference between pH values can be seen. While more mannitol was produced at higher pH, glycerol and erythritol production was significantly reduced.



	20°C, pH 3	20°C, pH 6	35°C, pH 3	35°C, pH 6	27,5°C, pH 4,5
Mannitol	0	92	130	437	0
Glycerol	1509	1697	4493	2386	3731
Erythritol	288	405	403	74	378

Figure 21: Polyols produced at different pH and temperature combinations

As can be seen in Table 15, more biomass was produced at lower temperatures and less biomass at higher temperatures, regardless of the pH. Yields show a correlation between pH and temperature. The most erythritol per gram of glucose was obtained at 20 °C and pH 6, and

35 °C and pH 3. Erythritol production per gram of biomass is also highest in these conditions. All yields were calculated using Equation 7 to Equation 9.

Table 15: Biomass and yields at different pH and temperature combinations. The standard deviation is given in grey. If no standard deviation was written, then only two values were taken for calculations because of an outlier.

	20 °C, pH 3	20 °C, pH 6	35 °C, pH 3	35 °C, pH 6	27,5 °C, pH 4,5
Biomass (g)	1,677	1,473	0,574	0,355	1,367
	±0,113	±0,094	±0,026	±0,033	±0,052
E/Glu (mg/g)	8,590	13,343	12,922	3,371	9,081
	±1,304	±3,453	±0,064		±1,053
E/X (mg/g)	17,137	27,283	70,338	22,026	27,631
	±1,881	±5,536	±4,241		±3,287
X/Glu (g/g)	0,500	0,485	0,184	0,158	0,329
	±0,024	±0,027	±0,005	±0,008	±0,018

A response contour plot was created for better result illustration, which can be seen in Figure 22. Ideally, the optimum (red) is in the middle. However, in this case, it can be seen that the optimal conditions for erythritol production are two corner points, each with different pH and temperature values.

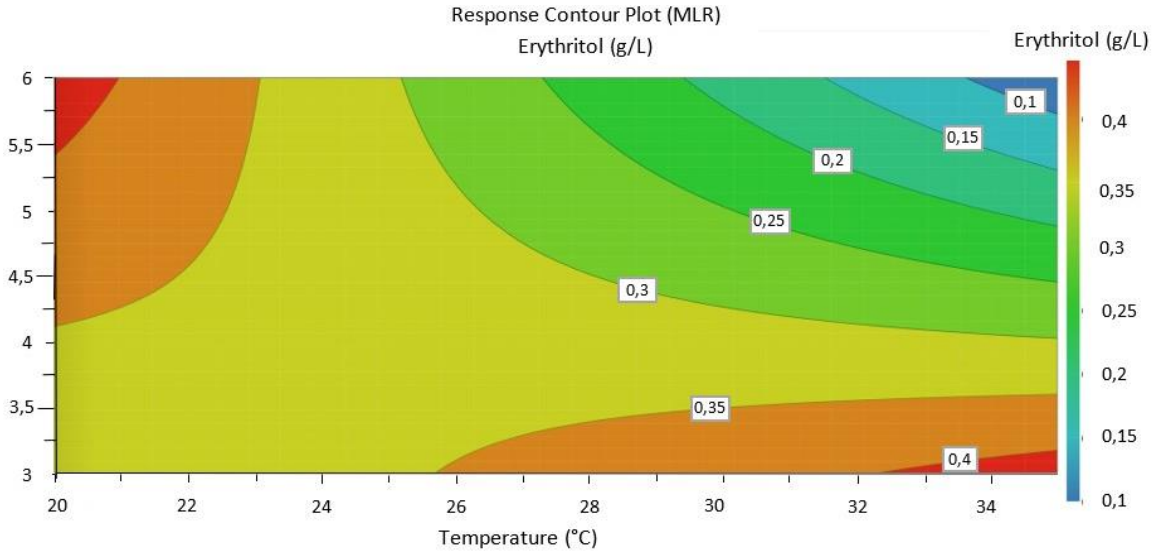


Figure 22: Response Contour Plot for produced Erythritol under different pH and temperature conditions

In Figure 23 to Figure 25 are the interaction plots for temperature and pH at different responses shown. If the two lines are crossed, there is an interaction between the parameters. This interaction is visible in the erythritol production and the yields of erythritol per gram of glucose.

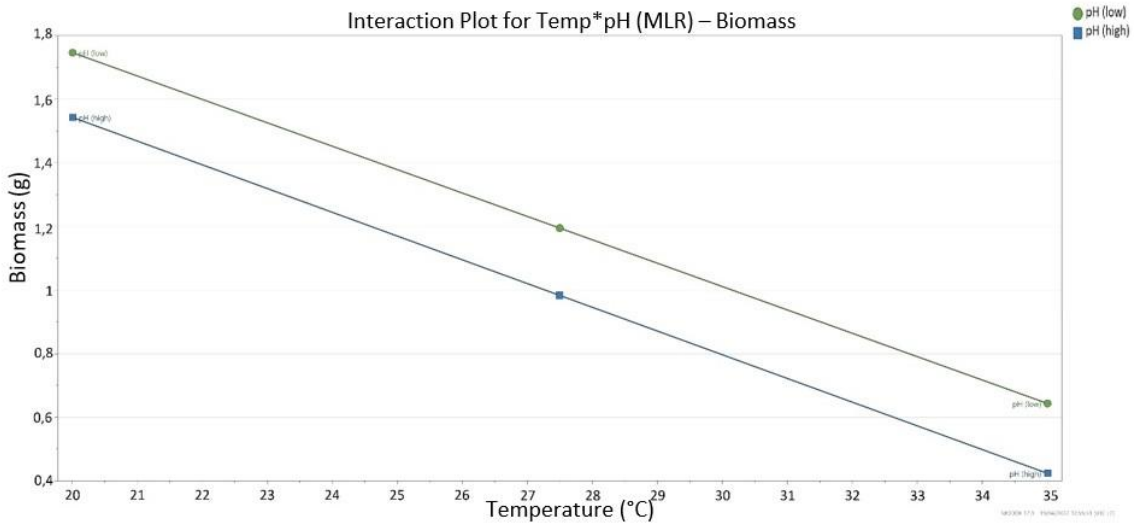


Figure 23: Interaction Plot showing the interaction between pH and temperature at biomass production

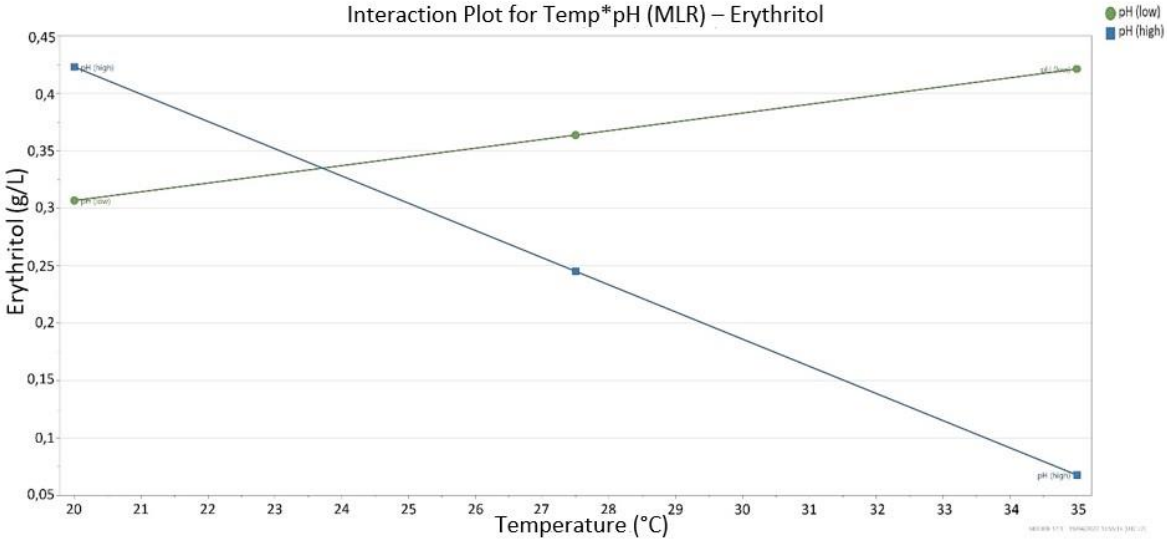


Figure 24: Interaction Plot showing the interaction between pH and temperature at erythritol production

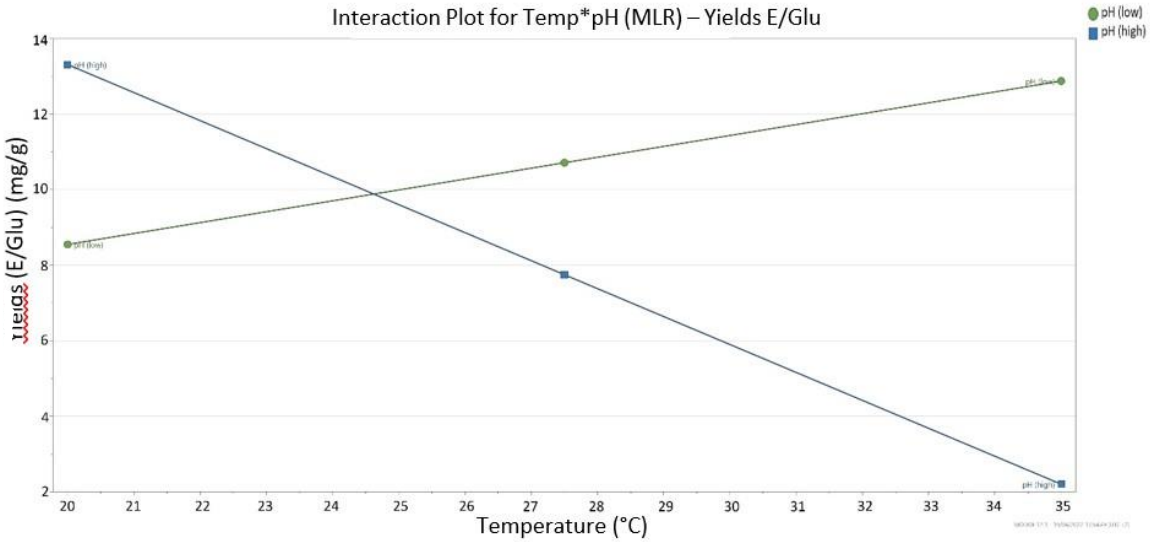


Figure 25: Interaction Plot showing the interaction between pH and temperature at erythritol production per gram glucose consumption

3.4.3 Confirm best conditions

For verification, the best conditions, namely 20 °C and pH 6, and 35 °C and pH 3, were again prepared and measured. Additionally, a flask was started at 20 °C and pH 3 but fermented at 35 °C for the last 24 h. As can be seen in Figure 26, the glucose consumption curves for the two optima found are similar to the first experiment (see Figure 19). In the condition where the temperature was changed after 72 h, there is a clear increase between 72 and 96 h. Before that, it was similar to the corresponding condition from section 3.4.2. Glucose consumption was calculated using Equation 6.

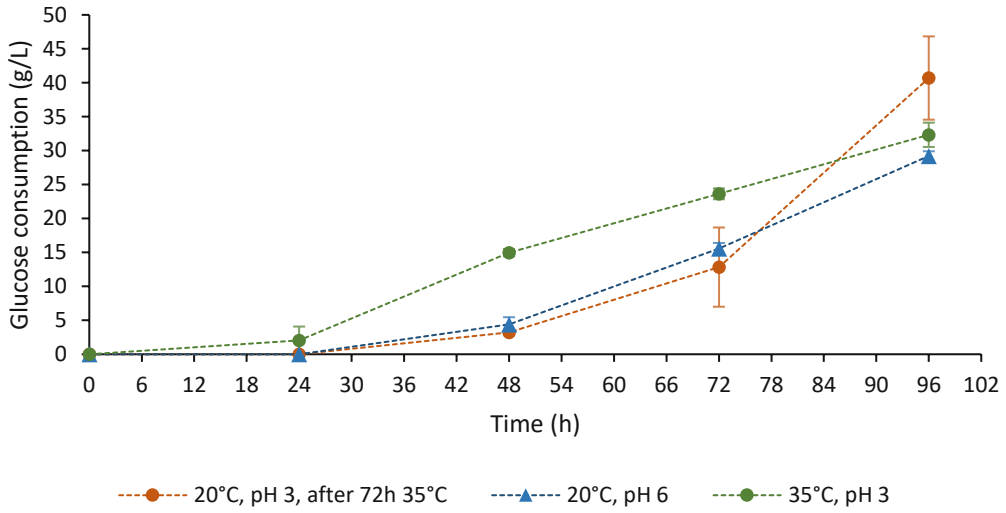


Figure 26: Glucose consumption over time at confirmation of the best pH and temperature combinations

In Figure 27, the erythritol production can be seen. The interpolated curve is less steep here than it was in Figure 20. At the end of the test series, only about half the amount of erythritol compared to the first run was detected. The same can be seen for the condition 35 °C and pH 3. For the condition where the temperature was changed, it can be seen that in the 24 h after change there is a significant increase in the amount of erythritol detected. However, this measurement point should be viewed cautiously as it is loaded with a large standard deviation.

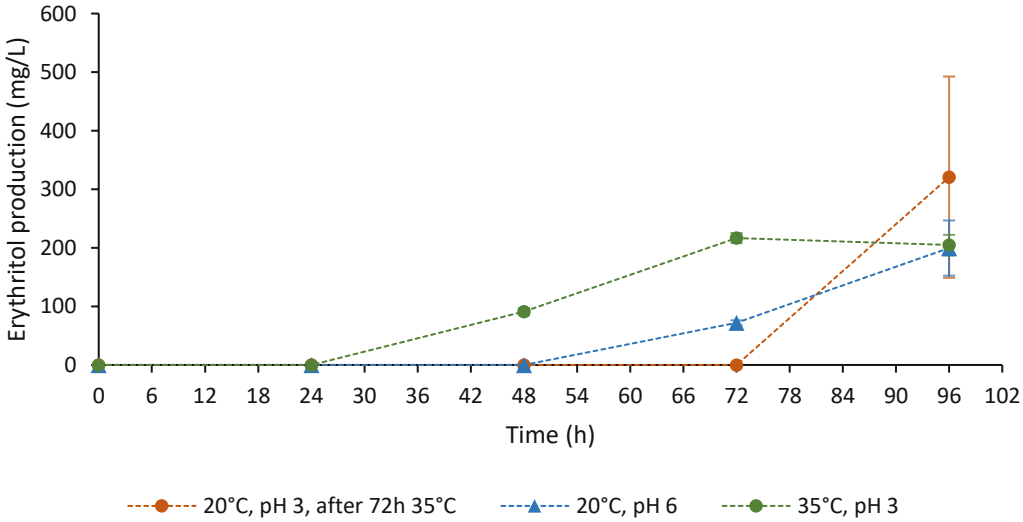
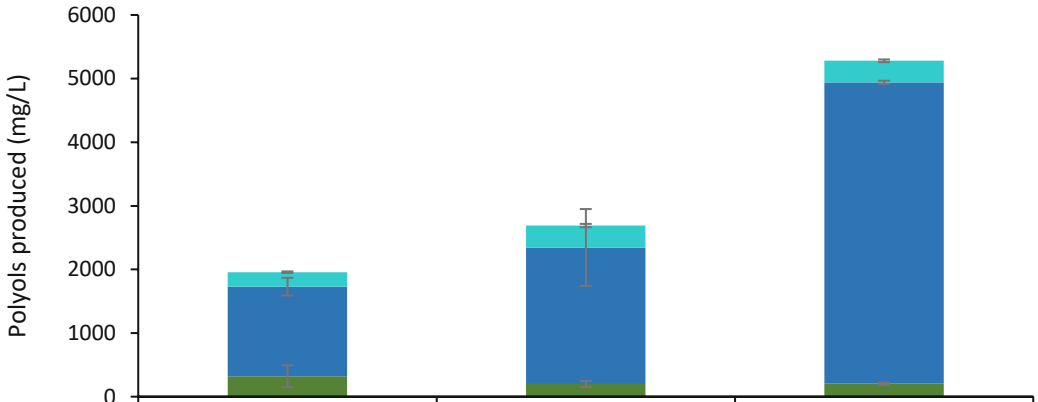


Figure 27: Erythritol production over the time at confirmation of the best pH and temperature combinations

Figure 28 shows that glycerol production at 35 °C and pH 3 is comparable to that in the first experiment. However, the amount of mannitol produced increased by 200 mg/L, while the amount of erythritol decreased by the same amount. Significantly more mannitol was produced at the 20 °C pH 6, but again by about 200 mg/L less erythritol. The most erythritol with the fewest byproducts was obtained with temperature change.



	20°C, pH 3, after 72h 35°C	20°C, pH 6	35°C, pH 3
Mannitol	228	345	339
Glycerol	1408	2146	4737
Erythritol	321	200	205

Figure 28: Polyols produced at confirmation of the best pH and temperature combinations

Biomass and yields were calculated using Equation 7 to Equation 9 and are given in Table 16. It can be seen that the biomass formation was similar to the first series of experiments in both optima. The biomass per gram glucose yields are also comparable. However, the lower erythritol production is reflected in the yields, which are reduced by nearly half. Yields at the temperature change condition are comparable to those at 20 °C and pH 6.

Table 16: Biomass and yields at the confirmation of the best pH and temperature combinations. The standard deviation is given in grey.

	20 °C, pH 3 after 72 h 35°C	**20 °C, pH 6**	**35 °C, pH 3**
Biomass (g)	1,385	1,240	0,601
	±0,098	±0,225	±0,020
E/Glu (mg/g)	5,935	6,705	6,358
	±0,245	±1,329	±0,857
E/X (mg/g)	16,363	16,080	34,115
	±0,761	±3,189	±3,974
X/Glu (g/g)	0,363	0,418	0,186
	±0,002	±0,027	±0,009

3.5 Fermentation time

3.5.1 Parameters

During one experiment in bioreactors, it was possible to extend the fermentation time to 96 h. Usually, foaming, overgrowth, or liquid loss problems occurred after 72 h. For some unknown reason, these problems only occurred after 96 h in this run. The conditions are shown in Table 17.

Table 17: Conditions for bioreactor with 96 h fermentation time

Parameter	Value
Temperature	28 °C
Aeration	13 PV
Stirring speed	300 rpm
Cultivation time	96 h
Impeller type	Marine blade
Total volume	0,5 L

3.5.2 Results

In Figure 29, the glucose consumption and the erythritol production can be seen. The lines between the individual measuring points are interpolated and do not reflect the actual course but serve to provide a better overview. For the calculation of glucose consumption, Equation 6 was used. The respective concentrations were measured in the supernatant of the samples. As can be seen, the courses are relatively similar up to 72 h, which was expected since the conditions were the same. However, it can be seen that both erythritol production and glucose consumption increased significantly between 72 h and 96 h fermentation time.

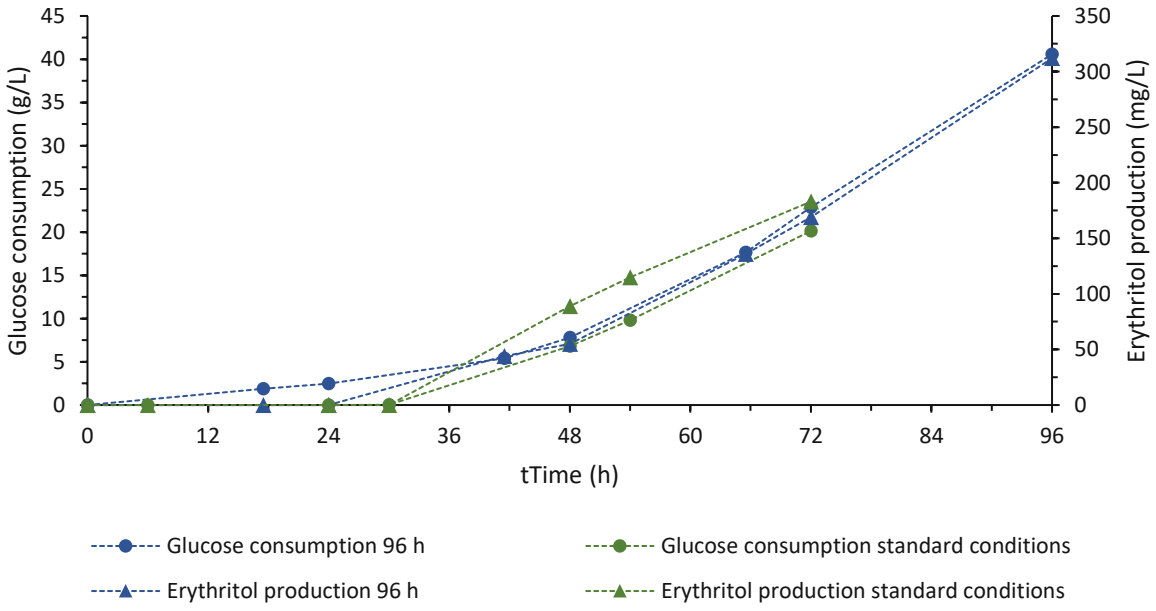


Figure 29: Glucose consumption and Erythritol production over time at 96 h fermentation times compared to standard conditions

The amount of polyol produced with extended fermentation time compared to standard conditions is shown in Figure 30. It is visible that relatively little glycerol and much mannitol can be measured with a longer fermentation time. Whether this is due to the longer fermentation time or other circumstances, such as the condition of the inoculum, cannot be conclusively determined, as it was not possible to retest the longer fermentation time for the reasons given above. However, the amount of erythritol produced is significantly increased after a 24 h longer fermentation time.

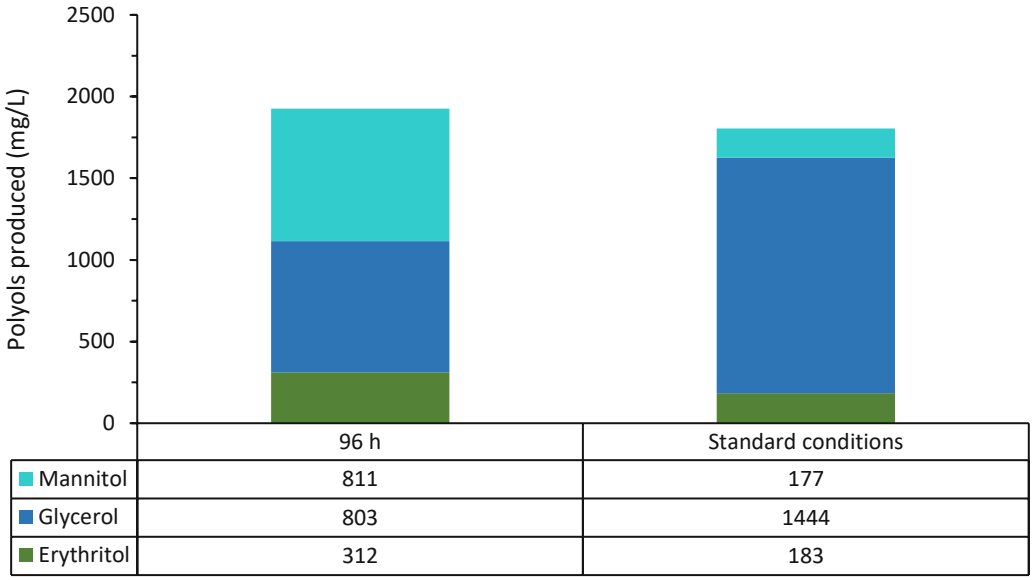


Figure 30: Polyols produced at 96 h fermentation time compared to standard conditions

Table 18 shows biomass and yields from a 24 h extended fermentation compared to standard conditions. Yields were calculated according to Equation 7 to Equation 9. The biomass is increased, but the yield biomass per gram of glucose is lower than in standard conditions. The yields of erythritol per gram of glucose are slightly lower at the longer fermentation time than under standard conditions. On the contrary, the yields of erythritol per gram of biomass increased.

Table 18: Biomass and yields at 96 h fermentation time compared to standard conditions

	96 h fermentation time	**Standard conditions (72 h)**
Biomass (g)	9,508	6,838
E/Glu (mg/g)	7,690	8,046
E/X (mg/g)	16,407	13,381
X/Glu (g/g)	0,469	0,601

3.6 Operation mode

As explained in section 1.3.3.1, the operation mode can influence the process. Therefore, a discontinuous fed-batch was performed. The following section compares different operation modes in bioreactors to investigate their influence on erythritol production.

3.6.1 Parameters

Three variations were performed: a discontinuous fed-batch with 30 g/L initial glucose concentration, a batch with the same initial concentration, and a discontinuous fed-batch with 50 g/L initial glucose concentration and a fermentation time of 96 h. The exact conditions are shown in Table 19. It should be noted that due to the in section 3.5.1 mentioned reasons, it was not possible to extend other fermentations to 96 h. The 50 g/L fed-batch fermentation was run simultaneously and started from the same plate as the reactor from section 3.5.

Table 19: Parameters for testing the influence of different operation modes

Parameter	50 g/L Fed-Batch	30 g/L Fed-Batch	30 g/L Batch
Temperature	28 °C	28 °C	28 °C
Aeration	13 PV	13 PV	13 PV
Stirring speed	300 rpm	300 rpm	300 rpm
Cultivation time	96 h	72 h	72 h
Impeller type	Marine blade	Marine blade	Marine blade
Operation Mode	Fed-Batch	Fed-Batch	Batch
Total volume (start)	0,5 L	0,5 L	0,5 L

3.6.2 Results

Figure 31 and Figure 32 show the time course of the measured glucose consumption and erythritol production, as well as interpolated lines between the measuring points for better clarity. In each case, measurements were taken by HPLC of the supernatant of the samples.

Glucose consumption, calculated using Equation 6, shows that the curves are relatively similar for the two fed-batch fermentations. The situation is different for batch fermentations. Here, a clear curve increase can be seen with a time delay. In total, more glucose was consumed in the batch fermentations than in the fed-batch fermentations.

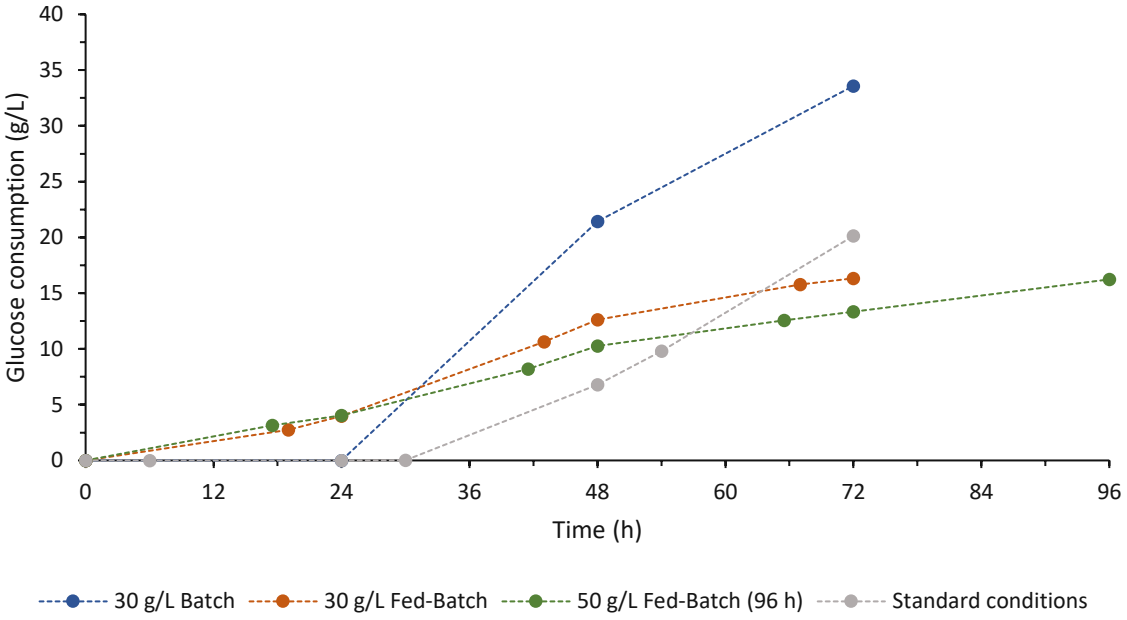


Figure 31: Glucose consumption over time at different operation modes compared to standard conditions

In the case of erythritol production, seen in Figure 32, erythritol was detected in the 30 g/L batch well before the other series of measurements, namely after 24 h. In the 30 g/L fed-batch, the measured erythritol concentration increased significantly within 6 h. However, since this increase seems questionable based on previous experience, this could also be due to a measurement error. The 50 g/L fed-batch detected the lowest amounts of erythritol over the entire period.

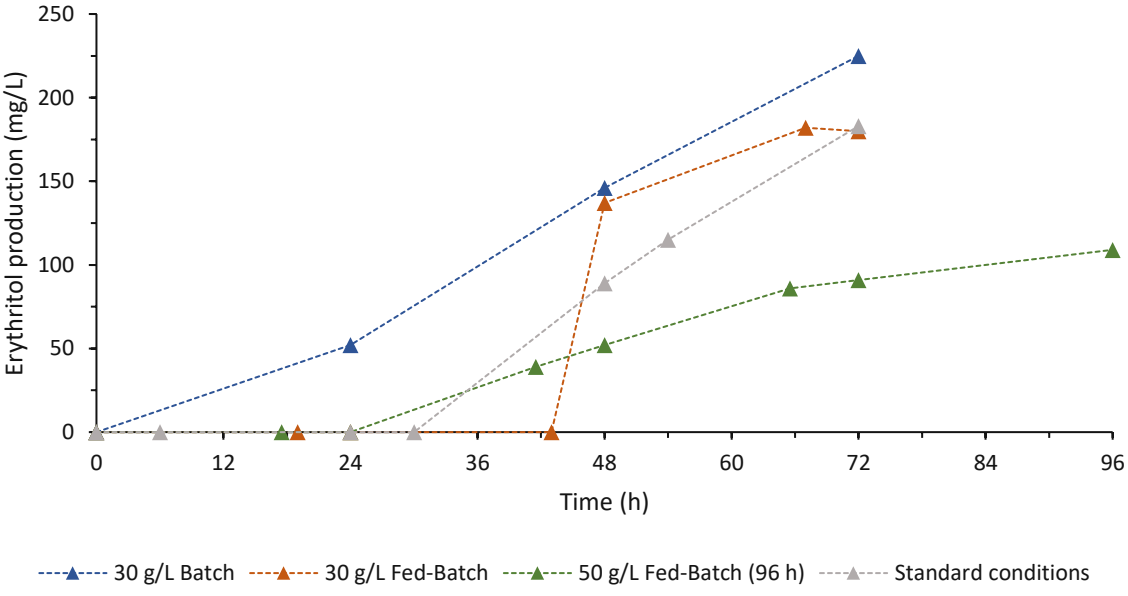
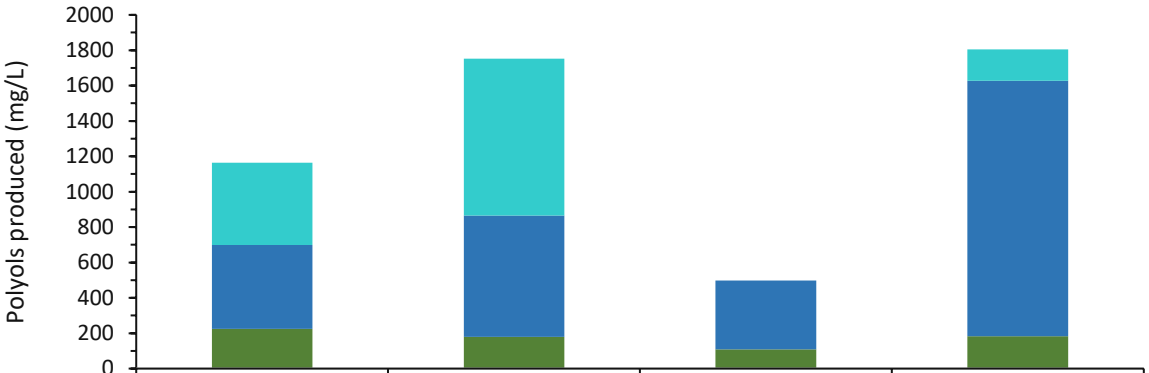


Figure 32: Erythritol production over time at different operation modes compared to standard conditions

The amount of polyol measured at the end of fermentation can be seen in Figure 33. It can be seen that all three conditions tested had significantly less glycerol compared to standard conditions. In the case of the two 30 g/L initial glucose concentration conditions, the same or higher erythritol concentrations were measured. In addition, mannitol production was increased in these two fermentations compared to standard conditions. As mentioned earlier, significantly less erythritol was produced in the 50 g/L fed-batch, despite extended fermentation time. Mannitol could not be detected at all in this fermentation.



	30 g/L Batch	30 g/L Fed-Batch	50 g/L Fed-Batch (96h)	Standard conditions
Mannitol	466	887	0	177
Glycerol	473	685	389	1444
Erythritol	225	180	109	183

Figure 33: Polyols produced at the end of different operation modes compared to standard conditions

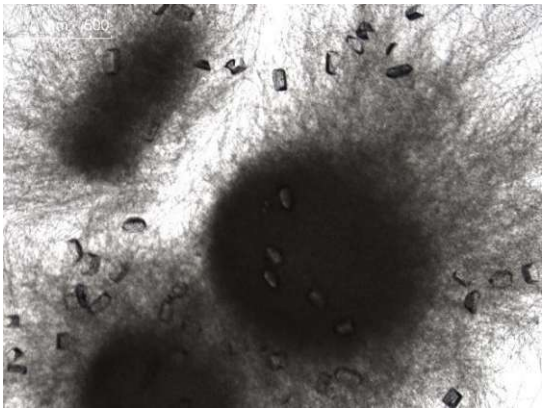
Table 20 compares biomass and yields of the different operation modes. The yields were calculated using Equation 7 to Equation 9. It can be seen that the biomass formation is reduced in the fed-batch fermentations in contrast to the two batch conditions. However, the Yields of biomass per gram of glucose are slightly higher in the fed-batch fermentations than in standard conditions. In the 30 g/L batch, much glucose has been consumed, as seen in Figure 31. Accordingly, the Yields based on glucose consumption are significantly lower than for the other conditions. However, the yields of erythritol per biomass are increased here and in the 30 g/L fed-batch compared to standard conditions. The 30 g/L fed-batch also showed the highest yields of erythritol per gram of glucose. The 50 g/L fed-batch produced less erythritol than the other fermentations, which is why the erythritol yields are lower.

Table 20: Biomass and Yields at different operation modes compared to standard conditions

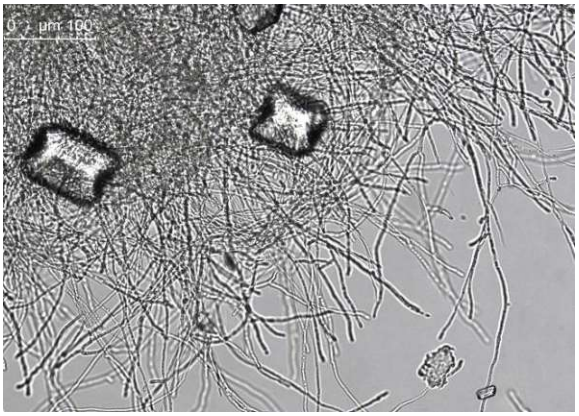
	30 g/L Batch	**30 g/L Fed-Batch**	**50 g/L Fed-Batch**	**Standard conditions**
Biomass (g)	6,508	5,397	5,541	6,838
E/Glu (mg/g)	6,699	11,024	6,714	8,046
E/X (mg/g)	17,286	16,676	9,836	13,381
X/Glu (g/g)	0,388	0,661	0,683	0,601

3.7 Morphology

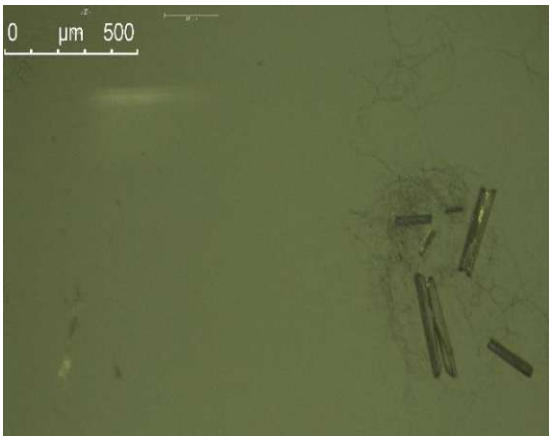
Figure 34 shows the microscope images of some of the experiments described above. Crystals can be seen in some of the images. What exactly these are could not be conclusively clarified. Since the pH value changes during fermentation and the crystals usually only appear in later samples, this could be a medium component that precipitates at higher pH values. However, this is only a conjecture and requires further investigation.



High stirring, high aeration, 50x magnification: dense pellets

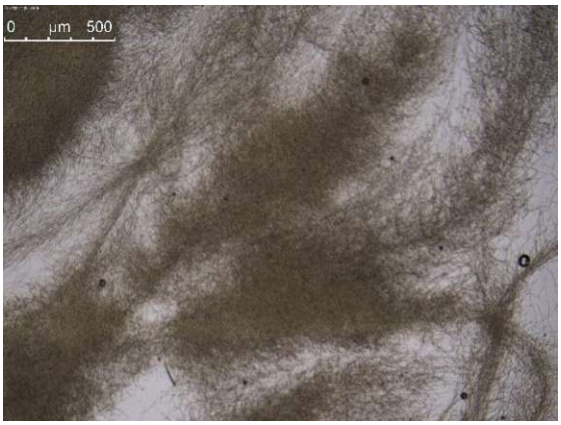


High stirring, high aeration, 300x magnification: dense pellets, intact hyphae



Moderate stirring, high aeration, 50x magnification: pellets

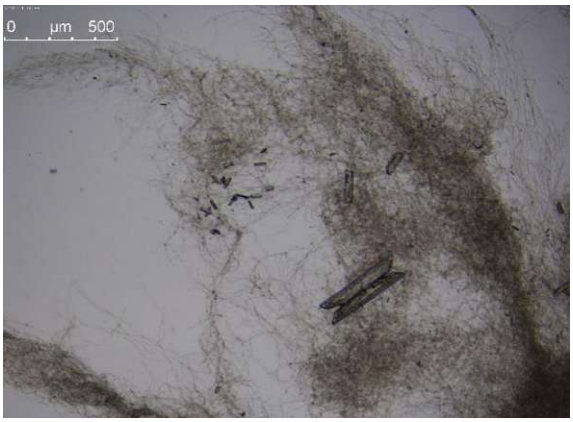
Moderate Stirring, high aeration, 300x magnification: pellets, intact hyphae



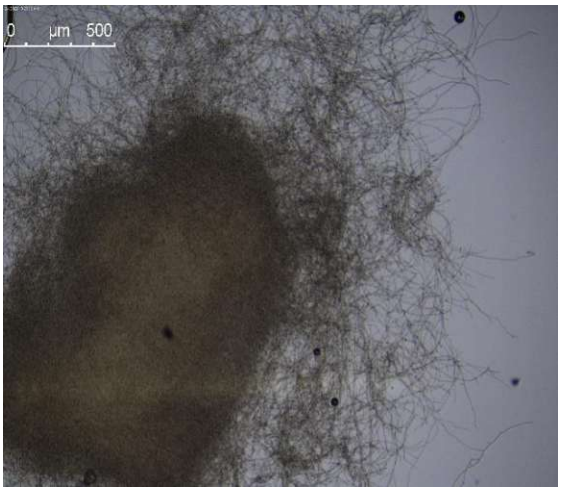
Elongated fermentation time, 50x magnification, dispersed

Elongated fermentation time, 300x magnification, dispersed, intact hyphae

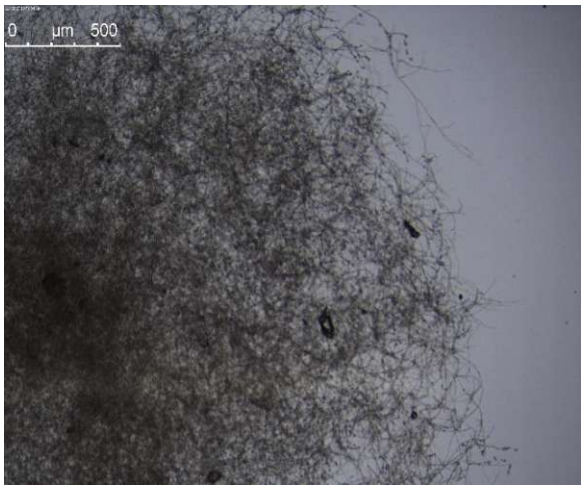
Reduced volume, 50x magnification, dispersed



30 g/L discontinuous fed-batch, 50x magnification, dispersed



Tween80 and antifoam, 50x magnification, pellets



Antifoam, 50x magnification, dispersed

Figure 34: Fermentation samples investigated under the microscope for their morphology, 48h fermentation time

4 Discussion

4.1 Reproducibility

Due to limited resources, the experiments in reactors were never performed in replicates. However, as described in section 3.1.2, the 0,5 L volume reactor was performed twice, giving at least a hint of reproducibility.

Erythritol production and glucose consumption are comparable. Also, the biomass formation and the resulting yields are similar. Only glycerol production differs significantly from each other. This can be due to many things. The two reactors were inoculated with different precultures from different spore suspensions. This could have had an influence. External influences that were not measured, such as air pressure, may also play a role. Replicates should be measured to determine reproducibility better. Moreover, at least three experiments should be performed under the same conditions to obtain a statistic validation.

4.2 Medium Influence

Domingues et al. also demonstrated in 2000 that Tween80 affects the morphology and thus significantly affects fungi's growth and metabolite production [60]. Due to its properties, it was added to shake flasks to prevent the spores from sticking to each other. In shake flasks, it was shown that the addition of Tween80 produced less biomass but more erythritol. However, the opposite effect was seen in bioreactors, although less pronounced with erythritol. Of course, it must be taken into account that the standard conditions were part of a different series of experiments, and therefore different external conditions were present. Nevertheless, it has already been shown in section 4.1 that the results of two test series under the same fermentation conditions are also comparable. The experiments showed that *T. reesei* generally behaves significantly differently in growth and polyol production in bioreactors than in shake flasks. Therefore, it is possible that Tween80 also has a different effect. In order to better assess the influence of Tween80, a series of experiments in bioreactors of two conditions, one with Tween80 and one without, each in triplicates, would be a good option. Unfortunately, this was not possible within the scope of this thesis due to a lack of resources.

The addition of antifoam was tested because the main problem in all previous fermentations was uncontrolled surface growth. With the help of a volume reduction, the fermentation time could at least be extended by 24 h despite this problem. Another attempt to solve the problem without antifoam was top-cooling (data not shown). A tube was wrapped around the top third of the reactor, through which cool water constantly flowed, thus cooling the glass surface. This was intended to reduce the formation of foam. Since this experiment was also unsuccessful, antifoam was added and its effect was tested. These tests showed a more homogeneous picture than with Tween80. The amount of polyol produced was reduced in shake flasks and bioreactors. Of course, it is not optimal to reduce the product formation, but the by-product amounts decreased more than the erythritol formation. Lower by-product formation can be beneficial depending on the possibilities and efficiency of the downstream process. However,

the biomass formation behaved differently. It was reduced in shake flasks but increased in the reactor. Without the addition of antifoam in bioreactors, the fungus often grew on the surface of the liquid, the foam, the agitator, and the probes. The addition of antifoam prevented this effect and the fungus grew in the nutrient-rich medium. Accordingly, it is not surprising that biomass production was increased in this case. Whether more biomass benefits the process is questionable, as the main problem during all fermentations in bioreactors was overgrowth.

In contrast to antifoam and Tween80, peptone does not affect the process significantly. Therefore, and due to its foam stabilizing properties, peptone was deleted from the fermentation medium in reactors.

4.3 pH and temperature

Stark has already evaluated in his thesis that adjusting the pH to below 6 during fermentation positively influences erythritol production [40]. It is already proven that initial pH and temperature increase the biomass growth rate in batch cultures [33]. A DoE was performed as a screening process to determine if initial pH and temperature significantly influence erythritol production. Temperature affects glucose consumption. A possible reason for this could be influenced metabolic activity [61]. Also, the initial pH influences erythritol production, but mainly in combination with temperature.

The interaction plots show a correlation between the two parameters in yields and erythritol production. This effect was already demonstrated in other fungi by Hallsworth and Magan in 1996 [62]. The correlation resulted in a response contour plot where optima can be seen in two corners. This and the yields showed that the current fermentation conditions are possibly not optimal and that productivity could be increased by adjusting this parameter combination. Ideally, two DoEs should be carried out, where the two optima are the new center points. This was not done due to time constraints, but an attempt was made to confirm the optima. However, only half as much erythritol could be produced during confirmation. Whether this is due to external circumstances cannot be conclusively clarified. To do this, the experiment would have to be repeated, but this was not done.

Another condition that was tested was a temperature shift after 72 h. This resulted in a significantly increased amount of erythritol with fewer by-products. In general, however, the fermentation time was relatively short at 96 h, compared to the 168 h fermentation time in Stark's experiments [40]. Therefore, the next step could be to perform the two already mentioned DoE around the determined optima with a fermentation time of 168 h to create better comparability with Stark's results. This would also allow the influence of the temperature shift to be better investigated. Moreover, standard conditions should be measured simultaneously during all experiments to allow better comparison.

pH and temperature are otherwise important process parameters in addition to their influence on metabolism. From a process-technical point of view, a lower pH is advantageous, as it can better prevent contamination by other microorganisms. The fermentation temperature and the resulting necessary heating and cooling of the reactor also determine the energy consumption and are thus directly included in the process costs. Moreover, they also influence, among other factors, morphology and therefore rheology. Rheology influences the power input for sufficient mixing and, therefore, the process costs [33].

4.4 Stirring and Aeration

Stirring and aeration serve, among others, to supply the microorganism with sufficient oxygen. For this purpose, shear is added to the air bubbles with the aid of the impeller, dispersing them into smaller bubbles. This increases the area per volume ratio of the bubbles and thus improves the k_La [44], which is a particularly important factor for *T. reesei*, as it is a strictly aerobic microorganism [63]. Compared to standard conditions, increasing stirring and aeration parameters, in this case, resulted in increased polyol production. However, this happened only up to a certain level; at too high stirring conditions, the polyol production decreased again. Vigorous stirring leads to high shear rates, which can harm mycelium formation and lead, for example, to hyphal fragmentations [32].

High stirring and aeration also led to increased biomass production. This has already been demonstrated in *Penicillium chrysogenum* [33]. Patel et al. saw this effect also in *T. reesei* [32]. However, it should be noted that their tested stirring speed interval ranged only from 200 to 400 rpm. This is clearly below the maximum of 700 rpm tested in this thesis. The desirability of more biomass production remains questionable since the main fermentation problem was overgrowth.

4.5 Fermentation time

The influence of fermentation time on erythritol production has already been proven [40]. Similar observations were shown in this work, but it was impossible to test fermentation times of more than 96 h. This was mainly because the fungus grew on the walls, in the foam and on the probes and clogged various inlets and outlets. In some cases, this resulted in increased pressure in the reactor, which led to a shutdown. When antifoam was added, fermentation could not be continued, mainly because the fungus had absorbed all the liquid in the reactor. Therefore, it was only possible to test a slightly longer fermentation time once. This resulted in increased glucose consumption and erythritol production. The erythritol amount increased by 71 % during the additional 24 h fermentation time. Due to the initial lag phase in the first approximately 24 h, this effect could become even more significant with longer fermentation times.

As already mentioned, shake flasks showed that a fermentation time of 168 h positively influenced the erythritol yields. Accordingly, further work should be done to solve the problems

mentioned above in order to ensure longer fermentation periods. With the help of antifoam, as mentioned, the uncontrolled surface growth could be controlled to a large extent. However, the addition created the new problem of liquid deficiency. One possibility would be a fed-batch, where concentrated glucose is not added once, as in this thesis, but where fermentation medium is added more frequently or continuously.

4.6 Operation mode

The data collected, see section 3.6.2, indicate that a lower initial glucose concentration positively affects erythritol production. The batch at 30 g/L resulted in an increased amount of erythritol under otherwise the same fermentation conditions, with even fewer byproducts. The 70 g/L initial glucose concentration from the standard conditions comes from Stark's previous experiments in shake flasks. At a fermentation time of 168 h, all glucose was degraded at the end so that erythritol could be used as a carbon source [40]. Therefore, an increased glucose concentration was a logical conclusion. However, his DoE also showed that the optimal glucose concentration is below 50 g/L, which is consistent with the data in this work.

Regarding erythritol yields, a fed-batch with 30 g/L initial glucose concentration showed the best results. The yields of erythritol per gram of glucose could be increased by 37 %. This effect has already been described for erythritol production in the yeast *Torula sp.* by Oh et. al.[64]. Furthermore, as already described in section 4.5, a fed-batch could help to overcome liquid deficiency problems. Moreover, as mentioned above, a lower initial glucose concentration could favor erythritol production, but longer fermentation times could also lead to the complete depletion of glucose in bioreactors. Fed-batch fermentation, whether operated continuously or discontinuously, could provide a remedy for this problem as well.

4.7 Morphology

The micrographs from Figure 34 show that different conditions resulted in different morphologies. A pellet growth form was more likely to be observed at a higher shear rate, i.e., at higher stirrer speed and higher aeration. This is not surprising and is already described in the literature [33]. The combination of Tween80 and Antifoam also resulted in pellet formation. However, to what extent the morphology influences erythritol production is not known. This would require many more experiments, which would be beyond the scope of this thesis.

As mentioned in the introduction, morphology also influences viscosity. Viscosity was determined once (data not shown) but showed no Non-Newtonian behavior or change in time, so it was not determined further. However, it could be important in the process design at higher resulting polyol concentrations or with longer mycelium growth.

5 Conclusion

This work aimed to transfer a process developed in shake flasks to a bench-scale bioreactor, trying to maintain the yields already achieved. Unfortunately, the erythritol yields from Stark's thesis could not be achieved. The maximum yields of erythritol per gram of glucose achieved in this work were 11,02 mg/g, while in shake flasks they were 15,27 mg/g. Yields of erythritol per gram biomass were even further away. These were 17,29 mg/g in bioreactors, compared to 80,06 mg/g in shake flasks.[40] The latter is mainly related to the good growth in bioreactors.

However, this good growth also brought most of the problems. In particular, foam formation and the resulting problems with overgrowth on the surface, the probes, and the foam itself prevented a longer fermentation time. This work and Stark's showed the positive influence of a more extended cultivation period. Foaming is a problem that only became relevant when the process was transferred to bioreactors. In shake flask experiments, foam plays a minor role. However, in shake flasks, erythritol production is not so different from that in bioreactors. In the shake flask experiments in this thesis and Stark's previous experiments, the erythritol produced after 96 h was around 300 mg/L. The amount of erythritol produced in the 96 h bioreactor was also in this range. Since the fermentation period in reactors was limited mainly by foaming and excessive growth, it is important to solve this problem soon. Attempts were made to prevent foaming by omitting foaming- or foam-stabilizing medium components such as Tween80 and Peptone. In the case of Tween80, it could not yet be determined whether omitting it harms or benefits the process more. Another way to overcome foaming is antifoam which has been tested in this thesis. Antifoam did help with preventing surface growth, but it created even more biomass, which in turn became a problem. Patel et al. investigated the effect of stirrer speed on foam formation in *T. reesei* fermentations. They concluded that at 200 rpm, the addition of antifoam was not necessary to make the foam controllable. This could also be a method to avoid antifoam [32].

Another way to control growth is a continuous fed-batch. In this case, a growth rate can be adjusted via the feed rate. The discontinuous 30 g/L fed-batch tested in this thesis showed a reduction in biomass and an increase in erythritol yields. When controlling growth, it could be possible to add antifoam without creating problems with massive biomass formation. In addition, a fed-batch has other advantages. Stark's DoE indicated that a glucose concentration below 50 g/L can benefit erythritol production. This assumption could be confirmed by the 30 g/L batch fermentation in this work. A fed-batch could lower the initial glucose concentration without causing problems with complete glucose depletion and the resulting consumption of erythritol by *T. reesei.* A lower glucose concentration is also advantageous if the medium is produced from industrial waste. Thus, a fed-batch could help to control the problem with overgrowth better, as well as to reduce the initial glucose concentration.

Despite not achieving similar yields as in shake flasks, some essential insights into erythritol production in bioreactors could be gained. For example, it was shown that stirring and aeration significantly affect erythritol production, growth and morphology. High stirring and, thus, high shear rates resulted in less erythritol and pellet formation. However, increased aeration and slightly increased stirring speeds resulted in more erythritol formation. As mentioned earlier, the two parameters exert a significant influence on morphology. As the process development progresses, morphology or changes in morphology during fermentation could have a more significant impact on the process than has been assigned to it in this work. Rheology changes have not yet been detected, but with longer fermentation time and higher polyol concentration, this may change and should be further investigated. A correlation between morphology and erythritol production could not be established so far but was not the focus when planning and performing the experiments and would extend the scope of this thesis. However, a change in morphology with media composition and shear rate could be observed.

In addition, the influence of initial pH and temperature on erythritol production could be demonstrated. Although it has not yet been conclusively determined what the optimum of these parameters is for erythritol formation, an influence on it and a correlation between the two parameters could be proven. Also, the possibility of a temperature shift gave positive results and could be pursued further. However, further DoEs are needed to screen for possible optima and fine-tune the parameters.

Finally, it should be noted that a major problem was limited resources. Since only one or two bioreactors were available simultaneously, replicates could not be measured. Of course, this makes it difficult to judge whether some effects are coincidences or results of parameter changes. The measurement of replicates would be desirable in order to be able to guarantee statistical validation. A fermentation with standard conditions should always be performed as a reference. If resources are available, this approach would be helpful to make the results more profound. Nevertheless, in this master's thesis, important insights into the influence of various parameters on erythritol production were obtained, which can be used for further research.

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7 List of Figures

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9 Abbreviations

Polyol	Polyalcohol
PPP	Pentose phosphate pathway
dH_2O	Deionized water
F-medium	Fermentation medium
Tracer	Trace elements solution
OD	Optical density
DoE	Design of Experiments
HCl	Hydrochloric acid
NaOH	Sodium hydroxide
HPLC	High Performance Liquid Chromatography
c	Concentration (g/L)
E/X	Yields of erythritol per unit biomass
E/Glu	Yields of erythritol per unit glucose
X/Glu	Yields of biomass per unit glucose