OPTIMIZATION OF STERILIZATION METHOD FOR CULTIVATION OF FILAMENTOUS FUNGI ON LEMON WASTE

BSc in Chemical Engineering – Applied Biotechnology

David Ljungberg
Oliver Conradsson

HÖGSKOLAN I BORÅS

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Authors: David Ljungberg & Oliver Conradsson
Supervisor: Akram Zamani, Kanishka Wijayarathna
Examiner: Steven Wainaina
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ABSTRACT

Consumption of citrus fruits and citrus juice production creates wastes, which could be valorized by using it for cultivating fungi. Before cultivation, the medium needs to be sterilized though autoclavation. Larger volumes used when autoclaving requires longer heating cycles and therefore runs the risk of degrading the medium to a greater extent. This research examines the effects of the volume lemon waste medium used while sterilizing. The aim is to find the largest volume still providing good growth for the filamentous fungus used, *Rhizopus Delemar.* Lemon waste was provided by Herrljunga Musteri AB and was pre-treated at 45°C for 2h. The liquid was strained and autoclaved in different volumetric series ranging from 200 – 10 000 mL, that was then used in 200 mL shake flask cultivations. A scale up in two 3,5 L bubble column reactors was also performed from the 10 000 mL autoclaved medium, after not observing severe impacts on growth. Testing was done by weighing biomass and HPLC analysis of sugars. The yield of the biomass in the shake flasks ranged from 0,11 – 0,14 g/g sugars and the biomass concentration ranged between 2,4 - 3,0 g/L. Overall, the volume of autoclavation seems to not too be of great concern when cultivating *R. Delemar* on lemon waste medium in the analyzed ranges.

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1 INTRODUCTION

1.1 Background

Lemon is a well-known citrus fruit. According to a market analysis by the United States Department of Agriculture (USDA) from 2023, the consumption of lemons and limes reached about 5,6 million metric tonnes in the EU during the season of 2022-23 and roughly 30 million tonnes globally. From fruit juice producers, citrus waste accounts for roughly 50-60% of the processed fruit (Wilkins, Surayawati, Maness & Chrz 2007). These figures are likely to cause a large amount of lemon waste. Lemon peels are among these wastes and contain some nutrients, for instance simple monosaccharides like glucose and fructose (Czech, Agniezka, Bozena & Piotr 2021). By exercising resource recovery, these nutrients could be extracted into an aqueous solution and the extracted nutrients from waste could then be utilized as a medium for the cultivation of filamentous fungi. Fungal biomass from wastes can be used to make an assortment of product such as vegan protein concentrate (Souza Filho, Nair, Andersson, Lennartsson & Taherzadeh 2018) or even fungal leather (Wijayarathna, K 2021). To cultivate a microorganism on a medium, however, it should be sterile. When autoclavation, steam sterilization (Centers for Disease Control and Prevention [CDC] 2016), is conducted on a medium, the liquid is exposed to high temperature over time. Heating glucose and fructose over longer periods of time has been shown to correlate with thermal degradation of the sugars and consequently, the formation and build-up of Hydroxymethylfurfural (HMF) (Woo, Kim, Hwang, Lee & Jeong 2015), (Woo et al 2010). HMF is known to inhibit microbial growth (Gencturk & Ulgen 2022), which is undesirable. Although the sterilization phase, where the high target temperature is maintained, remains equal for different volumes, this phase only makes up a portion of the whole procedure. The sterilization is preceded by a longer heat up phase and is followed by a sizeable cooling phase (Systec 2017). Autoclaving larger volumes will therefore require longer heating cycles, as it takes a longer amount of time to heat up to and cool down from the target temperature. This results in a greater risk of forming more potentially inhibitory compounds and degrading nutrients when autoclaving larger volumes.

1.2 Characterization and utilization of substrate

1.2.1 Lemon waste composition

Analysis of the sugar and dietary fibre contents in citrus fruit has shown that lemon peels and pulp have been found to contain some amount of fructose and glucose, in the range of about 0,6 g/ 100 g for peels and 1,2 g/ 100 g for pulp, respectively. Dietary fibres make up a considerable portion of the pulp and the peel respectively but the largest, above 60% by weight, in the peel. Lemon peels were also found to be on the lower end of the ascorbic acid content, averaging at about 7,8 mg/ 100 g, as opposed to the average of 30,3 mg/ 100 g in orange peels (Czech et al 2021). The outer coloured layer of the peel, referred to as the flavedo, carries oils in their pores. These oils contain many non-volatile compounds as well but does not make up a large portion of the oils, roughly 2% of lemon oils. A large fraction, about 90%, of citrus oils comes from a single compound, D-limonene (Kimball, D. 1999).

1.2.3 Cultivation on lemon based medium

The inhibitory effects of the peel oil from citrus wastes on fermentation has been demonstrated (Mizuki, Akao & Sauruwatari 1990). This assertion is further backed by a study on the inhibitory effects of fruit peel extracts, where *Citrus limon* extract gave rise to inhibition zones for all gram-negative bacteria tested and about half of the fungi (Rakholiya, Kaneria & Chanda 2014). In contrast to this, Bulkan et al showed in a 2022 study of the inhibitory effects of bioactive compounds that the yields of a filamentous fungi, *Rhizopus Oligosporous*, improved in the presence of 240 mg/L limonene. Cultivation of *Neurospora Intermedia* with 240 mg/L limonene, however, severely reduced biomass yield, seemingly indicating inhibition varies by fungi.

Utilization of citrus wastes for cultivation of filamentous fungi without additional nutrients has nevertheless been shown possible (Satari, Karimi, Taherzadeh & Zamani 2016). Extraction of sugars were conducted in a mixture of citrus waste and water, with the best ratio of grams citrus waste to mL water for extracting these sugars being found to be 3:5. Cultivations were done both in 1000 mL shake flasks and larger 3,5 L bench scale airlift reactors. The yields reported for *M. Indicus* when cultivating on unadulterated citrus waste medium in a shake flask showed comparable yields to cultivation in medium enhanced with additional nutrients, 0,36 g biomass/g consumed sugar in relation to 0,39 g biomass /g consumed sugar.

1.3 Non enzymatic browning

1.3.1 The Maillard reaction

So called non enzymatic browning can be a non-desirable set of reactions which degrades and may impact nutrients in foodstuff or form toxic compounds (Croguennec, Schuck, Brulé & Brulé 2016). One of the main paths to this type of browning is called the Maillard reaction. It refers to an intricate series of reactions initiated by a reaction between an amino group and a carbonyl group of a reducing sugar (Simpson et al 2012). Monosaccharides are therefore commonly involved, however, other compounds with carbonyl groups such as ascorbic acid or polyphenols could partake (Croguennec et al 2016). The intricacy of the series of reactions has been shown through how it gave rise to over 300 intermediates when heating a system with only two reactants, ribose and glycine (Hemmler et al 2017). The condensation reaction from the carbonyl and amino group leads to Amadori or Heyn rearrangements, reconfiguring their structure irreversibly into other isomers. The resulting ketosamines, from the Amadori rearrangements or further reconfiguration of the aldosamine Heyns products, provides a starting point for a broad range of intricate reactions (Croguennec et al 2016). The later stages of the Maillard reaction may include the formation of hydroxymethylfurfural (HMF) and Pyraline, culminating in the formation of dark brown polymers, melanoidins. These polymers have been suggested being formed from pyrroles or furans in polycondensation reactions (Simpson et al 2012). The activation energy of the reactions involved in the Maillard reaction is comparably high, therefore temperature has great impact on the rate of reaction. This relationship can be described using the Arrhenius equation and due to this strong correlation between temperature and rate of reaction, among other reasons, browning is highly temperature dependant (Croguennec et al 2016).

Equation 1:Arrhenius equation, where R = Perfect gas constant, T = absolute temperature,, Ea = activation energy, a = frequency factor (Croguennec et al 2016)

$$k_\theta = a \cdot e^{-\frac{E_a}{RT}}$$

The frequency factor incorporates the collision frequency and the steric factor, the exponential expression signifies the collisions producing enough energy to carry out the reaction (Zumdahl 2013).

1.3.2 Caramelization

Although the Maillard reaction is commonly linked to non-enzymatic browning, other reactions such as the thermal degradation of sugars, caramelization, belong in this category (Croguennec et al 2016). Caramelization is a term for reactions where, without involving amino groups, carbohydrates and sugars degrades under heat (Woo et al 2011). High temperature, above 120°C, is known to be ideal for this type of reaction (Simpson et al 2012). It has been shown to happen below this point as well in a study by Chen, Yang, Chen & Li from 2009 at 75-95°C. According to their findings, however, browning by caramelization has been shown to correlate with increasing temperature and time. Ring opening occurs as an initial part of the caramelization reaction (Simpson et al 2012). Rearrangements of sugars into their isomers during degradation, Lobry de bruyn-Van Ekenstein rearrangements, has been known since the late 19th century (Angyal 2001). The rate of isomerisation of glucose into fructose has been found to be about five times lower as opposed to the rate for the reverse reaction (Kocadağlı & Gökmen in 2016). Under acidic pH, dehydration reactions are promoted, which results in the formation of HMF. (Simpson et al 2012). The presence of salts in the heated system has been shown to enhance the yields of HMF at the expense of the dicarbonyls formed during degradation (Kocadağlı & Gökmen 2016).

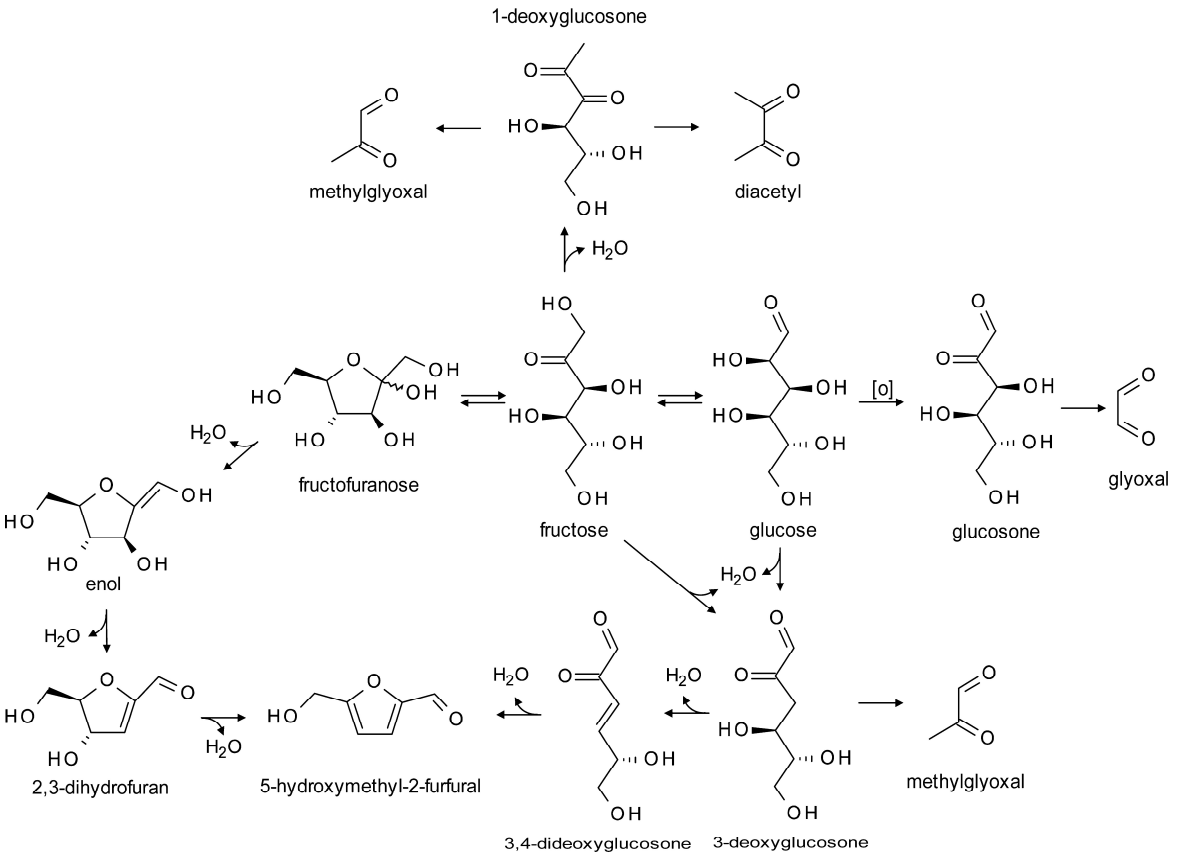


Figure 1. Mechanism of α-dicarbonyl compound and HMF formation from glucose and fructose degradation (Kocadağlı & Gökmen 2016)

Through dehydration reactions methylglyoxal will form coloring precursors, leading to colored high and low molecular weight compounds (Hormoki-Farkas, Örsi & Kroh 1997). Degradation is also present in ascorbic acid without interaction from nitrogenous compounds at first, but they do play a role in pigment formation (Croguennec et al 2016). In aerobic conditions the thermal degradation pathway of ascorbic acid includes furfural towards the end, the final product after reaction with amines being brown polymers (Simpson et al 2012).

1.4 Aim and scope

The aim of this thesis is to determine the optimal strategy for heat sterilizing lemon based medium for cultivation of filamentous fungi. The first objective is to examine if the volume of the medium used while sterilizing can influence the growth of the fungus in small scale conditions. Connected to this, the largest volume which can still provide good fungal growth is to be determined. A secondary objective, to investigate lower temperature sterilization over longer time as an alternative, is to be pursued depending on the data from the first objective. The final step of the process will be a scale up of the largest volume providing good growth in a bench scale bioreactor.

The thesis is only concerned with investigating optimal paths of heat sterilization for cultivation. No alternatives to heat sterilization are to be included, since it is not in the purview of this thesis.

3 MATERIAL AND METHOD

3.1 Material

Herrljunga Musteri AB provided the lemon waste used in the experiments, consisting mainly of peels with small amounts of attached pulp leftover from juice processing. The waste was kept prior to use in a freezer.

3.1.1 Chemicals

Sulfuric acid (1%), Sodium hydroxide (2 M and 10 M), D-glucose anhydrous (fisher chemical), Agar (Sigma-aldrich), Peptone (Sigma-aldrich), milli-Q water.

3.1.2 Microorganism

The microorganism used was *Rhizopus Delemar* (CBS 145940), a filamentous fungus that has its origin from the leaves of *Tectona grandis* in Indonesia, where the leaves are used in tempeh production (Westerdijk Fungal Biodiversity Institute n.d).

3.2 Equipment

The following equipment was used; Stirring plate (IKA, Color squid white), Analytical balance (KERN, AB1220 – 4NM), milli-Q water dispenser (Merck, Milli-Q IQ 7000), pH electrode (Mettler Teledo, Five Easy pH/mV), autoclave (SHP Steriltechnik AG, LABOKLAV 25) and (Systec, VB 150), blender (Waring commercial, Heavy duty blender), drying oven (Termaks), Bunsen burner, incubator (Binder), 65 L kettle (Digiboil), large scale (Ohaus, Defender 5000), mixer (Robot coupe, MP 450 XL FW ULTRA), hydraulic engine crane, HPLC (Waters, Alliance 2695 separations module), centrifuge (Thermo fisher, fresco 21), shaking water bath (Grant, OLS 200) and (Grant, OLS Aqua Pro), microscope (Axiostar plus), bubble column bioreactors (Belach Bioteknik), bioreactor control unit (Belach Bioteknik, Webant), pH-probes (Hamillton, Easyferm Plus PHI S8 225).

3.3 Preparation of agar plates

3.3.1 Making agar solution

For the preparation of 200 mL of agar solution, 4 g/L of peptone, 17 g/L of agar and 20 g/L of glucose was measured on an analytical balance. To a 500 mL beaker, 180 mL milli-Q water was added with a milli-Q water dispenser. While stirring at 490 rpm with a magnetic stir bar, the nutrients was slowly added. The solution was then pH-adjusted to 5,49 using 1 % sulfuric acid with the help of a calibrated pH electrode. The contents of the beaker were transferred to

a measuring cylinder and was topped off with distilled water for a 200 mL total volume, which was then transferred to a blue cap bottle and autoclaved at 121°C for 20 min. While working with aseptic conditions, (here referred to as working by a Bunsen burner with a disinfected workspace using 70 % ethanol), the sterilized agar solution was evenly transferred to 10 petri dishes and were then left to cool overnight and to check for contamination.

3.3.1 Inoculation of agar plates

With aseptic conditions 20 mL of autoclaved milli-Q water was transferred by syringe to a petri dish containing previously inoculated agar with the filamentous fungus *Rhizopus delemar*. Using an L-spreader, the fungus was gently spread out to release the spores from the surface to the suspension. A total of 13 mL of spore suspension was transferred using a syringe to a falcon tube. Each of the previously made agar plates were then inoculated with 0,1 mL of spore suspension and then evenly spread out. The inoculated agar plates were then placed in an incubator at 30°C for four days.

3.4 Preparation of medium

3.4.1 Blending of lemon waste

Five large bags of lemon waste were brought out of cold room and was blended to reduce the size of the peels to release the nutrients more easily. The blended waste was then transferred to four 2,2 L zip lock plastic bags and placed back in cold room at 4°C.

3.4.2 Dry weight measurements

A small amount of shredded lemon waste was added to three labelled aluminium cups and weighed on an analytical balance to measure the wet weight. The samples were then placed in a drying oven at 70°C overnight. The following day it was noticed that the aluminium cups were never weighed, and because of this the dry weight would give a false value. To remedy this, the dry lemon waste was scraped off onto new and tared aluminium cups. The samples were measured, and a dry weight/wet weight average was calculated, as shown in Table A1 in appendix A.

3.4.3 Heat-treatment of lemon waste

As the medium needed a 6 % lemon suspension with a total weight of 25 kg, the dry weight value calculated was implemented in the calculations of the lemon waste and water needed. Both calculations are shown in appendix A. This resulted in 14681 mL of milli-Q water being added using a milli-Q water dispenser to a 65 L kettle. On a large scale, 10,32 kg of blended lemon waste was measured and hastily added to the kettle which led to splashing and loosing

of some small amount of liquid. The 500 W, 1000 W and 2000 W heating of the kettle was then turned on and when the temperature dial showed 31°C the 1000 W and 2000 W heating was turned off. The temperature was then kept at 45°C for 2 hours while mixing every 15 min, shown in Figures 2 and 3. Specific time of mixture is shown in Table A2 in appendix A.

Figure 2. Heat-treating the lemon peels.

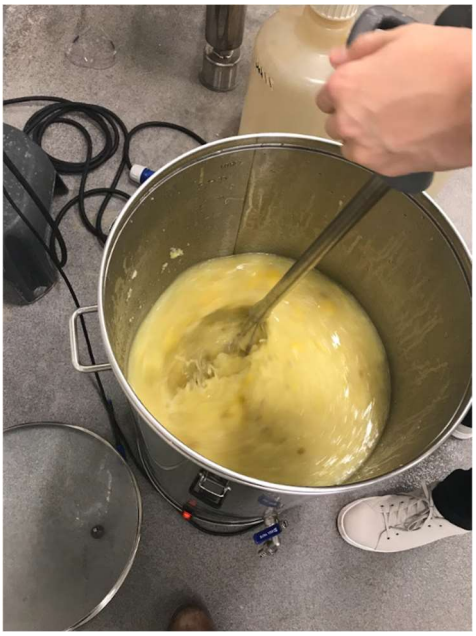


Figure 3. Mixing the kettle.

3.4.4 Straining the liquid

The heat-treated slurry was strained by squeezing the lemons by hand in a brew bag suspended over a 75 L bucket using a hydraulic engine crane, as shown in Figures 4, 5 and 6. Out of the 25 kg lemon waste slurry, roughly 17 L of liquid was yielded. Out of this 17 L, 1300 mL was transferred to a large 2000 mL beaker while the rest was poured into a large plastic container with a lid and placed in cold room at 4°C.

Figure 4. Suspended brew bag.

Figure 5. Transferring the liquid.

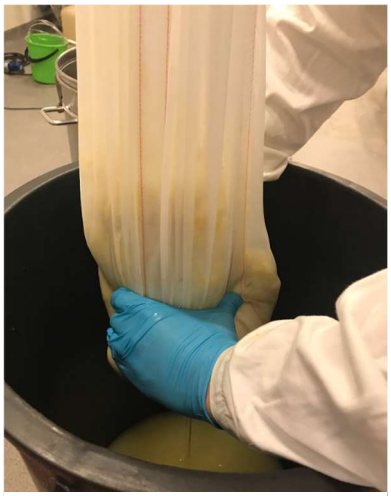


Figure 6. Squeezing out the liquid.

3.5 Autoclavation of medium

3.5.1 Autoclavation of 200 mL series

The pH of the strained liquid in the 2000 mL beaker was adjusted to 5,50 by adding 2 M sodium hydroxide and was then transferred by adding 200 ml to six 500 ml Erlenmeyer flasks, see Figure 7. The flasks were then autoclaved in two separate autoclaves, three in a small one (SHP Steriltechnik AG, LABOKLAV 25) named series B, and the others in a larger one (Systec VB 150), named series A, shown in Figure 8. The splitting of the 200 mL series into two was the result of the medium sized autoclave (Systec VX-95) unexpectedly breaking down, which had previously been planned to use. Autoclaved series A and B are shown in Figure 7.

Figure 7. 200 mL series, A and B, before and after autoclavation, with series A to the left.

Figure 8. Series A in Systec VB 150.

3.5.2 Autoclavation of 1000 mL, 5000 mL and 10 000 mL series

The large plastic container was taken out of cold room and was pH adjusted to 5,50 with 10 M sodium hydroxide. Three samples of liquid were transferred to aluminium cups and placed in a drying oven to check the dry/wet ratio, these measurements and following calculations are in appendix B. Using a measuring cylinder with a stated accuracy of ± 5,0 mL, 1000 mL and 5000 mL was transferred to a 1000 mL and 5000 mL blue cap bottle respectively. These were thereafter autoclaved (Systec VB 150) at 121°C for 20 min, shown in Figure 9 and 10. The large plastic container needed a remaining volume of 10 000 mL and was measured to roughly 10 400 mL, therefore approximately 400 mL of liquid was removed. The container was then autoclaved at 121°C for 20 min (Systec VB 150), as shown in Figures 11 and 12.

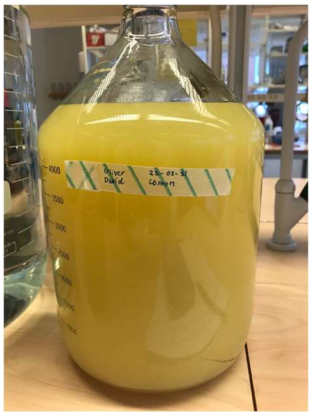


Figure 9. 5000 mL before autoclavation.

Figure 10. 5000 mL and 1000 ml autoclaved.

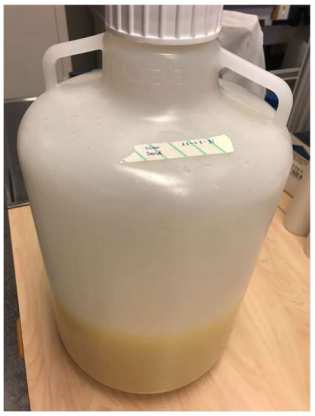


Figure 11. 10 000 mL before autoclavation.

Figure 12. 10 000 mL autoclaved.

3.6 Inoculation of medium

3.6.1 Inoculation of 200 mL series

Working with aseptic conditions, 20 mL of sterile milli-Q water was added to two inoculated agar plates and scraped with an L-spreader and the spore solution was extracted to a sterile falcon tube reaching a volume of 29 mL. Using an autopipette with sterile tips, 4 mL of spore solution was added to each of the six flasks. Two small 1,5 mL samples were taken from both A and B series and was transferred to Eppendorf tubes and centrifuged at 10 000 rpm for 10 min. The supernatant was transferred to new Eppendorf tubes and placed in the freezer awaiting HPLC analysis. Meanwhile the shake flasks were placed in a shaking water bath at 35°C and 110 rpm to grow the fungus.

3.6.2 Inoculation of 1000 mL, 5000 mL and 10 000 mL series

Because of evaporation during autoclavation the mediums had lost some liquid, therefore 65 mL of sterilized milli-Q water was added to the 1000 mL bottle and 1000 mL was added to the 10 000 mL container. The 5000 ml bottle had a marker showing the volume, this marker however, had disappeared during autoclavation. Therefore, the loss of volume for the 5000 ml bottle was interpolated between the values of the 1000 mL and 10 000 mL. The estimated loss of volume was found to be 480,5 mL, which was added to the 5000 mL bottle. These calculations are found in appendix A. For each series, 200 mL of medium was transferred to five sterile Erlenmeyer flasks. The process of inoculation, taking of samples and the placing in shaking water baths was identical to the 200 mL series. However, three liquid samples were taken from each series during inoculation to check the initial dry weight and was measured on an analytical balance. The samples were then placed in a drying oven at 70°C overnight. These measurements are shown in Appendix C.

3.7 Harvesting biomass

After 48 hours, the shake flasks of the 200 mL series were removed from the shaking water bath and the pH of each bottle was measured using a pH electrode. The pH ranged from 5,07 to 5,12 and the specific pH of each flask is shown in Table B1 in appendix B. However, one bottle from series A ruptured when being removed from the shaking water bath. The volume of each flask was measured, by separately pouring series A and B into two measuring cylinders and the measurements are shown in Table B3 in appendix B. The biomass was then sieved separately over large plastic measuring jugs. By straining (Figure 13), rinsing and pressing the biomass, remaining medium and water was removed before weighing each wet biomass. The biomass from series A was mixed, and the same was done with series B. Three samples were then taken from each mixture of biomass and was weighed and placed in a drying oven at 70°C, the final dry weight measurements are shown in appendix C. HPLC samples were prepared from the liquid separated from the biomass, just as previously done before. Harvesting of flasks which were made using liquid from 1000 mL, 5000 mL and 10 000 mL series was done identically. The pH and volumetric measurements are shown in appendix B.

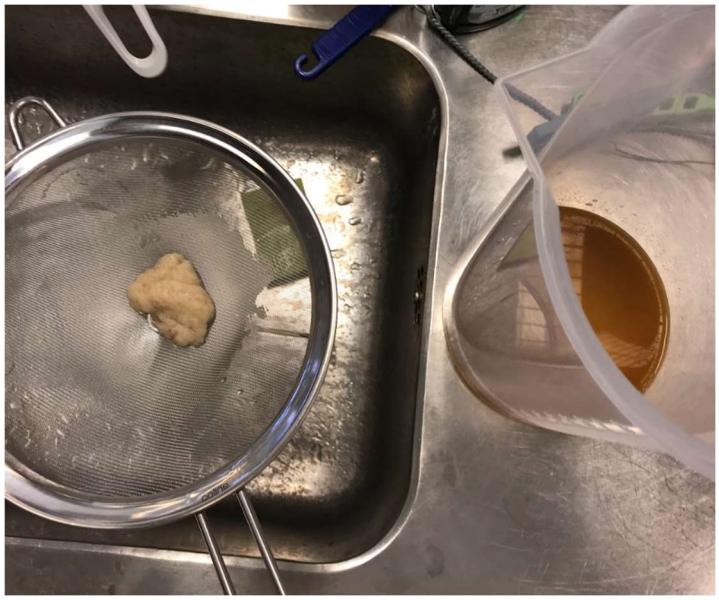


Figure 13. Straining the biomass.

3.8 Spore counting

Spore solution was made from one of the previously inoculated agar plates and 1 mL of this solution was transferred to a falcon tube containing 9 mL of milli-Q water, diluting the solution by 1/10. This was then used to create a dilution of 1/100. The suspensions were transferred to counting chambers and placed under a microscope. Number of spores counted and calculations regarding number of spores in the suspension that was added to each Erlenmeyer flask are shown in Table D8 in appendix D.

3.9 Scale up

The cultivation process was scaled up to a 4 L bubble column reactor using the remaining liquid from the 10 000 mL series. For this, two 4 L bubble column reactors (Belach Bioteknik) was assembled and tested for leaks and were then autoclaved. A new batch of agar plates was made and inoculated using the same method as before and some of the spore solution was diluted and used for spore counting. These counts are shown in Table D9 in appendix D. The agar solution used had the pH adjusted to 5,54 using 2 M Sodium hydroxide and 2 M sulfuric acid. For the reactors, two pH probes (Hamilton, Easyferm Plus PHI S8 225) were calibrated for the control unit (Belach Bioteknik, Webant). With the inoculated agar plates, 70 mL of spore solution was made for each reactor and was kept in 50 mL falcon tubes. When 3,5 L of sterile medium had been added to each reactor, the spore solution was added. The reactors were then hooked up to both heating and the aeration rate was set to 1 vvm, which led to the addition of 2 mL of antifoam in each reactor due to excessive foaming. The final process is shown in Figure 14. Under aseptic conditions HPLC samples were taken with a syringe at 0h, 18h, 21h, 24h, 42h, 45h, and 48h. Except for the first samples at 0h, pH was also measured during each sampling and adjusted at 24h and 45h, these measurements are shown in appendix B. Before the taking of each sample 10 mL had to be thrown to get a fresh sample from the tubing. The biomass was harvested after 48 hours by removing the biomass and straining out the liquid. Samples were taken identically to the smaller series, checking the pH, shown in appendix B. The dry weight and weight of the biomass was also measured identically, the measurements are shown in appendix C and D, respectively.

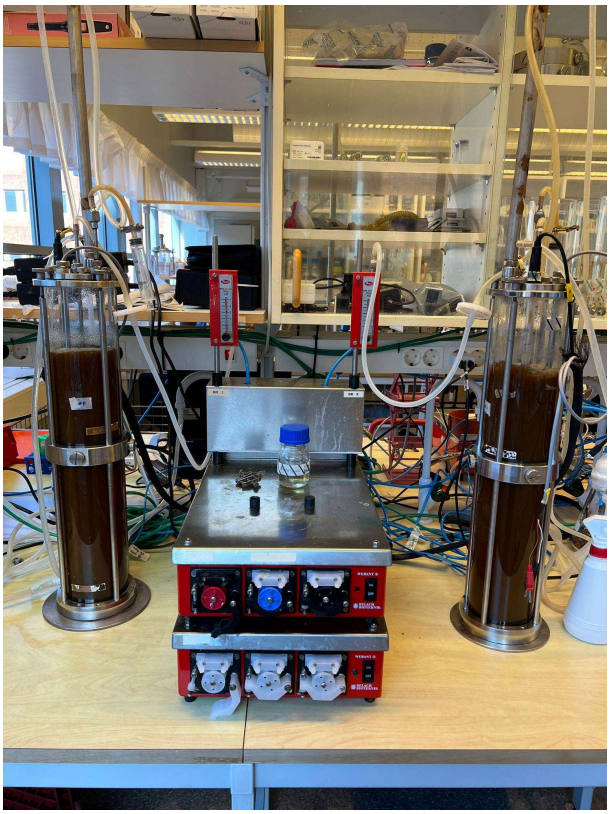


Figure 14. Inoculated reactors hooked up to heating and aeration.

3.10 HPLC analysis

The previously taken liquid samples were taken out of the freezer and categorically placed in Eppendorf tube racks, as shown in Figure 15. Once the samples had thawed, they were transferred with syringes and syringe filters to HPLC vials and placed in carousels, shown in Figure 16. The carousels were then placed in the HPLC machine shown in Figure 17 and each sample was analysed for 30 min. Resulting peaks from analysis by HPLC were analysed and some compounds of interest could be identified at their expected retention times from standards.

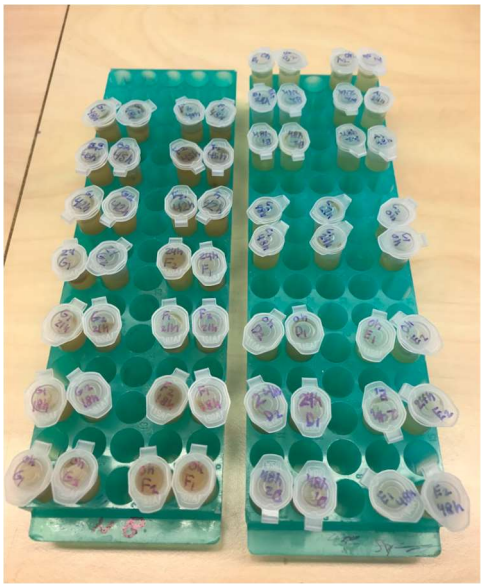
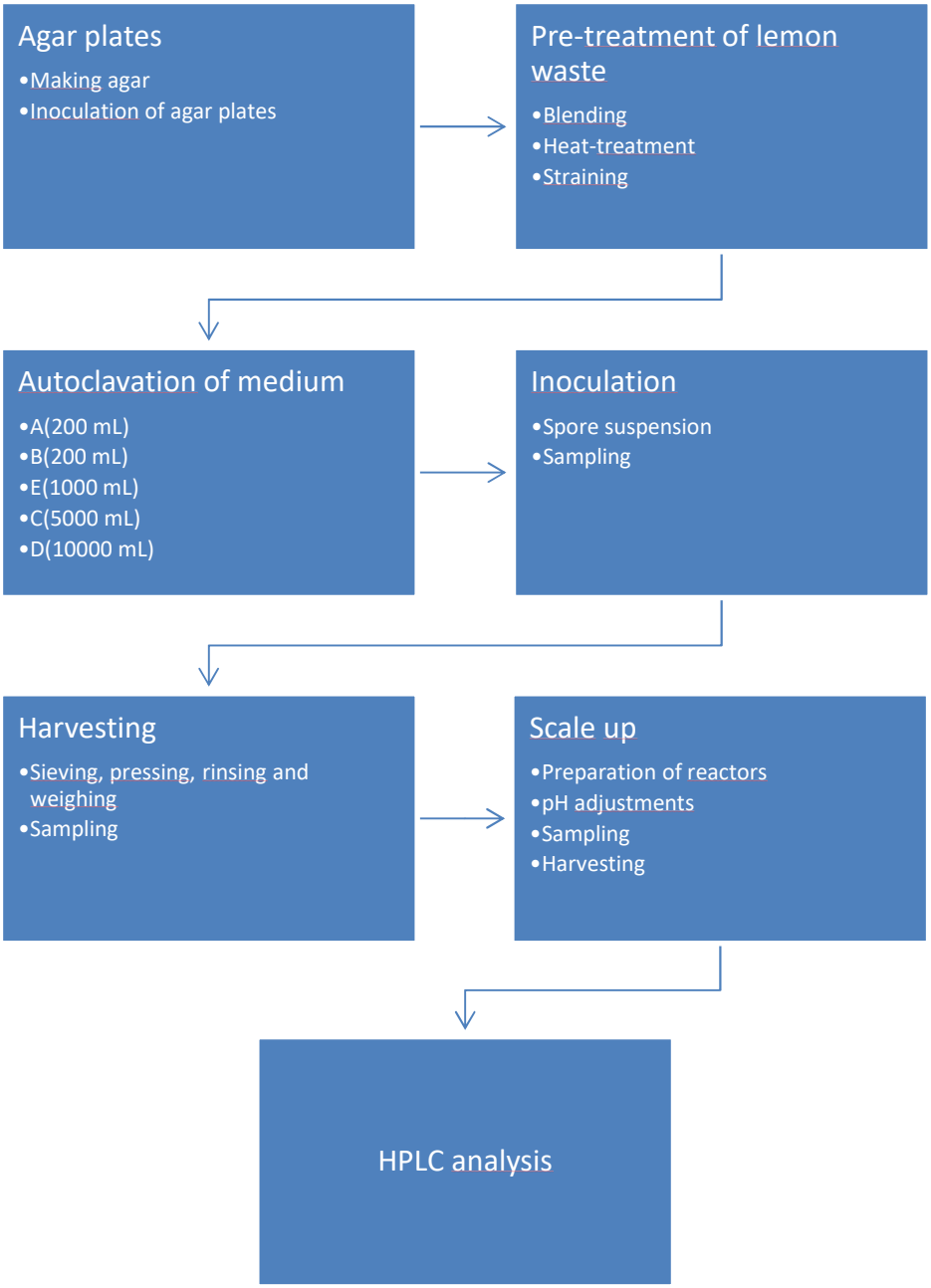


Figure 15. Eppendorf tubes in Eppendorf tube racks.

Figure 16. HPLC vials in carousels.

Figure 17. Waters, Alliance 2695 HPLC separations module.

3.12 Summary of experimental work



3.11 Method of significance testing

The standard deviations of the measurement series can be calculated by:

$$SD_{dataset} = \sqrt{\frac{\sum(x_i - \bar{x})^2}{n-1}}$$

Standard deviations are then pooled:

$$SD_{pool} = \sqrt{\frac{{SD_1}^2 \cdot f_1 + {SD_2}^2 \cdot f_2}{f_1 + f_2}}$$

Where f are the degrees of freedom, equal to n-1, of the respective datasets.

A t-test would be conducted using the following equation:

$$t_{dataset1\&dataset2} = \frac{\bar{x_1} - \bar{x_2}}{SD_{pool} \cdot \sqrt{\frac{1}{f_1} + \frac{1}{f_2}}}$$

By comparing t-values to tabulated critical values, based on the desired confidence interval and degrees of freedom, the null hypothesis could be evaluated (Simonsen 2005).

An assumption of this t-test is how the standard deviations of the data to be compared are equal (Simonsen 2005). The pooled variance t-test increases the risk of erroneously discarding the null hypothesis given unequal variances in conjunction with unequal sample sizes. Welch's t test utilizes the variances separately instead of pooling and is reported in literature by Hinkle et al to remedy these errors given unequal size of samples and variances (Adusah & Brooks 2011). Findings by Adusah & Brooks, however, would suggest that a welch t test is not to be used with small sample sizes, as it increases the error rate. Another study by Winter in 2013 seems to also put into question the usage of welch t test at very small sample sizes unless both the variance and sample sizes are unequal. Therefore, the unequal variance t test, Welch's t test, is to be used over the pooled t test only if variance and sample sizes differ.

Welch's t is derived from the following expression (Coombs, Algina & Oltman 1996):

$$t = \frac{\bar{x_1} - \bar{x_2}}{\sqrt{\frac{SD_1}{f_1} + \frac{SD_2}{f_2}}}$$

Obtaining the degrees of freedom, according to Moser and Stevens, for unequal variance t test can be more difficult and is calculated though the following equation (Ruxton 2006):

$$df = \frac{(\frac{1}{n_1} + \frac{\frac{SD_2{}^2}{SD_1{}^2}}{n_2})^2}{\frac{1}{n_1{}^2(n_1 - 1)} + \frac{1}{n_2{}^2(n_2 - 1)}}$$

To simplify processing the data, the Analysis ToolPak for excel was installed and used to perform t tests on the data.

4 RESULTS AND DISCUSSION

4.1 Observations from autoclavation

The mediums showed clear signs of browning through the discoloration post autoclavation. An increasingly brownish shade was observed as the volume of autoclaved medium got larger. This likely indicates that browning reactions has occurred to a greater extent in large volumes. Given the longer cycle time with heating and cooling, the result is not surprising.

4.2 Fungal cultivation

4.2.1 Shake flasks

After 24 h of cultivation the biomass had grown in form of suspended mycelium, and the amount of biomass seemed to have only increased slightly after 48 h. There was no visual difference of the biomass between the series. Table 1 shows the wet weight of the harvested biomass from the shake flasks from each series of autoclaved volume and is summated. As one shake flask from series A ruptured during harvesting, the sum of the biomass is much lower than the sum of series B.

Table 1. Wet weights of the biomass from cultivations of 200 mL medium from series A-E.

Autoclaved Volume (mL)	200	200	1000	5000	10000
index series	A	B	E	C	D
sample 1 (g)	6,3802	6,1576	4,0476	6,4006	4,8407
sample 2 (g)	7,024	5,8372	4,3152	5,555	4,687
sample 3 (g)	-	5,1484	4,5155	6,3415	4,4015
sample 4 (g)	NA	NA	4,5661	5,1737	4,1694
sample 5 (g)	NA	NA	5,115	5,0547	4,163
sum (g)	13,4042	17,1432	22,5594	28,5255	22,2616

With the average ratio of the dry/wet biomass samples located in appendix D, multiplied with the sum of the wet biomass from Table 1, an approximate total dry weight of each series biomass is achieved. These values are then used to approximate the biomass concentration in g/L, where series E shows the most amount of growth, as shown in Table 2.

Table 2. Biomass concentration of biomass for series A-E.

Series	Average Dry/wet ratio	Approximate dry weight (g)	Total volume samples (L)	Approximate Biomass concentration (g/L)
A	0,0835	1,1193	0,4	2,798
B	0,0870	1,4915	0,6	2,486
C	0,0911	2,5987	1	2,599
D	0,1069	2,3798	1	2,380
E	0,1335	3,0117	1	3,012

Based on the visualized trend in the graph of Figure 18, the biomass concentration seems to decrease as the autoclaved volume increases. Nevertheless, the discrepancies between sample group A and B, both autoclaved using 200 mL volumes, casts some doubt on this initial assessment. Furthermore, the 1000 mL datapoint showed an increase in the biomass concentration as compared to using 200 mL.

Table 3. Biomass concentration given standard deviations of dry/wet ratio.

Series	Approximate dry weight +1 SD dry/wet (g)	Approximate dry weight -1 SD dry/wet (g)	Approximate Biomass concentration +1 SD dry/wet (g/L)	Approximate Biomass concentration -1 SD dry/wet (g/L)
A	1,2171	1,0214	3,043	2,554
B	1,6218	1,3612	2,703	2,269
C	3,3569	2,6666	3,357	2,667
D	2,9210	2,2706	2,921	2,271
E	2,6380	2,1215	2,638	2,122

An aspect to keep in mind is how the data utilized in the graph is not raw data. The data has been processed using the mean of the dry weight fractions observed in separate measurements, possibly making it sensitive to error. The standard deviations of the dry weight fractions, as seen in appendix B, varies but is slightly larger for the large volumes. For the 1000 mL biomass the standard deviations were the largest, about twice the size of the deviation in both group A and B. This results in uncertainty on the calculated biomass concentrations, as displayed in Table 3 and Figure 18.

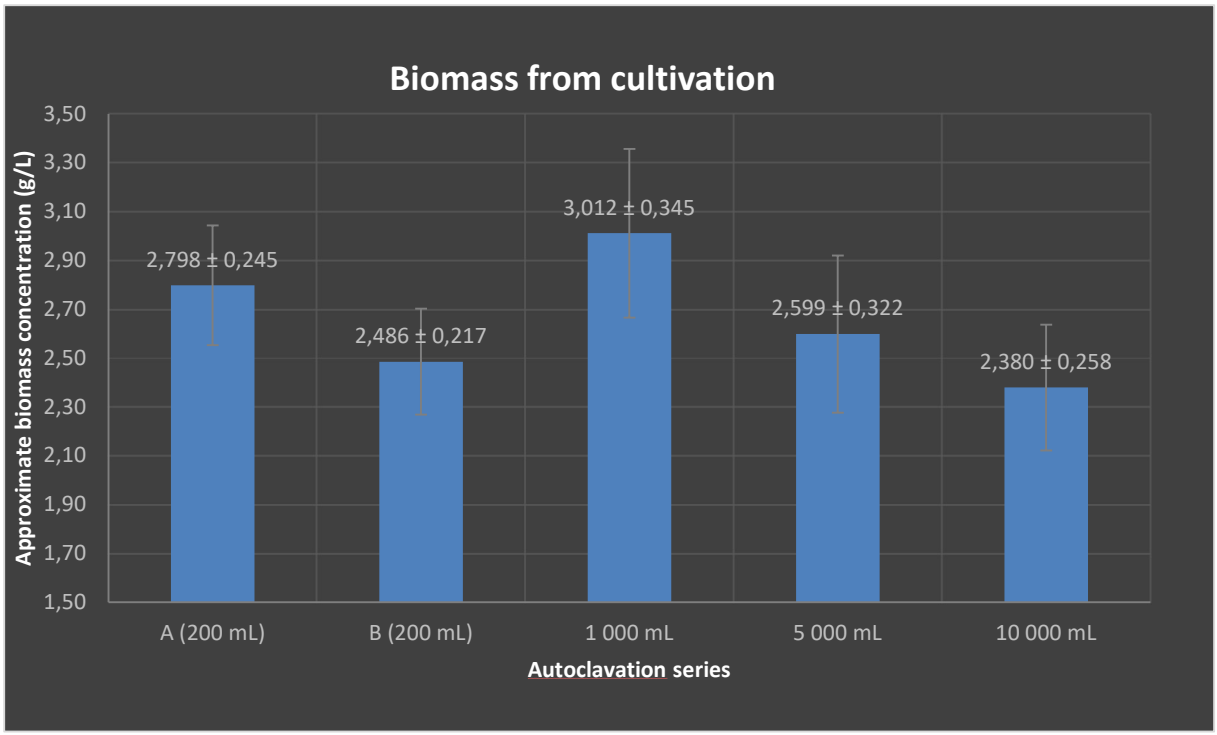


Figure 18. Graphical representation of calculated biomass concentration as a function of autoclaved medium volume with error bars accounting for respective dry/wet ratio uncertainties.

Analysis of initial sugar content detected by HPLC, as seen in Figures 19 and 20, showed how the highest sugar concentrations were found in series A. The standard deviation of series A was, however, the largest of all groups sampled. Slightly lower sugar concentrations found in series with larger autoclaved volumes is consistent with the increasing degree of browning observed. This overall trend seems to have been broken by the 10 L autoclavation through a small increase in sugar concentration. The visibly darker shade found in the 10 000 mL vessel clearly showed signs of increased browning and would be expected to be lower in sugar. It is possible that other compounds have participated to a greater extent in this medium. During the section *caramelization*, it was mentioned how ascorbic acid may also form brown polymers under thermal degradation. Another explanation could be variation in the concentration of these browning agents, including sugars. The large plastic container with ~17 L medium was shaken and tilted prior to moving medium into each respective autoclavation vessel with the intention of ensuring homogeneity. If this was not sufficient for a completely homogenous blend, it could explain the results. A small fraction of sedimented solids could have remained at the bottom parts despite our efforts to homogenize. Since the vessel used for autoclavation of 10 L was the same one used for storing the medium, the concentration of browning agents would increase in such a case. The larger standard deviation for 10 L autoclavation as compared to 5 and 1 L should also be kept in mind, as it indicates a wider spread on the data.

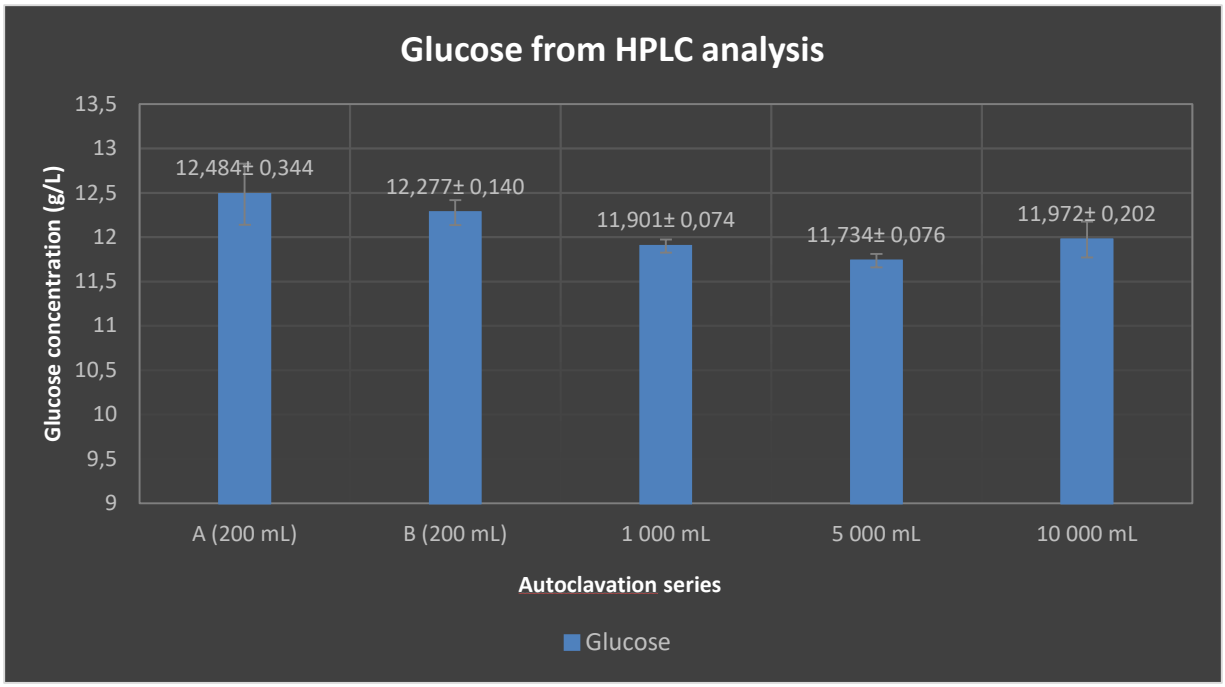


Figure 19. Average initial glucose concentration found in respective measurement series.

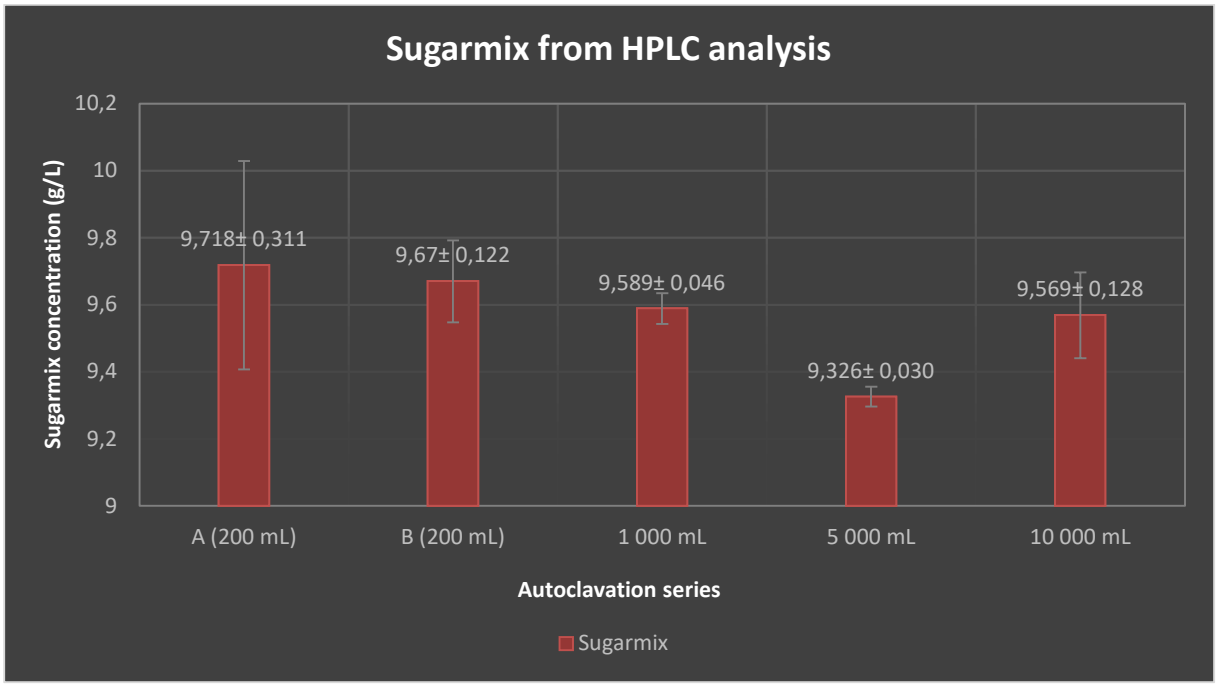


Figure 20. Average initial sugarmix concentration found in respective measurement series.

For shake flask cultivations no peaks from sugars could be detected at 24 h, which could imply it was all consumed by this time, therefore no sugar consumption profile could be graphed.

On Table 4 the yield of biomass/glucose and biomass/sugarmix is presented where series E shows the highest yield on both glucose and sugarmix. Series D shows the lowest yield in both cases.

Table 4. Yield of biomass based on initial sugars of series A-E

Series	Biomass concentration (g/L)	Glucose (g/L)	Sugarmix (g/L)	Yield (g biomass/ g sugars)
A	2,798	12,484	9,718	0,1260
B	2,486	12,277	9,670	0,1133
C	2,599	11,734	9,326	0,1234
D	2,380	11,972	9,569	0,1105
E	3,012	11,901	9,589	0,1402

4.2.2 Fungal cultivation in reactors

Just like the cultivations in the shake flasks, the biomass was grown in form of suspended mycelium. The amount of growth was the largest between 0 h and 18 h and slowed significantly during the following observations up until the harvest. Visually, more biomass had grown in reactor G than reactor F and is later confirmed by the wet weight measurements as shown in Table 5.

The calculated biomass concentration and subsequent yields for the reactors seemed to be an improvement over cultivation on 10 000 mL autoclaved medium in shake flasks, as shown in Tables 5 and 6. An issue with the data, however, is the high standard deviation of the dry to wet ratio in G. This deviation was found to be more than 8 times higher than the one found in the F series, likely owing to the one disproportionally high value. To avoid giving too much weight to such an outlier, this sample should be excluded.

Table 5. Biomass concentration of harvested biomass from reactor F and G.

Reactor	Wet weight	Average Dry/wet ratio	Approximate dry weight (g)	Total volume samples (L)	Biomass concentration (g/L)
F	58,7	0,1496	8,8715	3,5	2,509
G	76,8	0,2132	16,374	3,5	4,678
G	76,8	0,1581*	12,1421	3,5	3,469

*Deviant sample excluded

Table 6. Yield of biomass based on initial sugars of reactor F and G.

Reactor	Biomass concentration (g/L)	Glucose (g/L)	Sugarmix (g/L)	Yield (g biomass/ g sugars)
F	2,509	11,590	9,4415	0,1193
G	4,678	11,107	9,1455	0,2310
G	3,469*	11,107	9,1455	0,1713

*Deviant sample excluded

As previously mentioned, no peaks from sugars were detected at 24 h in the shake flask cultivations. A similar phenomenon was observed for some sugars in reactor cultivations but due to more frequent sampling, a profile of the decreasing sugars could be established as shown in Figures 21 and 22.

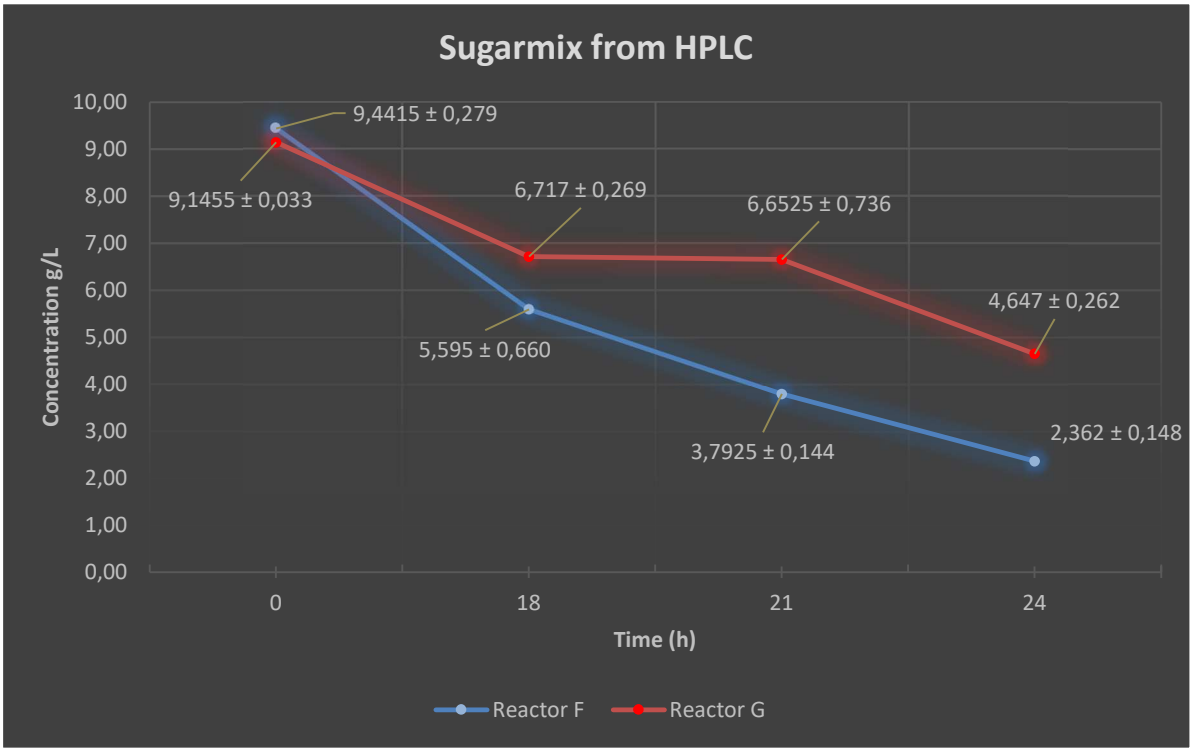


Figure 21. Average sugarmix concentration found in reactor F and G.

From the samples of reactor F, no glucose peaks were detected after the initial concentration sampling at the expected retention time. This could imply faster consumption rate, which seems somewhat consistent given the lower sugar mix concentrations found in Figure 21.

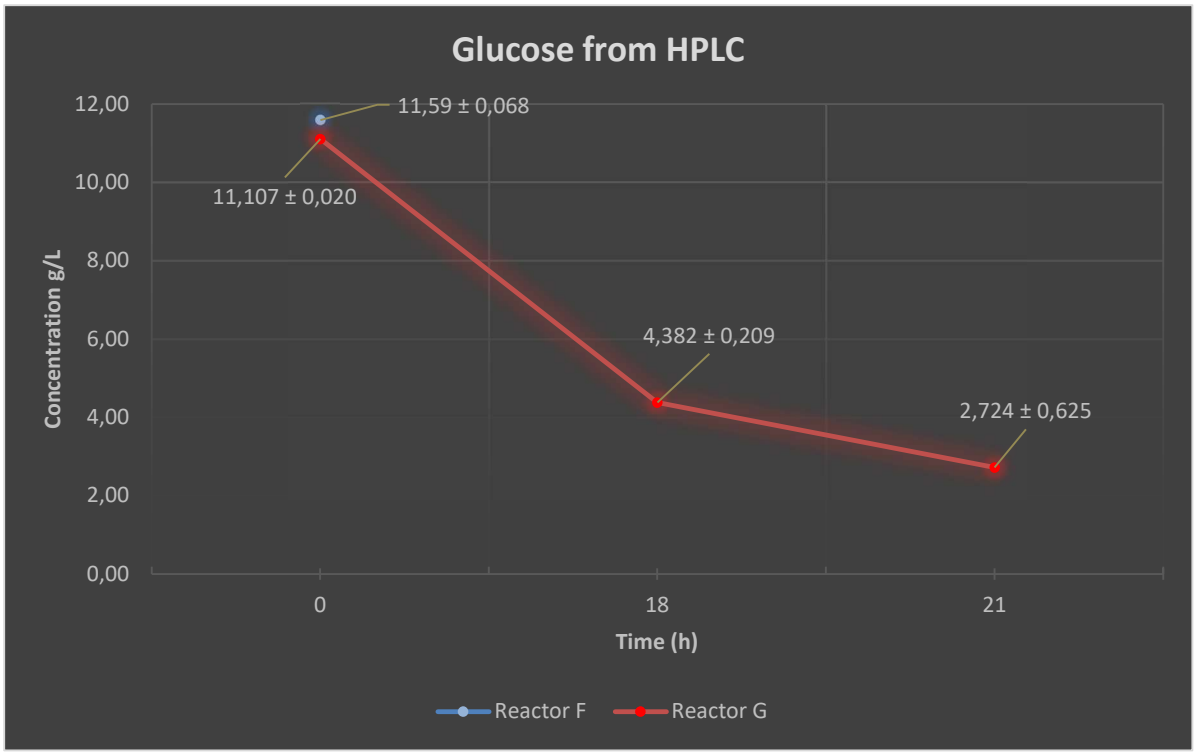


Figure 22. Average glucose concentration found in reactor F and G.

4.3 Significance testing

4.3.2 Analysis of significance testing

Most of the compared series of measurements did not cross the critical thresholds at an alfa value of 0,05 (95% confidence level), see Table E2 in appendix E. When comparing the data from the 1000 mL with any of the higher volumes, however, these critical levels were breached by a considerable margin. This would seem to imply dismissal of the null hypothesis, autoclaved volume does not affect fungal growth, is to be considered. Yet, it should be noted that the dry weights are derived from averaged dry per wet weight ratios from the respective series of cultivation. Application of this average, derived from 3 samples in a mix of the biomasses in the same series, could be questioned when done on individual wet samples. As mentioned previously, the standard deviation for the ratios of all higher volumes is larger than the averaged ratios from the 200 mL measurements. This could provide a source of error for the datapoints and leads to uncertainty of the findings as the true value of the dry weights could be closer to another.

The choice of test for hypothesis testing could be questioned. As mentioned by Winter in 2013, welch t test should not be conducted on small samples sizes without a violation of equality of variance and differing sample sizes. Furthermore, Winter mentions how the conventional t-test was designed with small sample sizes in mind by the originator of the test, William Sealy Gosset. For this reason, along with the increased error rates using welch t test for small sample sizes reported by Adusah & Brooks, the conventional t test was of preference. The interpretation

of what constituted violations in equality of variance and sample size could have been too lax. The only time an unequal variance t test was employed was when comparing A to C, at a variance ratio of above 2,3 and a 2:5 discrepancy in sample size. Another noteworthy candidate was when comparing B to C, at a variance ratio of above 1,6 and 3:5 discrepancy in sample size. Usage of Welch's t could have been merited in this case.

4.5 Comparison to other studies

Satari et al reported the preferred option for extracting sugars from citrus waste by a specific ratio of citrus waste to water. This ratio was found to be 3:5, favouring water. The ratio utilized for cultivations on the lemon medium was close to this ratio, about 10,3:14,7, which is roughly equivalent to 3,4:4,9. Recovery of sugars was found to be ranging from 19,7 to 30,3 g sugar per kg citrus waste. Since no HPLC sample was taken on the raw medium prior to the autoclavation, recovery for the present study is only an estimation. From lemon waste, an estimated 36,2 g sugar per kg lemon waste was obtained, see appendix E. This was unexpected as lemon had been characterized as on the lower end of citrus fruits in sugar content by Czech et al in 2021. Given this, the sugars were likely extracted to a larger extent with the comparatively longer pre-treatment. The biomass concentrations for reactor cultivations reported by the authors for *M. indicus* and *R. oryzae* were around 4,7 and 4,0 g/L respectively. Furthermore, their biomass yields ranged from 0,25-0,28 g / g consumed sugar. Yields and biomass concentrations on par with these were not achieved in the present study, about 0,12-0,17 g/g and roughly 2,5 to 3,5 g/L. Discounting the fact that the fungi used are not the same, the reactor chosen by Satari et al has its advantages over a bubble column, which was used in this study. Airlift reactors contain a draft tube and provides separation to up and downflowing streams. A more stable flow of liquid is achieved in airlift as compared to bubble column reactors and often improves the mixing (Doran 2012).

4.6 Discussion of results

The null hypothesis cannot be disproven without any reasonable doubt. There were significant differences in some the biomass data, but it could be slightly skewed since it is derived from a mean of the dry to wet ratios. Furthermore, the results from cultivation B in 200 mL autoclaved volume showed similar yields to the higher volumes autoclaved. Cultivation on remaining medium from 10 000 ml autoclavation in 4 L bubble column bioreactors ranged in yields. It ranged from around the same level as in shake flask to the highest yields encountered, seemingly proving how autoclaving medium in a larger vessel doesn't necessarily severely impact cultivation. Thermal degradation of sugars or other types of browning under sterilization of the medium may not be as relevant as expected, at least when the volume doesn't exceed 10 L.

In a study by Chen, Yang, Chen & Liu in 2009, fructose solutions of varying concentration were heated at different temperatures over extended periods of time. Their results showed very modest increases in the HMF formation at lower temperature in total and next to no increase in HMF for solutions with less than 1,11 M fructose for the first 20 hours at the highest temperature tested, 95°C. Given the relatively high molar mass for the sugars and the sugar concentrations found by HPLC, this high of a concentration is very unlikely to have been present. Even considering the general trend, as shown by Chen et al, that rate of formation for HMF increased with temperature, the low sugar concentration could greatly limit the speed of accumulation of these products. The long heating time required to start seeing any noticeable build-up of HMF, as shown by Chen et al, might mean the heating cycles for the volumes tested could possibly not have been long enough. A critical point where the formation of inhibitors became substantial enough to impact later cultivation was therefore not reached. Another possibility could be the resilience of the fungi used, *Rhizopus Delemar*, to HMF or other potentially growth impacting compounds formed.

5 CONCLUSION

The yield of the biomass in the shake flasks ranged from 0,11 – 0,14 g/g sugars in the tested ranges of 200 – 10 000 mL. Biomass concentrations from cultivation in shake flasks varied between about 2,4 to 3,0 g/L. Although some statistically significant differences were found for these values, there are factors that may have influenced or casts doubt on this result. Errors in the mean dry per wet weight ratio used to calculate dry weights, unequal variances and sample sizes to be compared, and a large discrepancy in yield for the two measurement series on 200 ml. Based on these findings the volume of autoclavation seems to not be too great of a concern when cultivating *R. Delemar* on lemon waste medium in the range of 200- 10 000 mL. As for other mediums richer in sugar, it is possible that a clearer divide emerges in this range of volumes.

Suggestions for further studies:

- How is the growth of *R. Delemar* affected by HMF?
- How much larger can the autoclaved volume be until the heat cycle becomes long enough to have a clear impact on growth for *R. Delemar*?
- Will the effects of autoclaved volume be more prevalent under cultivation on other citrus fruits?

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APPENDIX

APPENDIX A

The following tables shows the measurements and calculations made for this project.

Wet weight measurements for the calculations of the dry weight mean value

Table A1. Wet weight measurements of lemon waste with calculated mean value of dry weight/wet weight ratio.

Sample	Wet weight (g)	Dry Weight (g)	Dry weight/wet weight
1	4,2180	0,6026	0,142864
2	4,8900	0,7062	0,144417
3	5,6550	0,8415	0,148806
$\bar{x}$	-	-	0,145362

Calculations for 6 % lemon suspension in 25 kg medium

To get a 6 % lemon suspension in a 25 kg medium, 25000 g was multiplied by the fraction 6/100 to get a value of 1,5 kg of dry lemon.

$$\frac{6}{100} \cdot 25000 = 1500\ g\ \approx 1{,}5\ kg\ of\ dry\ lemon$$

This value was then multiplied by the mean value of dry weight measurements to get a value of 10319 g of wet lemon needed.

$$1500\ g\ dry\ lemon \rightarrow \frac{100\ g\ wet\ lemon}{14{,}53623\ dry\ lemon} \cdot 1500\ g\ dry\ lemon =$$

$$10319{,}04421\ g\ wet\ lemon$$

To get the amount of water needed the wet lemon value was deducted from the total weight of the medium, to get the value of 14681 g of water needed.

$$25000 - 10319 = 14680{,}955\ g\ water$$

Mixing of 65 L kettle

The kettle reached 45°C at 13:00 and was turned off at 15:00. The mixture at 12.45 was done while only reaching 41°C but was deemed necessary due to slow heating and minimise the chance of burning.

Table A2. Specific time of mixing the 65 L kettle

Mix	Time
1	12:45
2	13:00
3	13:15
4	13:32
5	13:47
6	14:03
7	14:19
8	14:35
9	14:51
10	15:02

Interpolation of evaporated volume

To get the estimated loss in volume of the 5000 mL bottle, the value had to be interpolated between the volume lost in the 1000 mL and 10 000 mL series. As the 1000 mL bottle lost approximately 65 mL and the 10 000 ml bottle lost approximately 1000 mL, the volume lost was calculated to 480, 5 mL.

$$\frac{1000 - 65}{10000 - 1000} \times 4000 + 65 = 480{,}5\ mL$$

Appendix B

pH measurements

Table B1. pH measurements series A - E

Autoclaved Volume	200 mL	200 mL	5000 mL	10 000 mL	1000 mL
Index series	A	B	C	D	E
1	5,12	5,12	5,12	5,10	5,09
2	5,07	5,10	5,14	5,09	5,14
3		5,09	5,10	5,11	5,15
4			5,13	5,11	5,16
5			5,14	5,07	5,15

Table B2. pH measurements for bubble column reactors F and G.

Sample (h)	F (pH)	G (pH)
18	4,85	4,87
21	4,83	4,68
24	4,75	4,74
24 (1st adjustment)	4,93	4,88
24 (2nd adjustment)	5,05	5,01
24 (3rd adjustment)	~ 5,20	~ 5,20
42	5,09	4,87
42 (adjustment)	5,21	5,18
45	5,27	5,22
48	5,26	5,21

Volumetric measurements

Table B3. Volumetric measurements series A - E

Autoclaved Volume	200 mL	200 mL	5000 mL	10 000 mL	1000 mL
Index series	A	B	C	D	E
1	188 ± 5	193 ± 5	202 ± 5	204 ± 5	201 ± 5
2	185 ± 5	198 ± 5	208 ± 5	203 ± 5	200 ± 5
3		192 ± 5	204 ± 5	205 ± 5	202 ± 5
4			204 ± 5	205 ± 5	201 ± 5
5			205 ± 5	204 ± 5	203 ± 5

Appendix C

Dry/wet weight measurements

Table C1. Measurements for liquid of strained lemon waste.

Sample ID	Weight cup (g)	Cup+ Wet weight (g)	Cup+ Dry weight (g)	Dry weight (g)	Ratio Dry/Wet weight
1	0,8444	5,7050	1,0319	0,1875	0,0386
2	0,8074	5,6361	0,9941	0,1867	0,0387
3	0,8487	5,2925	1,0209	0,1722	0,0387
$\bar{x}$	-	-	-	0,1821	0,0387

Table C2. 200 mL series final liquid A.

Sample ID	Weight cup (g)	Cup + Wet weight (g)	Cup+ dry weight (g)	Dry weight (g)	Ratio Dry/Wet weight
1	0,8427	4,4960	0,9915	0,1488	0,0407
2	0,8390	4,8887	0,9876	0,1486	0,0367
3	0,8365	5,0248	0,9387	0,1022	0,0244
$\bar{x}$	-	-	-	0,1332	0,0339
SD	-	-	-	-	0.0085

Table C3. 200 mL series final liquid B.

Sample ID	Weight cup (g)	Cup + Wet weight (g)	Cup+ dry weight (g)	Dry weight (g)	Ratio Dry/Wet weight
1	0,7986	4,9687	0,8975	0,0989	0,0237
2	0,7999	4,9974	0,8994	0,0995	0,0237
3	0,7971	4,6857	0,8892	0,0921	0,0237
$\bar{x}$	-	-	-	0,0968	0,0237

Table C4. 5000 mL series initial liquid.

Sample ID	Weight cup (g)	Cup + Wet weight (g)	Cup+ dry weight (g)	Dry weight (g)	Ratio Dry/Wet weight
1	0,8302	5,1440	0,9938	0,1636	0,0379
2	0,8422	7,1802	1,0820	0,2398	0,0378
3	0,8434	6,7485	1,0691	0,2257	0,0382
$\bar{x}$	-	-	-	0,2097	0,0379

Table C5. 5000 mL series final liquid.

Sample ID	Weight cup (g)	Cup + Wet weight (g)	Cup+ dry weight (g)	Dry weight (g)	Ratio Dry/Wet weight
1	0,8436	4,6695	0,9283	0,0847	0,0221
2	0,8434	4,7985	0,9311	0,0877	0,0222

3	0,8431	4,4139	0,9224	0,0793	0,0222
x̄	-	-	-	0,0839	0,0222

Table C6. 10 000 mL series initial liquid.

Sample ID	Weight cup (g)	Cup + Wet weight (g)	Cup+ dry weight (g)	Dry weight (g)	Ratio Dry/Wet weight
1	0,8084	3,6755	0,9174	0,1090	0,0380
2	0,8329	4,2115	0,9613	0,1284	0,0380
3	0,8392	3,5810	0,9433	0,1041	0,0380
x̄	-	-	-	0,1138	0,0380

Table C7. 10 000 mL series final liquid.

Sample ID	Weight cup (g)	Cup + Wet weight (g)	Cup+ dry weight (g)	Dry weight (g)	Ratio Dry/Wet weight
1	0,8197	4,3041	0,8989	0,0792	0,0227
2	0,8364	4,5132	0,9199	0,0835	0,0227
3	0,8351	3,8006	0,9252	0,0901	0,0304
x̄	-	-	-	0,0843	0,0253

Table C8. 1000 mL series initial liquid.

Sample ID	Weight cup (g)	Cup + Wet weight (g)	Cup+ dry weight (g)	Dry weight (g)	Ratio Dry/Wet weight
1	0,7989	3,2744	0,8934	0,0945	0,0382
2	0,8414	3,2876	0,9350	0,0936	0,0383
3	0,8077	3,8397	0,9235	0,1158	0,0382
x̄	-	-	-	0,1013	0,0382

Table C9. 1000 mL series final liquid.

Sample ID	Weight cup (g)	Cup + Wet weight (g)	Cup+ dry weight (g)	Dry weight (g)	Ratio Dry/Wet weight
1	0,8404	4,4834	0,9220	0,0816	0,0224
2	0,8157	5,0600	0,9109	0,0952	0,0224
3	0,8324	5,1574	0,9296	0,0972	0,0225
x̄	-	-	-	0,0913	0,0224

Table C10. Reactor F final liquid.

Sample ID	Weight cup (g)	Cup + Wet weight (g)	Cup+ dry weight (g)	Dry weight (g)	Ratio Dry/Wet weight
1	0,8266	4,2379	0,9046	0,0780	0,0229
2	0,8237	4,3761	0,9045	0,0808	0,0227
3	0,8191	5,4935	0,9258	0,1067	0,0228

x̄	-	-	-	0,0885	0,0228

Table C11. Reactor G final liquid.

Sample ID	Weight cup (g)	Cup + Wet weight (g)	Cup+ dry weight (g)	Dry weight (g)	Ratio Dry/Wet weight
1	0,8249	4,1078	0,9051	0,0802	0,0244
2	0,8285	4,6656	0,9223	0,0938	0,0244
3	0,8268	4,1349	0,9075	0,0807	0,0244
x̄	-	-	-	0,0849	0,0244

Table C12. Average consumed substrate per solids in media

Shake flask cultivations	A	B	C	D	E
Consumed substrate/ Solids in media	0,124*	0.388	0,414	0,334	0,414
Reactor cultivations	G	F	-	-	-
Consumed substrate/ Solids in media	0,358	0,400	-	-	-

* Large standard deviation in dry/wet ratio

Appendix D

Biomass measurements

Table D1. 200 mL series biomass A.

Sample ID	Weight cup (g)	Cup + Wet weight (g)	Cup+ dry weight (g)	Dry weight (g)	Ratio Dry/Wet weight
1	0,8433	1,3717	0,8889	0,0456	0,0863
2	0,8261	1,2494	0,8572	0,0311	0,0735
3	0,8394	1,1683	0,8692	0,0298	0,0906
x̄	-	-	-	0,0355	0,0835
SD	-	-	-		0,0073

Table D2. 200 mL series biomass B.

Sample ID	Weight cup (g)	Cup + Wet weight (g)	Cup+ dry weight (g)	Dry weight (g)	Ratio Dry/Wet weight
1	0,7983	1,2486	0,8418	0,0435	0,0966
2	0,8009	1,2738	0,8417	0,0408	0,0863
3	0,7986	1,3474	0,8414	0,0428	0,0780

x̄	-	-	-	0,0424	0,0870
SD	-	-	-		0,0076

Table D3. 1000 mL series biomass E.

Sample ID	Weight cup (g)	Cup + Wet weight (g)	Cup+ dry weight (g)	Dry weight (g)	Ratio Dry/Wet weight
1	0,8215	1,2871	0,8800	0,0585	0,1256
2	0,8155	1,4688	0,8938	0,0783	0,1199
3	0,8167	1,2435	0,8828	0,0661	0,1549
x̄	-	-	-	0,0676	0,1335
SD	-	-	-		0,0153

Table D4. 5000 mL series biomass C.

Sample ID	Weight cup (g)	Cup + Wet weight (g)	Cup+ dry weight (g)	Dry weight (g)	Ratio Dry/Wet weight
1	0,8302	1,1886	0,8686	0,0384	0,1071
2	0,8246	1,2652	0,8607	0,0361	0,0819
3	0,8234	1,2692	0,8609	0,0375	0,0841
x̄	-	-	-	0,0373	0,0910
SD	-	-	-		0,0114

Table D5. 10 000 mL series biomass D.

Sample ID	Weight cup (g)	Cup + Wet weight (g)	Cup+ dry weight (g)	Dry weight (g)	Ratio Dry/Wet weight
1	0,8360	1,2213	0,8782	0,0422	0,1095
2	0,8262	1,3984	0,8946	0,0684	0,1195
3	0,8229	1,4586	0,8811	0,0582	0,0916
x̄	-	-	-	0,0563	0,1069
SD	-	-	-		0,0116

Table D6. Reactor F biomass

Sample ID	Weight cup (g)	Cup + Wet weight (g)	Cup+ dry weight (g)	Dry weight (g)	Ratio Dry/Wet weight
1	0,8224	1,6636	0,9535	0,1311	0,1558
2	0,8203	1,4761	0,9173	0,0970	0,1479
3	0,8210	1,5664	0,9291	0,1081	0,1450
x̄	-	-	-	0,1121	0,1496
SD	-	-	-		0,0056

Table D7. Reactor G biomass

Sample ID	Weight cup (g)	Cup + Wet weight (g)	Cup+ dry weight (g)	Dry weight (g)	Ratio Dry/Wet weight
1	0,8239	1,2701	0,8950	0,0711	0,1593
2	0,8229	1,4877	0,9272	0,1043	0,1569
3	0,8259	1,6396	1,0223	0,1964	0,2414
x̄	-	-	-	0,1239	0,2132
SD	-	-	-		0,0481

Spore counting

Table D8. Spore counts for shake flasks.

ID	1/10	1/100
1	18	7
2	15	15
3	22	16
4	14	15
5	7	17
x̄	14,6	

Calculation for the 4 mL spore suspension with a mean value of 14,6, gave 58,4 million spores.

$$\frac{14{,}6 \cdot 10^2\ (Spores)}{0{,}1\ (mm^3)} \cdot 4000(\mu l) = 58{,}4 \cdot 10^6\ Spores$$

Table D9. Spore counts for scale up reactors.

ID	1/10	1/100
1	44	9
2	23	4
3	39	5
4	28	7
5	27	3
x̄	18,9	

Calculation for the 70 mL spore suspension with a mean value of 18,9, gave the number of 1,3 billion spores.

$$\frac{18{,}9 \cdot 100\ (spores)}{0{,}1(mm^3)} \cdot 70\ 000(\mu l) = 1{,}323 \cdot 10^9\ spores$$

Appendix E

Sugar concentrations and estimated sugar recovery

Table E1. Initial sugar concentration quantified by HPLC & standard deviations

Group index	A	B	C	D	E
Glucose sample 1 (g/L)	12,243	12,376	11,681	11,830	11,849
Glucose sample 2 (g/L)	12,729	12,178	11,788	12,116	11,954
Glucose mean (g/L)	12,484	12,277	11,734	11,972	11,901
SD (g/L)	±0,344	±0,140	±0,076	±0,202	±0,074
Sugarmix sample 1 (g/L)	9,5	9,757	9,305	9,499	9,537
Sugarmix sample 2 (g/L)	9,94	9,584	9,347	9,68	9,602
Sugarmix mean	9,718	9,67	9,326	9,589	9,569
SD	±0,311	±0,122	±0,030	±0,128	±0,046

Average sugar concentrations from B were used to estimate sugar concentration in raw medium as 200 ml autoclavation were shorter and concentrations found in B has lower standard deviations.

Calculation of sugar recovery were based on the roughly 17 L medium obtained in total and the weight of wet lemon waste, about 10,3 kg.

$$\frac{17(L\ medium)\cdot(12{,}277+9{,}67)(\frac{g}{L}\ sugars)}{10{,}3\ (kg\ lemon\ waste)} \approx 36{,}2\ \text{g sugar /kg lemon waste}$$

Significance testing

Table E2. Results from t tests using analysis ToolPak.

Series tested	t Stat	P(T<=t) one-tail	t Critical one-tail	P(T<=t) two-tail	t Critical two-tail	Ratio variances
A vs B	1,603	0,104	2,353	0,207	3,182	1,393
A vs E	1,037	0,174	2,015	0,347	2,571	1,148
A vs C	1,067	0,182	2,353	0,364	2,306	2,322
A vs D	2,232	0,038	2,015	0,076	2,571	1,228
B vs E	0,575	0,293	1,943	0,586	2,447	1,213
B vs C	0,575	0,293	1,943	0,586	2,447	1,667
B vs D	0,468	0,328	1,943	0,656	2,447	1,134
E vs C	2,359	0,023	1,86	0,046	2,306	2,022
E vs D	4,276	0,001	1,86	0,003	2,306	1,070
C vs D	1,166	0,139	1,86	0,277	2,306	1,890

HÖGSKOLAN
I BORÅS

Besöksadress: Allégatan 1 · Postadress: 501 90 Borås · Tfn: 033-435 40 00 · E-post: registrator@hb.se · Webb: www.hb.se