Utilization of Organic Fractions in Municipal Solid Waste via *Trichoderma Reesei*

Tyler Rickabaugh
Matriculation Number: 70455874

Ostfalia University of Applied Sciences
Tampere University of Applied Sciences

Examining professors:
Prof. Dr. Ing. Thorsten Ahrens
Prof. Dr. Elke Wilharm

Declaration of Authorship

I, Tyler Rickabaugh, hereby certify that this thesis has been composed by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a Bachelor's degree. This project was conducted at the Ostfalia University of Applied sciences from 06/2017 to 08/2017 towards fulfillment of requirements of Ostfalia University of Applied Sciences and Tampere University of Applied Sciences for the degrees of B.Eng. in Environmental Engineering & Bio and Environmental Engineering under the supervision of Prof. Thorsten Ahrens.

Date: _____Signature of candidate: _____

Abstract

The aim behind this thesis work is to test the feasibility of *Trichoderma Reesei* having the capacity to produce biomass in a substrate derived from organic fractions found in waste from washed municipal solid waste. Previous projects at Ostfalia Hochschule have utilized this wash water from municipal solid waste using *Bacillus amyloliquefaciens*, and have shown success in the fermentation process of this microorganism. The hypothesis explored in this thesis is can *T. Reesei* produce biomass in the wash water, and if so can this application be upscaled to a 75l bioreactor.

The results show that *T. Reesei* can successfully grow in pure wash water, while enzyme production is low without an added carbon source such as dead leaves collected from the forest bed. Parameters used in the cultivation process on a laboratory scale showed they can be transferable in upscaling methods. *T. Reesei* was able to produce biomass in both the 1.5l and 75l bioreactors. Despite contamination issues, *T. Reesei* proved to be a resilient fungus, producing mycelium in unideal conditions.

CONTENTS

Abbreviations

l	-	Liter
g/l	-	Gram per Liter
mg/l	-	Milligram per Liter
ml/l	-	Milliliter per Liter
rpm	-	Revolutions per minute
ml	-	Mililiter
µl	-	Microliter
h	-	Hour
min	-	Minute
°C	-	Degrees Celsius
MSW	-	Municipal Solid Waste
DNSA	-	Dinitrosalicylsäure
PDA	-	Potato-Dextrose-Agar
DOC	-	Dissolved Organic Content

1 INTRODUCTION

This thesis, worked on by Jan Manicke and myself, examines the plausability of cultivating the fungi *Trichoderma Reesei* in an organic fraction effluent derived from municipal solid waste (MSW). Along with this, the thesis explores the capacity for upscaling the cultivation process, starting in Erlenmeyer flasks, then moving to 1.5l bioreactors, and finally to a 75l bioreactor. The focus of the thesis is to only produce biomass, while enzyme production of the fungi is only lightly touched on. The following information within this thesis illustrates the methods used and results found cultivating *T. Reesei* in MSW effluent.

2 Growing T. Reesei in Nutrient Broth

2.1 Background

The fungi *T. Reesei* was discovered over 70 years ago on the Solomon Islands during World War II (Bischof, R, et. al., 2016). It was here where the Natik Army Research Laboratories were able to isolate the extracellular cellulases producer that was causing havovic on military equipment. With the ability to produce cellulase in order to hydrolyse cellulosic material into easily fermentable monosaccharides, allowed *T. Reesei* to become one of the most greatly used microorganisms in the enzyme industry (Paloheimo, M, et. al., 2016).

T. Reesei is a soil borne Ascomycete fungi that produces long thin cellular filliaments called hyphae (Singh, A, et. al., 2014). At the tip of the hypal is where asexual spores and enzymes are secreted from (Nevalainen, H; Peterson, R; 2014). Mycelium, which is comprised of bundles of hyphae fillaments, acts as a network for the fungi and is also where reporduction occurs (Celine, 2017). This mycelium can be seen with the naked eye, and will be used as an indicator for growth throughout this thesis (Celine, 2017).

Before attempting to cultivate *T. Reesei* in MSW wash water, a complete understanding of how *T. Reesei* grows in optimal conditions, as well as the proper surrounding environment needed to grow, was necessary. In order to achieve this, the first task was to grow *T. Reesei* in optimal substrate (ie. nutrient broth) and environmental conditions, for these fundamental steps would be used throughout the entire thesis work.

To produce biomass from *T. Reesei*, parameters such as pH, carbon to nitrogen ratio, temperature, agitation, and aeration need to be taken into consideration (Singh, A, et. al., 2014). The pH of the substrate used has the ability to influence growth of the mycelium, enzyme production, and sporulation (Li, C, et. al., 2013) (Singh, A, et. al., 2014). A pH range of 4-6 is ideal for optimal growth of *T. Reesei* (Li, C, et. al., 2013). Temperature ranges of 25°C and 35°C, and aeration and agitation speeds of 150rpm also create optimum growing conditions for the fungi (Singh, A, et. al., 2014).

The script used to create the nutrient broth was provided by Prof. Wilharm, while the method procedures that were performed in the laboratory to create the nutrient broth was overlooked by Caren Dobberphul. The script used for this process can be found in Appendix 1.

The cultivation of *T. Reesei* was to be performed in Erlenmeyer flasks as well as on agar plates. Cultivation on agar plates is used to generate spores on an potato-dextrose-agar medium, whereas cultivation in Erlenmeyer flasks is used to produce larger amounts of biomass.

2.2 Methods

2.2.1 Creation of Potato-Dextrose-Agar Plates

The Potato-Dextrose-Agar (PDA) is used to create *T. Reesei* spores on agar plates. The recipe used for creating the PDA as well as the ingredients needed was provided by Prof. Wilharm and can also be found in Appendix 1.

To create the potato infusion, 200g of sliced potatoes are boiled for one hour in one liter of water. Once the time is up, the potatoes are pressed through a fine sieve into a 1000ml glass jar. The appropriate amount of glucose and agar were added to the potato infusion, and placed onto a mixing plate to create a homogeneous mixture. Afterwards the PDA was placed into the Autoclave at 121°C and 200kPa for 15 minutes for sterilization.

When the Autoclave is complete, the PDA needs to be mixed again due to the agar settling on the bottom, and then it can be poured into agar plates. The PDA aqag was then poured into plastic petri dishes and let to solidify. As soon as the agar has completely solidified it can be inoculated with the *T. Reesei* culture.

2.2.2 Inoculation of PDA Plates

To inoculate the agar plates, 100µl of liquid culture was poured onto the middle of the plate, and then spread throughout the plate with a sterilized glass spreader, while spinning the plate to ensure a nice even spread. This entire process was done while working under a Bunsen burner to prevent unwanted contamination. After inoculation, the agar plates are placed in the incubator at 30°C for 10 days, and then visually analyzed for results.

For harvesting the newly created spores after 10 days of inoculation, 5ml of 0.14 M of NaCl solution was pipetted onto the PDA plates to suspend the spores. Afterwards, the plate was tilted to the side, and 1.5ml of the liquid pipetted into an Eppendorf cap with 15% vol/vol of glycerol before storing in the freezer at -18°C to be used later.

2.2.3 Creation of Nutrient Broth

As stated in the background, the recipe used to create the nutrient broth was found on the script that was provided by Prof. Wilharm, which can be located in Appendix 1, along with the ingredients needed to make the liquid medium and trace element solution.

In the liquid medium, NH_4Cl and Urea are added to provide a nitrogen source for *T. Reesei* (Sternberg, D; Dorval, S; 1979). KH_2PO_4, found also in the liquid medium, acts as a buffer within the broth. This is needed due to *T. Reesei* creating acidic conditions when consuming ammonia during it's growth phase (Sternberg, D; Dorval, S; 1979) .

When creating the liquid medium, all substances were added into a 500ml glass jar except for the cellulose. The cellulose was added separately into Erlenmeyer flasks to ensure that each flask had the appropriate amount of cellulose. In a separate 500ml glass jar, the trace element substrate was prepared. These trace elements are needed for cellulase production (Ferreira, S, et. al., 2009). Following the procedure via the script given, a 100 fold concentration of trace elements was added into the 500ml glass jar of liquid medium. The jar was then placed on a mixing plate, and stirred continuously to ensure all ingredients had been dissolved.

After mixing was completed, the pH was measured via a calibrated pH meter. The pH of the nutrient broth should be right at 4.5, however if the pH is higher, HCl can be used to lower the pH to the appropriate level. A pH lower than 4.5 will result in growth inhibition of the fungi, reducing the amount of biomass produced (Sternberg, D; Dorval, S; 1979).

2.2.4 Cultivation in Erlenmeyer Flasks

For the first cultivation batch, three 250ml Erlenmeyer flasks were used, capped off with cardboard tops. In each flask 50ml of nutrient broth was added, along with 0.625g of cellulose. All three flasks were then placed into the autoclave at 121°C and 200kPa for a duration of 15 minutes for sterilization.

Once the Autoclave was complete, the flasks were placed in a cooling bath to reduce the temperature of the nutrient broth to a suitable temperature for inoculation. When the flasks finished cooling, a volumetric pipette was used to inoculate each flask with exactly 50µl of *T. Reesei* inoculant provided by Caren Dobberphul. Throughout the entire inoculation process, work was done under a Bunsen burner to ensure unwanted contaminates entering into the flasks. Also every time the Erlenmeyer flask was opened and closed, the lid was always torched with the flame to prevent contamination.

After successfully inoculating the flasks, they were placed on a shaking plate set to 100rpm at 30°C, and checked every 24 hours to ensure there was growth in the flasks. The results of the cultivation were verified by looking at samples of the nutrient broth under a microscope at 400x magnification to identify the hyphal growth of the fungi.

2.3 Results

2.3.1 Cultivation in Erlenmeyer Flasks

The nutrient broth created in the laboratory provided the perfect environment for the *T. Reesei* to grow. After 2 days on the shaking plates, the *T. Reesei* started to produce clusters of mycelium that could be seen with the naked eye in the flasks. Samples from this biomass were taken and analyzed under the microscope at 400x magnification to confirm the growth that was seen in the flask was in fact *T. Reesei*. The results can be seen in Figure 1 and Figure 2.

The thin long filaments seen in Figure 1 are the *T. Reesei* hyphae. It is from the tips of these hyphae where the cellulase enzyme is secreted into the solution (Nevalainen, H; Peterson, R; 2014). In Figure 2, the white clusters at the bottom of the flask show the mycelium bunching together, representing a positive growth in the medium.

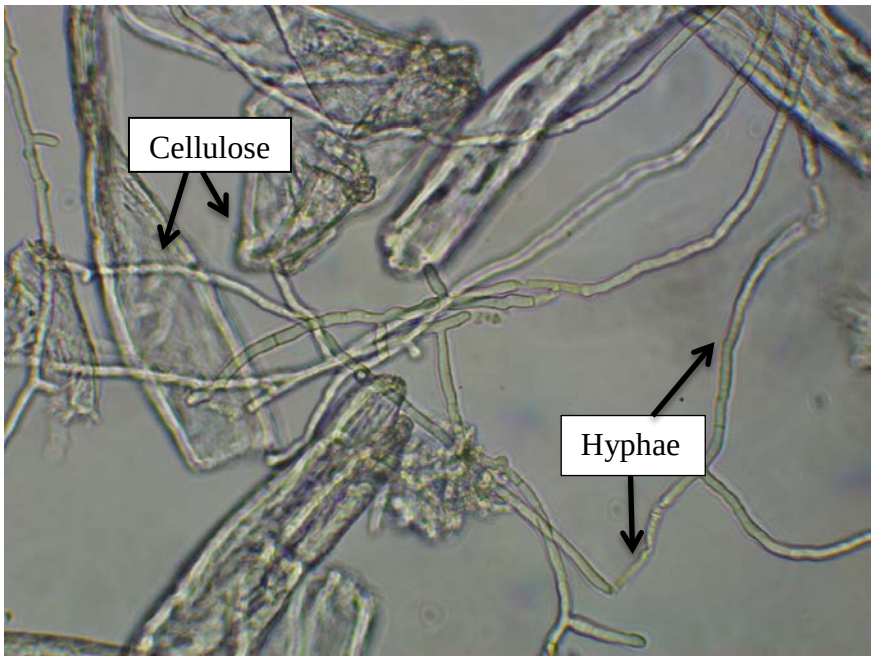


Figure 1. T. Reesei under the microscope at 400x resolution. (Rickabaugh,T.; Manicke, J, 2017)

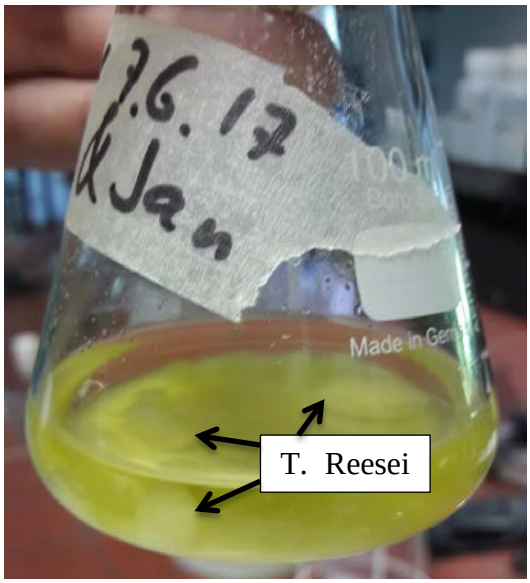


Figure 2. Visible growth of T. Reesei mycelium in Erlenmeyer Flasks. (Rickabaugh,T.; Manicke, J, 2017)

2.3.2 Inoculation of PDA Plates

Looking at the agar plates after 10 days of incubation time in the incubator, only one out of the three agar plates appeared to have visible growth. The result from this agar plate can be seen in Figure 3. The yellow coloring in the PDA plate is the hyphae from *T. Reesei* starting to spread throughout the PDA. These new spores can be extracted from the plate using a NaCl solution and can be stored in the freezer at -18°C for future inoculations.

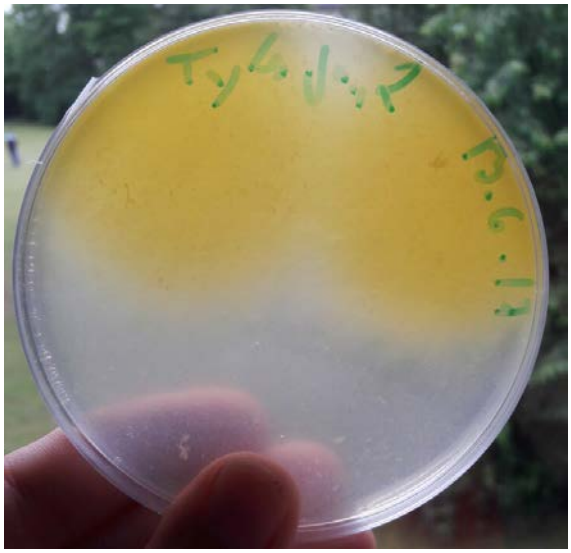


Figure 3. PDA Plate Inoculated with T. Reesei.

2.4 Discussion and Conclusion

The aim of this task was to become familiarized and comfortable working with *T. Reesei* as well as understanding the methods of cultivating *T. Reesei* in nutrient broth, which was

successful. These methods will be used throughout the entire thesis, for this is how the stock culture will be created and stored during the entire thesis, and it was imperative that the methodology for growing *T. Reesei* in nutrient broth was fully understood.

It was found that the cultivation of *T. Reesei* using the Erlenmeyer flasks was a simpler method in producing biomass compared to cultivation with the PDA plates. The risk of contamination is lower using the flasks, as well as producing more biomass that can be used as inoculant in the future. It was decided that the cultivation of the stock culture would be performed via Erlenmeyer flasks.

Growing *T. Reesei* on PDA plates is an good method for creating pure cultures, seeing as it is easy to isolate and harvest spores. However, when moving to larger volumes of substrate, larger volumes of inoculant are needed than the 1.5ml of inoculant produced from the PDA plates. When working with larger substrate volumes, it is more desirable to use a larger inoculant volume in order to speed up the fermentation process. By using the Erlenmeyer flasks, it is easy to produce liters of hyphae and spores that can be transfered into a new substrate and let to grow.

Assessing the success rate of *T. Reesei* biomass is performed via microscopy and by looking at the flasks with the naked eye to identify the mycelium. This is a simple method used to draw conclusions, however it does not give specified biomass volume production values. To depict performance values in future testing, new methods should be considered such as weighing of dry mass, enzyme testing, etc. This method is acceptable for this thesis work, for the main aim of the thesis is to produce biomass in MSW wash water, not to increase the efficiency of biomass production in wash water.

3 Wash Water Production

3.1 Background

In 1993 the European Union passed the Landfill Directive requiring EU states to reduce the amount of biodegradable municipal waste that was going to landfills (Fischer, C., 2013). Due to this piece of legislation, Germany is looking for new ways to reduce the carbon content entering into its landfills.

To reduce the amount of carbon entering into the landfill, waste management companies are looking into washing the MSW before placing it into the landfill. This process has the ability to reduce the leachate entering into the surrounding environment, as well as reduce the amount of emissions being released into the atmosphere from the decomposing waste (Cossu, R, Lai, T, 2011). According to Cossu and Lai's study (2011), they have found that wash water derived from similar waste samples used in this thesis to have upwards of 7700mg/l of dissolved organic carbon (DOC) after 3 hours of washing which could be exploited for the cultivation of *T. Reesei*.

Previous experiments have successfully been conducted at Ostfalia utilizing the organic fractions found in wash water to cultivate the microorganism *Bacillus amyloliquefaciens* to produce 2,3-butanediol (Prayoga, A, Walpole, R, 2017). The methodology used by Prayoga and Walpole differs greatly from what was used by in Cossu and Lai's study, however the main principle of capturing the surface organic fraction on MSW from waste washing remains the same.

In order to produce the wash water that will be used in the cultivation of *T. Reesei*, the methodology used by Prayoga and Walpole will be utilized. Their methods allow for the production of larger quantities of wash water that will be needed for the upscaling section of the thesis.

3.2 Methods

The methods followed for creating the wash water were provided from previous projects at Ostfalia (Prayoga, A, Walpole, R, 2017). The total amount of wash water obtainable on this laboratory scale is 25l derived from 1kg of MSW. The MSW is collected from En-

tsorgungszentrum Salzgitter GmbH, with 10kg of waste being collected per visit and stored on 25l buckets.

To create the washing station, a 50l tub is used as the base of the station. Resting on top of the tub, sits a 25l bucket with 2cm holes drilled in the entire bottom of it to allow the water to flow back into the tub. The bucket is drilled onto two pieces of wood that support the bucket onto the 50l tub. Inside the 50l tub a water pump was placed that had a hose attached to it, which was used to pump the water from the tub into the bucket. The flow rate used with the water pump is 8000l/h. Figure 4 shows how the design was set up in the lab.

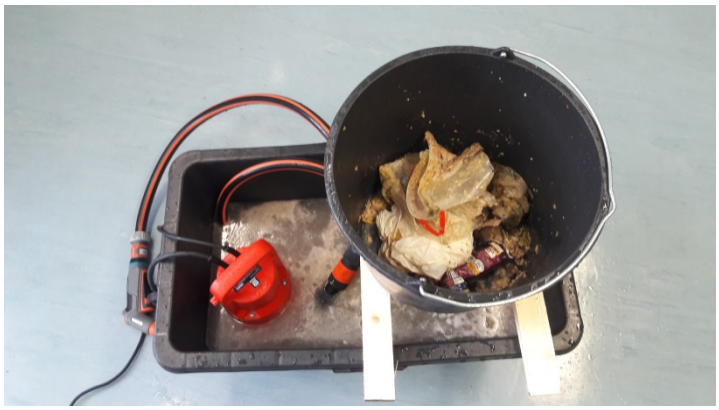


Figure 4. Waste washing station in laboratory.

The tub is filled up with 25l of tap water, and 1kg of MSW is weighed out and placed into the bucket. The water pump is turned on, forcing water out of the hose into the bucket washing away any surface organic content. The MSW undergoes this washing process for 15 minutes, during which the waste is simultaneously stirred with a pitchfork. After 15 minutes, the water in the tub is collected; the pH is tested with a calibrated pH meter, and then stored at room temperature until needed.

3.3 Results

The wash water was produced successfully via the laboratory scale washing methods. Approximately 25l of wash water was produced with a pH of 7.40, which is well within a suitable range for *T. Reesei* to grow in.

3.4 Discussion and Conclusion

By following the washing methods provided by Prayoga and Walpole allowed for a successful production of wash water. However, because MSW was being used to produce the wash

water, the contents of the waste will vary. This can alter the available organic content in the wash water, as well as the pH, creating an inconsistent substrate. This inconsistency can have an effect on the performance of the *T. Reesei* and other microorganisms.

The production of wash water is a simple method on a laboratory scale, which could be a useful procedure for waste management sites to use as a pre-treatment of MSW entering the landfill. Directive 1999/31/EC is a directive passed by the EU that requires waste management companies to treat the waste that comes in before it goes to the landfill (European Commission, 2016). Treatment requirements state the waste must go through physical, thermal, chemical or biological process before it can proceed to the landfill (European Commission, 2016).

Washing of MSW would classify as a pre-treatment process under this directive, by reducing the amount of available organic material leaching into the surrounding environment. Apart from reducing the impact of the surrounding environment, MSW washing creates a cheap liquid substrate that could be used in the cultivation and fermentation process of microorganisms.

When it comes to MSW washing treatment, waste management facilities have mostly used washing treatments on ash remnants from the incineration plants. This ash contains high concentrations of heavy metals, salts, chloride and organic pollutants, which can have hazardous effects on the environment if left untreated (Lam, C, et. al., 2010). By washing the ash with a combination of water and acids, it helps reduce the amount of heavy metals and salts within the ash (Lam, C, et. al., 2010). However, if waste management facilities would like to go one step further in reducing contaminates found in the ash, incorporating a pre-treatment of washing the solid waste before incineration is feasible.

4 Nutrient Broth to Wash Water Ratio

4.1 Background

In Section 2.3 of this thesis, it has been shown that *T. Reesei* is capable of growing in ideal conditions like the nutrient broth. However, it is unknown how the fungi will perform using the wash water as its carbon source. The aim behind this experiment is to see if it is possible to grow *T. Reesei* in the wash water.

Varying ratios of nutrient broth and wash water was used in this process to try to identify how well *T. Reesei* will grow. By using different ratios of nutrient broth to wash water, it can then be said that the fungi will be able to grow in pure wash water, or wash water with a certain percentage of nutrient broth added.

The main purpose behind this cultivation is to successfully grow *T. Reesei* biomass in the flasks with varying wash water to nutrient broth ratios. Because biomass is the only concern and not the type of products that *T. Reesei* creates, only a visual analysis of the final fermentation was used to determine the success rate of the experiment.

4.2 Methods

For this part of the experiment, a total of six different ratios of wash water to nutrient broth were used. With each different ratio of substrates, a total of three flasks were prepared. Table 1 helps show the ratios of wash water to nutrient broth that were used in each flask, along with the corresponding amounts of cellulose in each flask.

Table 1. Breakdown of Wash Water to Nutrient Broth Ratios.

	Ratio			
No.	Washwater	NB	m(Cellulose)	β(Cellulose)
1.1	100%	0%	0 g	0 g/l
1.2	100%	0%	0 g	0 g/l
1.3	100%	0%	0 g	0 g/l
2.1	80%	20%	0.125 g	2.5 g/l
2.2	80%	20%	0.125 g	2.5 g/l
2.3	80%	20%	0.125 g	2.5 g/l
3.1	60%	40%	0.25 g	5 g/l
3.2	60%	40%	0.25 g	5 g/l
3.3	60%	40%	0.25 g	5 g/l
4.1	40%	60%	0.375 g	7.5 g/l
4.2	40%	60%	0.375 g	7.5 g/l

4.3	40%	60%	0.375 g	7.5 g/l
5.1	20%	80%	0.5 g	10 g/l
5.2	20%	80%	0.5 g	10 g/l
5.3	20%	80%	0.5 g	10 g/l
6.1	0%	100%	0.625 g	12.5 g/l
6.2	0%	100%	0.625 g	12.5 g/l
6.3	0%	100%	0.625 g	12.5 g/l

The inoculation of the Erlenmeyer flasks was performed the same way as described in Section 2.2.4 of this thesis. However instead of using 50µl of inoculant as done in Section 2.2.4, 1ml of inoculant was used. A higher inoculant volume was used for the wash water to ensure that there were enough fungi cells within the solution to promote growth. A total of 50ml of substrate was used per flask. After the flasks were inoculated, they were placed on a shaking plate, shaking at 100rpm with the temperature at 30°C.

To obtain the success of growing *T. Reesei* in the varying substrates, the flasks were monitored every 24 hours for 7 days, recording what was visually seen inside the flasks.

4.3 Results

It was visibly apparent that the *T. Reesei* was able to grow more rapidly in the Erlenmeyer flasks containing 100% wash water, compared to the Erlenmeyer flasks that had 100% nutrient broth. After 24 hours the *T. Reesei* produced a visible amount of biomass in the flasks containing between 60-100% wash water. Within 48 hours all of 18 flasks had produced a visible amount of biomass. Figures 5, 6 and 7 show the growth rate of *T. Reesei* after 24 hours.

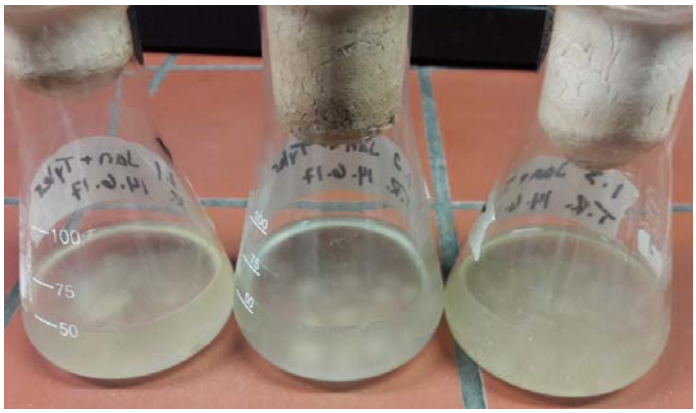


Figure 5. Growth of T. Reesei after 24h in 100% Wash Water. (Rickabaugh,T.; Manicke, J, 2017).

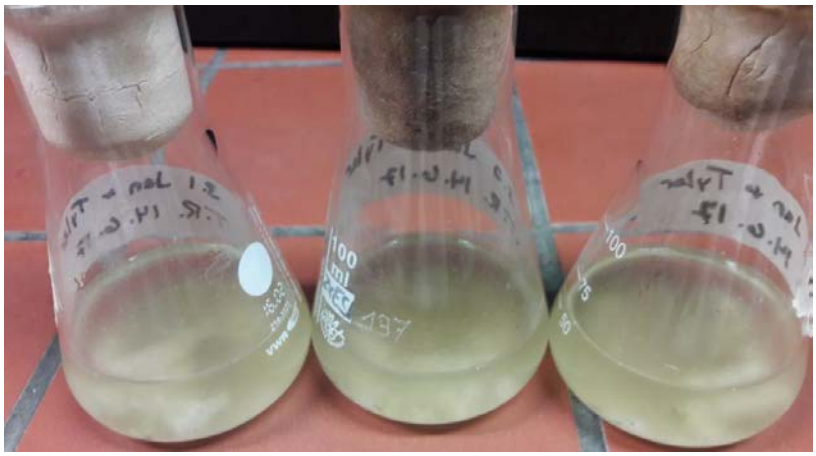


Figure 6. Growth of T. Reesei after 24h in 60% Wash Water : 40% Nutrient Broth. (Rickabaugh,T.; Manicke, J, 2017).

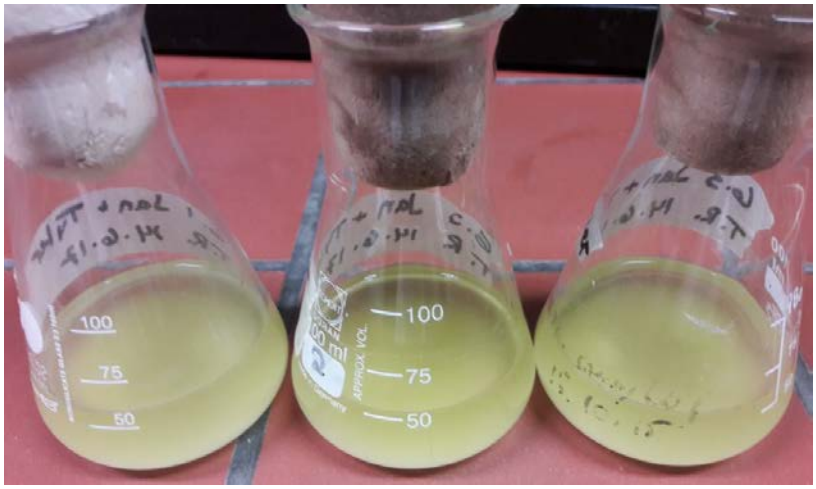


Figure 7. Growth of T. Reesei after 24h in 100% Nutrient Broth. (Rickabaugh,T.; Manicke, J, 2017).

4.4 Discussion and Conclusion

T. Reesei showed the capacity to grow in all the varieties of wash water to nutrient broth mixtures. Referring back to the aims of this experiment, the main objective was to see if it was possible to produce biomass in wash water, and if so in what concentration. The results are visibly conclusive in that, no matter what concentration of wash water to nutrient broth, the fungi was capable of growing.

It was observed that the *T. Reesei* was able to grow at a faster rate in 100% wash water compared to 100% nutrient broth. *T. Reesei* has shown to produce larger quantities of mycelium in soluble carbon sources such as glucose and lactose, compared to insoluble carbon sources such as cellulose (Dashtban, M, et. al., 2011). The high DOC content shown to be found in MSW wash water thanks to Cossu and Lai's experiments would help explain why *T. Reesei* was able to grow at a faster rate in wash water compared to the cellulose rich nutrient broth.
Enzyme production from *T. Reesei* could be effected though when growing in pure wash water compared to nutrient broth. When monosaccharides such as glucose are a readily available

carbon source for the fungi, cellulase production is inactive (Dashtban, M, et. al., 2011). Cellulase needs an inducer, such as the presence of cellulose, in order for the transcription of the enzyme to occur (Dashtban, M, et. al., 2011).

Further investigation into enzyme production needs to be taken into consideration to determine if *T. Reesei* is producing cellulase in 100% wash water, and if so to what extent. Because the wash water contains easily degradable carbon sources, the fungi may produce substantially less cellulase than found in 100% nutrient broth, if any at all.

5 Nutrient Broth with Glucose as Carbon Source

5.1 Background

It was discovered that *T. Reesei* grows faster in pure wash water in comparison to pure nutrient broth with cellulose as the carbon source. This was thought to be because of the easily degradable organic material within the wash water. In order to prove this theory, cellulose was replaced with glucose in the nutrient broth mixture.

The aim of this experiment was to analyse visually the growth rate of *T. Reesei* in pure nutrient broth with glucose, to see if it had comparable growth rates as found in pure wash water.

5.2 Methods

The preparation of nutrient broth followed the same procedures used in Section 2.2.3 of this thesis, as well as the inoculation procedures found in Section 2.2.4. However instead of using 50µl of inoculant as done in section 2.2.4, 1ml of inoculant was used. Another varying step in this method is the replacement of cellulose with glucose. The replacement amount is 1:1. The flasks were autoclaved at 121°C and 200kPa for 15 minutes before inoculation. Along with this the flasks were also placed onto a shaking plate at 100rpm in 30°C for incubation.

All three flasks were monitored every 24 hours for 7 days, with the visible results recorded.

5.3 Results

After 24 hours on the shake plate, all three Erlenmeyer flasks showed visible growth. Figure 8 shows what the flasks looked like after 24 hours of growth.

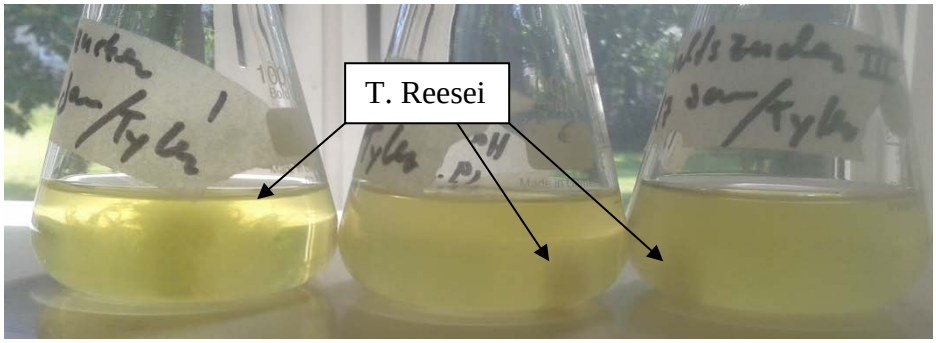


Figure 8. Growth of T. Reesei after 24h in Pure Nutrient Broth and Glucose. (Rickabaugh,T.; Manicke, J, 2017).

5.4 Discussion and Conclusion

It was observed that *T. Reesei* was able to grow in nutrient broth with glucose at comparable rates found in pure wash water. From this result, it is conclusive that the reasoning for *T. Reesei* growing quickly in pure wash water was due to the easily degradable organic material in the substrate. One thing that is left undetermined however is if the fungi are producing cellulase as it normally would with cellulose as its carbon source.

6 Wash Water with Leaves as Carbon Source

6.1 Background

T. Reesei has proven it has the capability to grow quite rapidly in pure wash water along with nutrient broth containing glucose. The aim of this section is to observe if *T. Reesei* has potential to grow in pure wash water with a different carbon source than the powdered cellulose in the nutrient broth, along with the easily degradable organic substances found in wash water. Alongside testing the growth feasibility, enzyme production will be tested to see if the fungi are producing cellulase in pure wash water and wash water with leaves.

The alternative carbon source used in the wash water will be dead leaves collected from the forest bed. All plant cell walls are comprised of cellulose, which is needed for *T. Reesei* to produce cellulase ("Plant Structure and Function", accessed 2017). Forest bed litter is a readily available carbon source that could be utilized in producing enzymes from *T. Reesei*.

T. Reesei has shown to have more enzymatic production when insoluble carbon sources such as cellulose are available compared to dissolved glucose (Dashtban, M, et. al. 2011). Cellulose acts as an expression inducer for the fungi, promoting transcription of enzyme (Dashtban, M, et. al. 2011). When monosaccharides such as glucose are available, *T. Reesei* will consume this carbon source without the assistance of cellulase.

It is undetermined yet if the fungi will produce cellulase when it is exposed to the organic content within the wash water. Enzyme testing will be able to demonstrate whether *T. Reesei* has the capacity to produce cellulase in pure wash water, and if so to what extent. Along with pure wash water, dead tree leaves will be added in different volumes compared to the cellulose volumes used in section 4 of this thesis.

The results from the enzyme test will depict the amount of reducing sugars available for consumption. Monosaccharides such as glucose classify as a reducing sugar because it has a free aldehyde or ketone group available for oxidation ("Reducing Sugar", 2004). Polysaccharides such as cellulose do not have an available aldehyde group for oxidation ("Nonreducing Sugar", 2004). As *T. Reesei* excretes cellulase out of their hyphae, it acts as an endo-active enzyme, cleaving the ends off the polysaccharides, creating available reducing sugars (Jarle Horn, S, et. al, 2012). The available reducing sugars that are found in the substrate can then be used as a carbon source for microorganisms to produce biomass.

6.2 Methods

6.2.1 Cultivation of *T. Reesei* in Wash Water with Dead Leaves

The preparation of the Erlenmeyer flasks for inoculation and cultivation followed the same methods that were described in Section 2.2.4 of this thesis. For this cultivation experiment, a total of nine Erlenmeyer flasks were prepared all with 50ml of pure wash water. Along with the wash water, three different weights of leaves were added with the wash water to provide an added carbon source for *T. Reesei* to utilize. Table 2 breaks down the weight of dead leaves used per flask.

Table 2. Dead Leaf Weights added to Wash Water Flasks

Dead Leaf Weights in Wash Water Flasks		
Samples	Volume of Wash Water (in ml)	Weight of Leaves (in grams)
1.1	50	0
1.2	50	0
1.3	50	0
2.1	50	0.3125
2.2	50	0.3125
2.3	50	0.3125
3.1	50	0.625
3.2	50	0.625
3.3	50	0.625

6.2.2 Enzyme Test

To test for the enzyme activity of cellulase from *T. Reesei* in wash water, the script found in Appendix 1 was used. The necessary chemicals needed to create the reagents as well as the needed equipment for this process are also found in Appendix 1.

Along with the reagents, a calibration curve is needed to reference the final values of the measured samples against the known cellulase concentrations. To produce a calibration curve, two different glucose solutions needed to be prepared. The first solution is the glucose stock solution containing 6g of glucose per liter of deionized water. This solution was then diluted at a ratio of 1:10 in deionized water, creating the glucose standard solution. Various volumes of the glucose standard solution were pipetted into test tubes that contained various volumes of deionized water. These volume variations can be seen in Table 3.

Table 3. Glucose Concentration Values.

Concentration [g glucose/L]	**mL glucose-standard solution [600 mg glucose/L]**	**mL water**
0,15	2,5	7,5
0,1	1,0	5,0
0,075	1,0	7,0
0,06	0,6	5,4
0 (Blind)	0	10

To produce a calibration curve, each concentration sample had to be prepared in the following manner. First, 0.5ml of each concentration sample was pipetted into a 1.5ml Eppendorf cap along with 0.75ml of the DNSA reagent that was prepared. Appendix 1 explains that the nitro group of dinitrosalycylacid are reduced by phenol and Na_2SO_3 acting on the reducing sugars, resulting in the color of the solution turning from yellow to brown.

After the substances were in the Eppendorf cap, small holes were punched through the lid of the cap to release the pressure that is built up inside during incubation. Next, the caps were placed into an Eppendorf Thermo Mixer C incubator for 12 minutes at 100°C. Once the time was finished, the caps were placed into an ice bath to cool down, and then 350µl of each sample was pipetted into a 96-well plate. The 96-well plate was then run through Thermo Scientific Multiscan Go photometer machine, and tested at 540nm. The results were collected and analysed in Microsoft Excel.

After the calibration curve had been made, the wash water samples were tested for a positive enzyme concentration. First 0.5ml of solution was pipetted from each flask into a 1.5ml Eppendorf cap and placed into a centrifugation machine for 5 minutes at 15000rpm to remove and suspended cells in the solution. The tube was removed from the centrifugation machine after 5 minutes, and 450µl of cell free supernatant was pipetted out into a new Eppendorf tube. Along with the supernatant, 50 µl of 1% CMC solution was pipetted into the tube, and then the tube was placed into the Eppendorf Thermo Mixer C incubator for one hour at 50°C. When the hour long incubation was finished, 0.75ml of DNSA reagent was pipetted into each Eppendorf tube sample. These samples are then placed back into the incubator for 12 minutes at 100°C. Following the incubation, the tubes were placed in an ice bath to cool off, and then 350µl of solution was pipetted from the caps into a 96-well plate and tested in the photometer the same way as the calibration samples were tested.

In order to determine the cellulase concentration in the wash water samples, negative control samples must be created in a relatively similar manner as done for the calibration curve. First, 0.5ml of liquid substrate was pipetted from each flask and placed into a 1.5ml Eppendorf cap, which was then centrifuged for 5 minutes at 15000rpm to remove any suspended cells in the solution. Next, 450µl of supernatant was pipetted from the centrifuged caps into a new 1.5ml Eppendorf tube, along with 50µl of 1% CMC- reagent and 0.75ml of DNSA reagent. These prepared samples then went through the same incubation, cooling, and testing procedures as the glucose samples for the calibration curve.

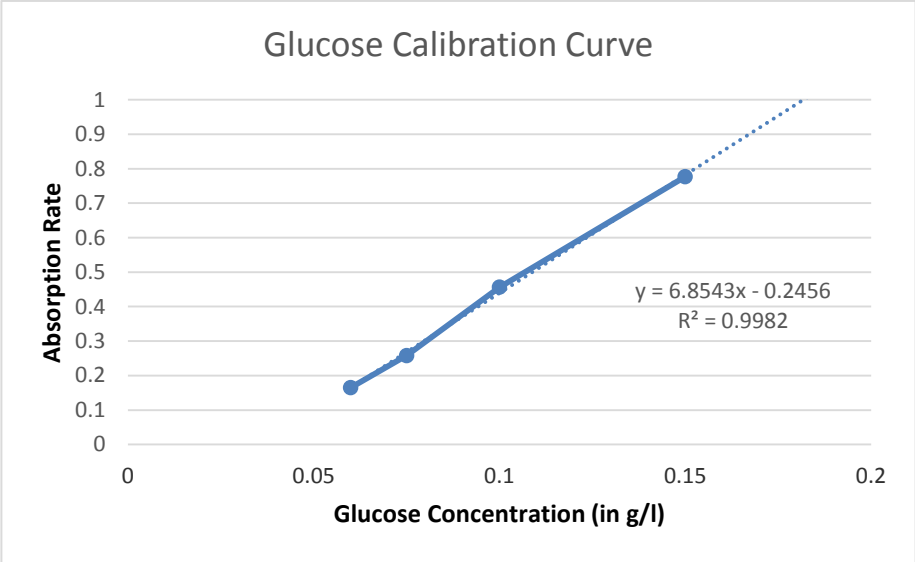
A positive control sample comprising of a commercial cellulase enzyme is also prepared for testing. To prepare the positive control sample, a dilution of 1:5000 of commercial cellulase in 0.14 M NaCl was prepared in a test tube, then 450µl of the solution was pipetted into a 1.5ml Eppendorf tube. The sample was then treated the same as the positive enzyme containing wash water, going through the same incubation, cooling, and testing methodology.

To calculate the available reducing sugars in the samples, the negative absorption values from the photometer were subtracted from the positive enzyme concentration tests. These values were then plotted into the calibration curve chart to determine the enzyme concentration levels in the solutions.

6.3 Results

The results from the glucose calibration can be seen in Chart 1. Based on the results from the calibration testing, Microsoft Excel was able to calculate the equation for the line. The absorption values of the wash water samples were cross referenced against the line equation to compute the glucose concentration in the sample.

Chart 1.Glucose Calibration Curve. (Rickabaugh,T.; Manicke, J, 2017).



The data in Table 4 shows that the flasks that contained more leaves in the substrate also contained more reducing sugars due to the available cellulose in the substrate. All of the samples containing only wash water showed to have zero available reducing sugars, for all of the reducing sugars available in the wash water were consumed in the production of hyphae. The average amount of reducing sugars produced by *T. Reesei* in sample 3 is roughly half the amount of reducing sugars found in the nutrient broth samples. Sample 3 had a 1:1 ratio of dead leaves in wash water to cellulose in nutrient broth. It is to be noted though that the positive absorption rate for the nutrient broth was outside the acceptable range limit for calculation, which could hinder the accuracy of the results.

Table 4. Reducing Sugars in Samples. (Rickabaugh,T.; Manicke, J, 2017).

	Reducing Sugars Concentration			
Sample	**g/l**	**Avg (in g/l)**	**µmmol/l**	**Avg (in µmmol/l)**
1.1	-0.035		-194.444	
1.2	-0.031		-170.372	
1.3	0.030	-0.012	166.157	-66.220
2.1	0.111		615.105	
2.2	0.140		776.804	
2.3	0.104	0.118	578.631	656.846
3.1	0.098		543.536	
3.2	0.245		1362.000	
3.3	0.197	0.180	1096.473	1000.669
NB1	0.391		2174.385	
NB2	0.292	0.341	1619.989	1897.187

6.4 Discussion and Conclusion

It is conclusive from the results that there is little enzyme production from *T. Reesei* in pure wash water. Enzymatic production only seems to occur when there is an added carbon source in the wash water such as dead leaves. This reiterates the point made is Section 6.1 of this thesis that when *T. Reesei* is exposed to dissolved sugars such as glucose it is unlikely for the fungi to produce cellulase (Dashtban, M, et. al. 2011).

Due to the absorption values of the positive control for both nutrient broth samples exceeding the acceptable ranges of 0.1-0.8, the final reducing sugars concentration is inaccurate. This creates difficulties in comparing the wash water samples containing dead leaves to the nutrient broth containing powdered cellulose. However, a conclusion derived from the results

show that by adding additional carbon sources such as dead leaves does increase enzymatic production of *T. Reesei*.

The methodology for testing the amount of reducing sugars available in different substrates was very tedious work, which opened up room for errors. The absorption values from the positive control of nutrient broth solutions were out of the acceptable range limits due to improper dilution rates. Other samples also needed to be diluted in order to be in an acceptable range for the photometer, however finding the proper dilution rate was challenging. This created a trial and error method of trying to find the correct dilution rate which resulted in wasted time and resources in the laboratory.

On top of dilution rates, ensuring the exact amount of reagents and solution to samples were the same for each test posed challenges. By adding more or less volume of reagent or solution in a sample could alter the absorption values. Instead of using glass pipettes, using an adjustable volume pipette guaranteed that the exact amounts of solution and reagents were added.

7 Upscaling to 1.5 Litre Reactor

7.1 Background

T. Reesei has shown that it has the capacity to produce visible amounts of mycelium in pure wash water in Erlenmeyer flasks. The next aim of this thesis was to see if this process can be upscaled using larger volumes of wash water. Before the cultivation process moved to a large 75l bioreactor though, testing needed to be performed in an intermediate step by cultivating *T. Reesei* in a 1.5l bioreactor.

It is important to create the same growing environment throughout the upscaling process that was used while working in the laboratory. Parameters to take into consideration while upscaling are aeration, agitation, temperature, and pH (Smith, C, 2014).

For the 1.5l upscaling, *T. Reesei* was cultivated in nutrient broth and wash water with dead leaves to test if the fungi had the ability to produce biomass in larger volumes. It was grown in the nutrient broth first due to the substrate's containment of optimal nutrients. Afterwards the fungi was inoculated in wash water to test the cultivation feasibility.

To confirm the results, samples were taken from the bioreactors and analysed via microscopy to see if there was any visible growth of the hyphae.

7.2 Methods

7.2.1 1.5 Litre Reactors with Nutrient Broth

For the cultivation of *T. Reesei* in 1.5l reactors, two Lenz 1.5l glass bioreactors with 3 neck flat flange lids were used. Two of the three neck pieces were closed off using cappers, while one neck had a Lenz gas washing bottle head that was used to supply oxygen to the fungi. Attached to the output valves of the gas washing bottle head were two Acro® 50mm Vent Devices with PTFE Membrane filters from Pall Corporation. Figure 9 helps show the design setup used in the fermentation process.

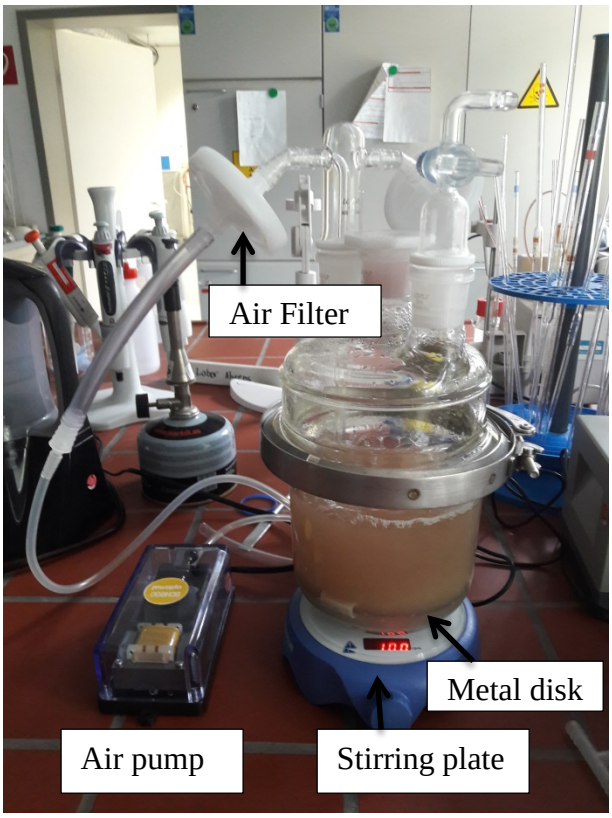


Figure 9. 1.5l bioreactor setup.

A stainless steel disk with millimetre holes drilled in it sat 1cm off the bottom, with a magnetic stirrer placed underneath the disk. The reasoning behind the metal disk was to create a barrier between the magnetic stirrer and the fungi, to ensure that the shear strength of the magnetic stirrer would not disrupt the growth of the fungi.

The preparation of the nutrient broth was conducted the same way as found in Section 2.2.3. For this test 750ml of nutrient broth was used for both reactors along with 9.375g of powdered cellulose. The cellulose amount is derived from the script provided by Wilharm and can be found in Appendix 1.

Before inoculation, the glass bioreactors containing the nutrient broth is placed into the autoclave at 121°C and 200kPa for duration of 15 minutes for sterilization. It needed to be ensured that when placing the bioreactor into the autoclave, the membrane filters were tightly secured on the gas washing bottle head with autoclaveable tubing, for these filters are prone to slip off the valves during autoclaving.

After autoclaving, the bioreactors were placed in a cool water bath to reduce the temperature of the nutrient broth to room temperature for inoculation. 15ml of inoculant was pipetted into each bioreactor, maintaining the same inoculate to liquid substrate ratio used in laboratory scale. A Bunsen burner was used during the inoculation process to help prevent any unwanted contaminates entering into the solution

The bioreactors were then set on a magnetic stirring plate with the speed set to 100rpm, duplicating the agitation speed used during cultivation in flasks. A Schego Optimal membrane pump was connected to the membrane filter on the gas washing bottle head, pumping 250l/h of air into the reactor. For this set up, the air pump acted as an oxygen supply source as well as an agitator mixing up the cellulose rich solution.

Samples were extracted from the reactors after 48 hours and analysed under the microscope to see if any hyphae growth was visible.

7.2.2 1.5 Litre Reactors with Wash Water

The same methodology found in Section 7.2.1 was used for cultivating *T. Reesei* in wash water. To produce the wash water, the same procedures used in Section 3.2 were used. Dead leaves were added into the wash water at a 1:1 ratio of cellulose in nutrient broth. After 48 hours of cultivation, samples were analysed via microscopy to detect hyphae growth.

7.3 Results

The results from both cultivation cycles were successful in producing mycelium. At a magnification of 400x, the hyphae could be seen growing in the nutrient broth and wash water. Figure 10 shows the hyphal growth in nutrient broth after 48 hours surrounded by cellulose, while in Figure 11 shows the hyphal growth in wash water containing leaves.

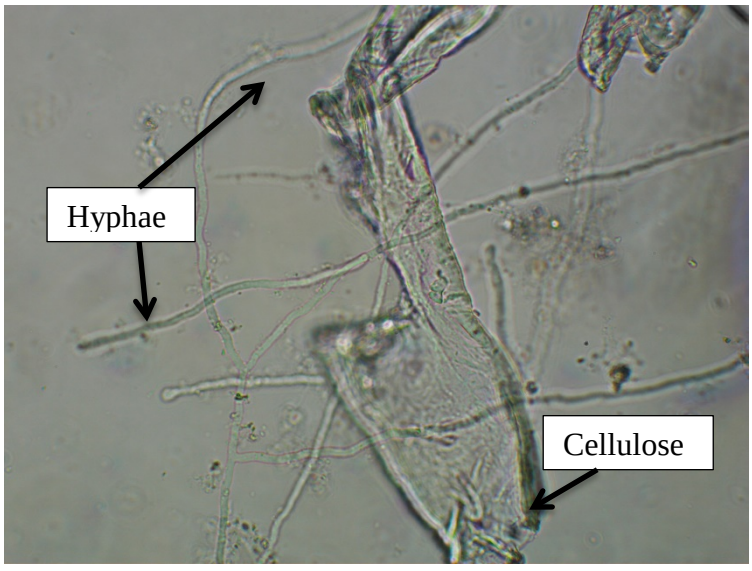


Figure 10. *T. Reesei* after 48h cultivation in 1.5 l reactor with nutrient broth. 400x magnification. (Rickabaugh,T.; Manicke, J, 2017).

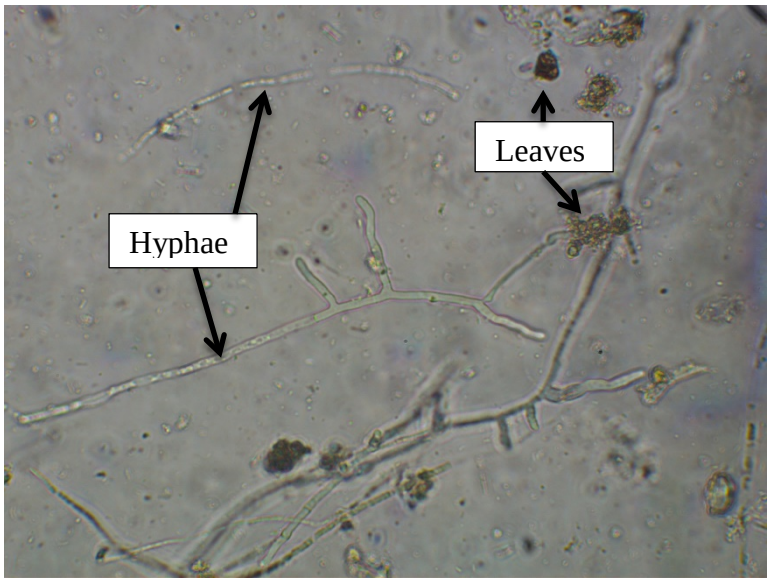


Figure 11. *T. Reesei* after 48h cultivation in 1.5 l reactor with wash water with leaves at 400x magnification. (Rickabaugh,T.; Manicke, J, 2017).

7.4 Discussion and Conclusion

T. Reesei has shown that it has the capacity to be upscaled to a 1.5l bioreactor using nutrient broth and wash waster as a liquid substrate. This experiment was only to test the plausibility of producing biomass in larger volumes, which has shown to be successful. Due to the main focus of this testing being only on producing biomass, enzyme testing was not performed.

Working with a larger bioreactor compared to the Erlenmeyer flasks used in the lab created its own challenges. After autoclaving the reactors, the cellulose and leaves had a tendency to settle underneath the metal disk, preventing the magnetic stirrer to revolve freely. Due to this issue, the fungi were limited to the agitation provided from aeration.

On top of the agitation issues, the aeration pipe in one of the bioreactors seemed to become clogged after 24 hours of fermentation. It was discovered that as time proceeded in the fermentation process, the fungi seemed to settle inside the glass pipe attached to the gas washing bottle head. Even though the air pump was turned off midway through the cultivation process, the fungi still showed to have growth.

Despite the challenges faced during this cultivation test, *T. Reesei* was still able to produce biomass, showing the true resilience of these fungi.

8 75l Bioreactor Testing

8.1 Background

Ostfalia Hochschule is currently in the developmental process of creating a 75l bioreactor facility in Ilsede, Germany, which has had mixed results from previous attempts at cultivating different microorganisms. The pilot plant has been able to successfully cultivate yeast within the reactor, however due to a temperature control issue was unsuccessful in the cultivation of *Bacillus amyloliquefaciens* (Prayoga, A, Walpole, R, 2017). The aim of the final upscaling process is to test the plausibility of *T. Reesei* producing biomass in the developmental bioreactor owned by Ostfalia.

As demonstrated in Section 7 of this thesis, *T. Reesei* has shown to have the capacity for upscaling using optimal substrates such as nutrient broth along with the use of MSW wash water. Although *T. Reesei* proved it was capable of producing biomass in wash water, nutrient broth was the substrate of choice for the 75l bioreactor. Due to poor weather conditions, harvesting MSW from the landfill was not possible, hindering the team from producing wash water for the final experiment.

Analysis of the fungi was conducted the same way as done throughout this entire thesis, via microscopy. By identifying hyphae in the substrate it could be determined if the developmental bioreactor is suitable for cultivating fungi such as *T. Reesei*.

8.2 Methods

The reactor used for this upscaling method is an AMS Technology Vorcarburierkessel 75l bioreactor with a maximum input capacity of 75l. For this experiment nutrient broth with a volume of 75l was used. Preparation of the nutrients for the nutrient broth was prepared in the laboratory at Ostfalia Hochschule, following the same recipe that was used in Section 2.2.3 by weighing out the approximate amounts of nutrients and placing them into a 1000ml glass jar. Glucose was substituted for cellulose at a 1:1 ratio to accelerate the growth of the fungi in the reactor.

All the contents of the nutrient broth were poured into the bioreactor and 75l of tap water was added, then the reactor was autoclaved at 121°C for 20 minutes at 200kPa to kill off any unwanted microorganisms. After the reactor cooled to 40°C, 1.5l of *T. Reesei* inoculum was poured into the reactor, using a butane lighter to sterilize the opening of the reactor. A KNF Lab Mini Diaphragm Vacuum Pump model number N 811 KN_.18 was used to provide aera-

tion to the reactor at a flow rate of 11.5 l/min. The lid of the reactor was covered with high strength tape, securing the aeration hose in the reactor and sealing off the top preventing contamination. For agitation, a Tschamber stirrer with a maximum stirring speed of 260rpm was set to 15% of maximum power, which was recommended by the facility operator.

Fermentation took place for 120 hours before samples were taken. After collecting the samples, they were transported immediately back to the laboratories at Ostfalia Hochschule for microscopic analysis.

8.3 Results

Microscopic analysis proved that there was in fact hyphal growth within the solution, as shown in Figure 12. Along with hyphae, it was discovered that there was also yeast and bacteria in the solution. Nevertheless, the contaminated solution did not seem to hinder the growth of the fungi.

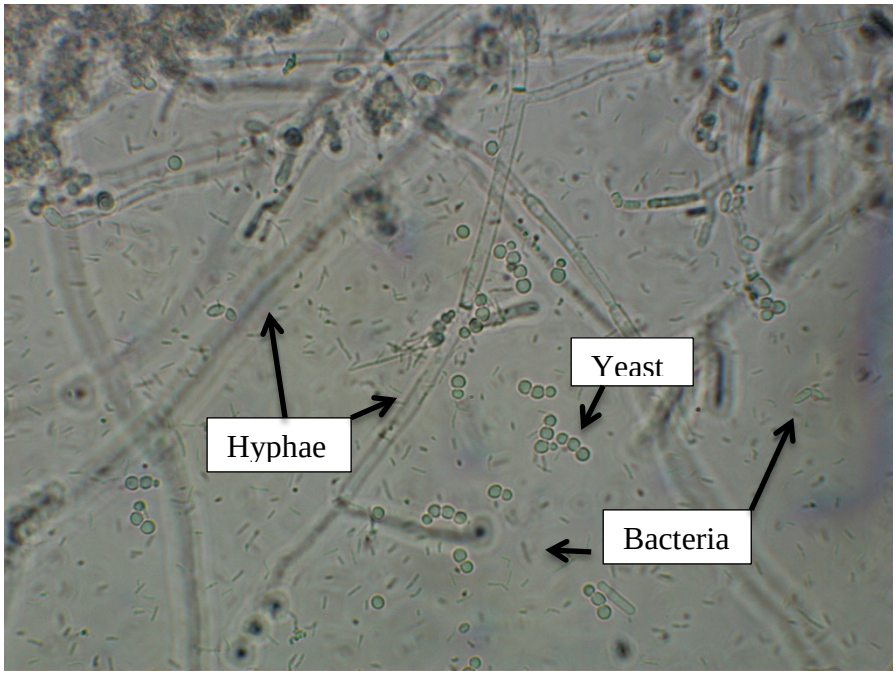


Figure 12. 400x Magnification of 120h fermentation in 75l bioreactor at Ilsede, Germany. (Rickabaugh,T.; Manicke, J, 2017).

8.4 Discussion and Conclusion

The feasibility of cultivating *T. Reesei* biomass at the developmental facility in Ilsede, Germany was successful. After 120h of fermentation, the microscopy results proved that the reactor was capable of growing the fungi.

A closer look at the results however shows that there was severe contamination inside the bioreactor. The contamination could be due to a poor seal from the tape used to cover the lid and seal the aeration hose in place, or from the autoclave improperly sterilizing the bioreactor. Further investigation into the source of contamination needs to be conducted before coming to conclusions.

To determine if the contamination source is internal or external, the fermentation process described in Section 8.2 should be repeated without using the inoculant and aeration pump to see if the contamination appears again in the microscopic analysis. Because yeast was found as a contaminate in the sample, it could be used as a contamination indicator, for yeast has the capacity to grow in both aerobic and anaerobic conditions and will not have any growth inhibition from the removal of the air pump (Snoek, I, Steensma, Y, 2007).

9 Conclusion

As stated in the introduction, the main aims of this thesis were to successfully cultivate *T. Reesei* in MSW wash water, and test the feasibility of upscaling this process to a final volume of 75l in the pilot bioreactor at Ilsede, Germany. From the results presented in this thesis, it can be concluded that *T. Reesei* can in fact produce biomass in MSW wash water, as well as have the capacity to upscale to larger quantities.

It was discovered that while *T. Reesei* can grow in pure wash water, there is minimal enzyme production unless an added carbon source such as dead leaves are added. Based on the enzymatic production tests, the cultivation of *T. Reesei* in wash water containing dead leaves produced half the amount of reducing sugars of what was found in *T. Reesei* cultivated in nutrient broth containing cellulose. However, the results gathered from the nutrient broth enzyme tests were out of range for the photometer to calculate accurate results. Further enzymatic testing should be conducted to gather firmer results.

Parameters used in the cultivation methods performed in the laboratories were transferable to larger working volumes when upscaling. *T. Reesei* was able to grow quite well in both the 1.5l and 75l bioreactors, with microscopic analysis showing hyphal growth in every sample. Although some samples showed to have contamination, the fungi showed no growth inhibition within the reactors. The focus of the thesis was only to produce biomass, and enzyme production was only touched on, however contamination inside the solution could have an effect on the down streaming process later on.

In conclusion, the concept of growing *T. Reesei* biomass in MSW wash water has proven to be successful. Along with biomass production, it has been demonstrated that the methodology used in the production of *T. Reesei* biomass on a laboratory scale has the capacity to be upscaled to larger volumes. Research in increasing enzyme production *T. Reesei* in wash water as well as elimination in contamination at Ilsede will be beneficial to this process in the future.

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APPENDICES

Appendix 1. Nutrient Broth and Enzyme Test Script

T. reesei cultivation and cellulase production and analysis

Material & Methods:

Dry substrates for cellulases:
cellulose
CMC (carboxymethylcellulose)
Frozen maize silage (biogas substrate)

Commercial enzymes (liquid at 4°C):
cellulases from *T. reesei* ATCC26921

Media:
T. reesei liquid medium:

cellulose	12,5 g/L
NH_4Cl	equimolar (referred to N) to 3.9 g/L $(NH_4)_2SO_4$
KH_2PO_4	2.0 g/L
$MgSO_4 \times 7\ H_2O$	0.3 g/L
$CaCl_2 \times 2\ H_2O$	0.4 g/L
Urea	0.3 g/L
Tween-80	0.2 mL/L
Bromkresolpurpur	5 mg/L

Trace elements:

$FeSO_4 \times 7\ H_2O$	5.0 mg/L
$MnSO_4 \times H_2O$	1.6 mg/L
$ZnSO_4 \times 7\ H_2O$	1.4 mg/L
$CoCl_2$	2.0 mg/L

The trace elements are provided as 100-fold concentrate. The pH-value of the medium is adjusted to pH 4,5 with HCl. Autoclave after dissolving all ingredients (program 10: 15 min 121°C)

Plate-medium (for sporulation):
Potatoe-Dextrose-Agar (PDA):
1 L potatoe infusion (see below)
20 g/L glucose (see below)
15 g/L Agar
Autoclave and add sterile (filtered) glucose after autoclavation, pour in plates.
For the potatoe infusion: slice 200 g potatoes and boil for 1h in 1L water, press through a fine sieve and autoclave.

Cultivation:

Inoculation from spores:
Inoculate 50 mL liquid culture with 250 µL from a resuspended spore culture (fresh or frozen). Alternatively, plate 100 µL of above spore culture on plates. Incubate at 30°C.

Inoculation from liquid culture:

Take up to 1/10 of biomass-containing culture volume and transfer it under sterile conditions into fresh sterile medium. Incubate on shaking platform at 30°C.

Preparation of spore cultures:
Strike liquid culture on a potatoe-dextrose-agar plate and incubate at 30°C for ~ 10 days.
Harvest spores by pipetting 5 ml 0,14 M NaCl-solution on the plate. Detach spores by gently circling the plate. Tilt the plate to collect spore suspension, transfer the spores into 1,5 mL Eppendorf caps. Add 15% (vol/vol) glycerol and store at -18°C.

Preparation of liquid culture supernatants:
- centrifuge liquid culture (e.g. 1 mL) at maximum speed for 15 minutes to pellet the cells
- transfer supernatant to new tube except ~200 µL at the bottom of the tube

analysis Cellulase-Activity-tests

1. Test of endoglucanase activity / DNSA-Test

Principle: (endo-)glucanolytic enzymes produce glucan fragments with reducing ends on one side. The concentration of the reducing ends is estimated by the DNSA test. In the presence of phenol and Na_2SO_3 the reducing sugars reduce the nitro group of dinitrosalycylacid, thereby turning the colour from yellow to brown.

reagents: Carboxymethylcellulose (CMC)
Tris
HCL
3,5-Dinitrosalicylsäure
Phenol
Di-sodiumsulfite Na_2SO_3
Potassium-sodium-Tartrat
NaOH

equipment: water and ice bath at 100°C and 0°C
Photometer at 640 nm

DNSA-Reagent: **wear gloves while working with DNSA-reagent**
40 g Potassium-sodiumtartrat x 4 H_2O
100 mg Na_2SO_3
400 mg Phenol (be careful: toxic!)
2 g 3,5-Dinitrosalicylsäure, dissolved in 100 mL of 2% NaOH
add water to 200 mL, store in the fridge.

Glucose stock solution [6 g glucose/L]: take 1,5 g glucose and dissolve in 250 mL deion. water
glucose-standard solution for the calibration curve (1:10 dilution from 6 g/L glucose stock solution in deion. water)

Concentration [g glucose/L]	mL glucose-standard solution [600 mg glucose/L]	mL water
0,15	2,5	7,5
0,1	1,0	5,0
0,075	1,0	7,0
0,06	0,6	5,4
0 (Blind)	0	10

Perform test:

a.) Calibration curve:
- Mix 0,5 mL of each glucose standard solution with 0,75 mL DNSA-reagent in an 1,5 mL reaction tube
- Punch holes with a fine needle in the cover of the pipet tubes to avoid their springing open during boiling
- Incubate for 12 minutes in boiling water bath
- Transfer in ice-water-bath for a few minutes

- Transfer 350 µL in wells of a 96-well plate and measure plate at 540 nm

b.) Test

- Pipet 50 µL of 1% CMC-solution into 1,5 mL reaction tube
- Add 450 µL sample (cell-free supernatant)
- Incubate for 1 h at 50°C (heat block)
- Add 0,75 mL DNSA-reagent, boiling, cooling and measuring as a.)

c.) Controls

medium background (treat like sample without incubation = prepare duplicates of each sample and boil and measure before (background control) and after incubation (test)):

- take 450 µL sample to 50 µL of 1% CMC-solution into 1,5 mL reaction tube and immediately add 750 µL DNSA-reagent, boiling, cooling and measuring as a.)

positive control: commercial enzyme solution

- prepare a 1:5000 dilution of the commercial enzyme in 0,14 M NaCl (dilute in 2 steps: 1:100 and 1:50 from the 1:100 dilution) and store it at 4°C
- take 450 µL of the 1:5000 dilution and treat like a sample (see b.)
- the absorption of the positive control should lay in the linear range of Absorption, optimal between 0,4 and 0,6. Prepare new dilutions if necessary.

The absorption of the medium background will be subtracted from the absorption of the respective samples before calculating the enzyme activity.

Absorbancy values should be between 0,1 and 0,8. If necessary, dilute samples with 0,14 M NaCl.

Calculation:

Absorption values per g glucose are converted into µmol glucose = µmol reducing sugars.

1 Unit activity = 1 µmol reducing sugar/min

For calculating the specific activity you have to refer to 1 mL sample/enzyme control.