UPSCALING PRODUCTION OF 2,3-BUTANEDIOL BY *BACILLUS AMYLOLIQUEFACIENS* UTILISING WASTE WASHING TREATMENT EFFLUENT FROM MUNICIPAL SOLID WASTE AS SUBSTRATE

Adhitya Prayoga - 70455878

Robert Walpole - 71455881

Ostfalia University of Applied Sciences

Tampere University of Applied Sciences

Examining professors:

Prof. Dr. Ing. Thorsten Ahrens

Prof. Dr. Ekkehard Boggasch

Supervisors:

Dipl.-Ing. Sylvia Drescher-Hartung, MBA

Faika Bozankaya, B.Eng., MRes

Declaration of Authorship

We, Adhitya Prayoga & Robert Walpole, hereby certify that this thesis has been composed by us, that it is the record of work carried out by us and that it has not been submitted in any previous application for a Bachelor's degree. This project was conducted by us at the Ostfalia University of Applied sciences from 05/2017 to 07/2017 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 & Prof. Ekkehard Boggasch.

Date: _____ Signature of candidate: _____

Date: _____ Signature of candidate: _____

Acknowledgements

Adhitya Prayoga

I would like to express my thanks to a good friend and my co-author, Robert Walpole, whose exceptional work ethics had inspired and contributed in the writing of this thesis. My parents, Jatmiko Danurejo and Tri Asih Istiani, for their lessons in persistence and perseverance which I applied throughout my life. My best friend and love, Riena Juliannisa Surayuda, whose moral and emotional support had always helped me through the tough times. And most importantly, to me; you did it!

Robert Walpole

Many thanks to Adhitya Prayoga, it has been an immense pleasure working with somebody with such an enormous work ethic, it also stands to your character that we somehow didn't manage to get bored of each other's company during the course of the year; "Grandison for life". To Erin, Sanna & my family: thank you all for the love & support that has gotten me through my time apart from you all. Finally, to me; good job!

From both authors

Thanks to Thorsten, Silvia & Jens, whose guidance, support & enthusiasm was essential to the development of this paper. Special thanks to Faika, who has gone above and beyond her role as supervisor, due to her this paper appears far more professional that it would have without her specialised knowledge, attention to detail & constant nagging.

Abstract

The hypothesis that 2,3-Butanediol can be produced by *Bacillus amyloliquefaciens*, using water used to wash municipal solid waste was first investigated by Liebig & Gerlitz. Their work gave promising results and was a prime candidate to be replicated and verified, which was done by a project team. The team went further and included up-scaling as a part of their scope. This current paper implements their recommendations and further strives to increase the yield by methodological improvement, while continuously increasing the process scale.

Results were successful with a yield of 295,66 mg l-1 2,3-butanediol achieved using a facility with a maximum TRL level of 4. The timing of extraction is crucial due to the reverse transformation of 2,3-butanediol to acetoin. Several sources of inhibition were identified, especially pH, and these must be considered in future production. The current maximum yield was not achieved under ideal parameters, thus leaving room for the further exploration of upscaling under optimum conditions to improve yield and volumetric production.

Table of Contents

1. Introduction

The thesis works detailed hereafter shows the methods and results whereby it was aimed to upscale the production of 2,3-butanediol (2,3-BD) from the water created when municipal solid waste (MSW) is washed. This was done utilising the metabolic pathway of the bacteria: *Bacillus amyloliquefaciens*. Similar work was previously done once at flask scale (Gerlitz, 2017; Liebig, 2017), to verify the hypothesis that this was possible, and a replication work undertaken to conclusively verify those thesis works (Walpole, et al. 2017), it is from this point that the current paper continues with upscaling.

1.1 2,3-Butanediol

Biotechnological production of bio-based chemicals using renewable materials instead of fossil-based sources are experiencing an increase in interest due to various policy incentives at different levels (Hatti-Kaul, et al. 2007). One of these bio-based chemicals that is sought after commercially is 2,3-BD. 2,3-BD is a colourless and odourless liquid with various industrial applications. The first recorded industrial interest of 2,3-BD dates to World War II as an organic intermediate for synthetic rubber production (Fulmer, Christensen and Kendali 1933). Currently, 2,3-BD garners commercial interest as a bio-fuel with a heating value of 27.2 kJ g^{-1}, comparable to other renewable liquid fuels such as methanol (22.1 kJ g^{-1}) and ethanol (29.1 kJ g^{-1}) (Flickinger 1980). Other popular uses of 2,3-BD include use as antifreeze agents (utilising the low freezing point of -60 °C), flavouring agents (the diacetyl formed gives a “buttery” flavour), high quality aviation fuel, and potential applications for pharmaceuticals, perfumes, explosives, printing inks, and plastics (Garg and Jain 1995)

1.2 Bacillus amyloliquefaciens

In the interest of economic gain and ease of complexity, biotechnological production of 2,3-BD can be perceived as more attractive when compared to the traditional chemical production method (Rehm 1970). Several different microorganisms have previously been identified as 2,3-BD producers. From this group of microorganisms, only a few had the ability to produce it in substantial quantities. One of them, *Bacillus amyloliquefaciens*, shows promise in industrial 2,3-BD production with a yield of 0,33 g 2,3-BD per 1g glucose (Ji, Huang and Ouyang 2011). It is favourable and safer to work with *Bacillus amyloliquefaciens* due to the GRAS (Generally Regarded as Safe) status of the bacteria when compared to some of the other 2,3-BD producers such as *Klebsiella oxytoca* or *Enterobacter aerogenes* which are regarded as class 2 (pathogenic) microorganisms (Yang, Rao, et al., Production of 2,3-butanediol from glucose by GRAS microorganism Bacillus amyloliquefaciens 2011). In addition, unlike other Bacillus species, *Bacillus amyloliquefaciens* can be considered advantageous in terms of industrial production since it does not produce undesirable by-products such as glycerol and ethanol in substantial quantities, indicating a more efficient carbon utilisation and requiring a simpler down streaming process (Alam, et al. 1990).

1.3 Utilisation of municipal solid waste

Since the implementation of a total ban on non-pre-treated MSW to landfills in Germany on the 1st of June 2005 (Fischer 2013), interest on research of treatment methods of MSW has risen. Even more recently, with the expiration of the transitional period of the EU Landfill Directive (Directive 1999/31/EC) in 2009, strict limits on organic substance concentrations in landfill disposal is pushing more research in recovering nutrients from waste (Umweltbundesamt 2016). One of the requirements of the German Closed Cycle Management Act was to separate recoverable

substances (i.e. nutrients such as organic carbon, nitrogen, etc.) from the waste before landfilling (Nelles, Grünes and Morscheck 2016). While one of the numerous aims of this piece of legislation is to reduce the pollution from leachate emission, this created a response from the academic world which could be observed in the rise of utilising waste to produce useful products.

One such research involves the washing of waste to reduce the leachable fraction of the waste before landfilling (Cossu and Lai, Washing of Waste Prior to Landfilling 2012). Further research indicates that this "waste washing treatment" (WWT) was observed to remove over 65% of the dissolved organic carbon (DOC), chemical oxygen demand (COD), and Total Kjeldahl Nitrogen (TKN) from the MSW fraction (Cossu, Lai and Pivnenko, Waste washing pre-treatment of municipal and special waste 2012). Research on utilising waste as a substrate in fermentative biotechnological production of biofuels is not a totally ground-breaking idea as exemplified by several papers (Feng, et al. 2014) (Jain, et al. 2016) (Mozumder 2015). If the removed DOC, COD and TKN are present in the WWT effluent, the potential of the effluent as a substrate for the biotechnological production of a useful product (i.e. 2,3-BD) using a suitable microorganism (i.e. *Bacillus amyloliquefaciens*) is promising.

This study assesses the efficiency of nutrient transfer from solid waste to the wash water effluent, the utilisation of said effluent as a substrate to biotechnologically generate valuable biofuel using a GRAS microorganism as a major continuation of other works conducted previously (Gerlitz, 2017; Liebig, 2017), and expands to assessing the feasibility of upscaling the process for economic gains.

The research was conducted in the laboratory facilities of the environmental engineering department of the faculty of supply engineering of Ostfalia University of Applied Sciences as well as the pilot plant facility belonging to the same institution located in Ilsede.

To physically assess the success of the upscaling attempts, the same methods of testing were employed as in earlier papers using the same substrate type (Walpole, et al. 2017) Biomass concentration was observed and charted via obtaining an optical density (OD) value, similarly the health of the biomass was visually inspected by microscopy with 3 assessment parameters, firstly to determine the activity of the biomass, secondly to determine the density of the biomass and also finally to show that no contamination by other microbes had occurred. Having ensured the biomass was in good physical standing, the product generation then needed to be determined, this was done in 2 separate yet conjoined stages by a GCMS machine. The GC (gas chromatography) identifies the products by vaporisation and separation, and then the subsequent analysis and quantification of the products in the MS (mass spectrometry). Another method of testing undertaken to monitor the operational conditions was measurement of the pH. A pH of under 4 would be lethal to the bacteria, who have an operational range of pH 5.5 to 6.5 and an optimal activity value of 5.9 (Welker and Campbell 1967).

2. Materials and Methodology

2.1 Microorganism

The microorganism used throughout these experiments was *Bacillus amyloliquefaciens*, strain ATCC 23350. The microorganism was maintained in a stock culture of 50 ml volume in a flask and was cultivated in a 36 °C IKA KS 3000i control incubator and agitated at 100 rpm. The stock culture was renewed at least every week and inoculated with a ratio of 1:50 (v/v ratio) from the previous stock culture. In addition to this stock culture, single strike colonies were plated and incubated at 36 °C for 24 hours, and then kept in a refrigerator at 8 °C.

2.2 Substrate

2.2.1 Stock culture

The medium for the stock culture was composed of 5 g l^{-1} peptone (VWR Chemicals: Peptone from meat), 2,5 g l^{-1} yeast extract (AMRESCO: Yeast Extract, Bacteriological), 2 g l^{-1} NaCl (Safrisalz: “Tafel Salz”), and 60 g l^{-1} household sugar (Gut & Gunstig: “Feiner Zucker”). The ingredients were weighed using a Sartorius BP 300 S scale and mixed with an IKAMAG RCT magnetic stirrer until dissolved. The medium was autoclaved to ensure sterilisation in a Systec DE-65 autoclave device held at 121°C for a duration of 15 minutes at a pressure of 2 atm.

2.2.2 Agar plates

The agar substrate was prepared as described above with the addition of 12 g l^{-1} agar (AMRESCO: Agar, Bacteriological). After sterilisation, while the liquid was still warm and thus viscous it was poured into empty plastic petri dishes and allowed to solidify upside down in order to prevent condensation forming on the agar. The agar plates were created to allow inoculation of a new culture from a single unstressed colony if anything should negatively affect the stock culture.

2.2.3 Molasses medium

For the biotechnological fermentation, medium was prepared as described above however with the replacement of the sugar content with 60 g l^{-1} molasses (Nordzucker: Melasse).

2.2.4 Waste Washing Treatment Effluent

2.2.4.1 Waste sampling

Waste was obtained from the Entsorgungszentrum GmbH Salzgitter centre, see: Picture 1. The sampling was done three days before the first waste washing treatment process. At the waste management centre, the following safety precautions were followed: breathing masks, protective footwear, reflective safety vests and protective gloves were worn. Care was also taken to avoid any hazardous waste such as medical waste, toxic or sharp items. The waste was collected with a pitchfork and stored in sealable containers, with a storage volume of approximately 60 litres. The container was filled with approximately 10 kg of MSW. The sample was kept in the same airtight container, and was stored in a basement room with no sunlight exposure and a relatively cool temperature.

Picture 1. The MSW from which the sample was taken from

2.2.4.2 Waste Washing Treatment Effluent Production

Approximately 1 kg of waste was weighed using a scale (Sartorius BP300S). It was placed into a 20-litre bucket with 10 mm Ø holes drilled to the base, which was then placed atop a frame above a 60 litre volume fish crate. The number of holes is dependent on the diameter of the container; however, it should be sufficient to allow drainage of the wash water without filling the container, this will be assisted by manual agitation of the waste while washing to move obstructions to the drainage holes. The base crate was then filled with 25-litres of water into which the pump (Hellweg Basic SP3230) and piping were connected and inserted. The piping was directed to the waste and the pump activated. It was washed for a duration of 5 minutes while being stirred manually with a trowel, as in accordance with results from previous works (Walpole, et al. 2017). The effluent was then passed through a 2mm sieve to protect the bioreactors mechanical parts from erosion and sampling tubes from particulate clogging.

2.3 Analytical methods

2.3.1 Biomass concentration via optical density

Optical density (OD) was measured using a spectrophotometer (UV/Vis spectrometer UV2). The absorbance was set at 620 nm. Dilutions of 1:2, 1:5, 1:10 and 1:20 were made on both controls and samples when needed for a base comparison to be deducted against samples taken. The reason for dilution is that the machine is only accurate to a maximum result of 1.000, anything higher requires a dilution to achieve a value under this figure. This method of testing was a continuation of that undertaken by (Walpole, et al. 2017), which quantifies biomass in a sample by hindering the light passing through a cuvette, with more hindrance relative to a higher density of the biomass in suspension. As it was a method used previously it also allowed for ease of comparison of results of previous tests.

2.3.2 pH measurement

The acidity/alkalinity levels of the samples were measured using a pH meter (WTW pH 3210). The pH meter was calibrated using two pH buffers (WTW; 4,01 pH buffer TEP 4 & 7,00 pH buffer TEP 7). Three different samples were measured separately and the average of the results was calculated. Results taken from the bioreactor were mathematically adjusted to correlate to those of the pH meter in the lab as there was a constant discrepancy of +0.36pH between readings taken from the bioreactor when compared to those of the pH meter.

2.3.3 Product generation via GCMS

2.3.3.1 Sampling

A volume of 2 ml was taken from the various testing setups and placed into a 2ml reaction cap. The reaction caps were then centrifuged (Eppendorf model: 5424 R) for 5 minutes at 15000 rpm. The supernatant of the samples was then filtered using a 0,45µm diameter filter to remove any remaining microorganisms in suspension and then placed into the GCMS vials for testing. For storage, the samples were frozen at -18°C until testing if it could not be done immediately.

2.3.3.2 GCMS method settings

Product generation analysis was done via GCMS (Shimadzu GCMS-QP2010 Plus, Column: Stabliwax-DA capillary column (26.9m, 0.32mm., 0.5 µm film thickness; Restek). The method settings of the GC and the MS can be found on tables 1 and 2 respectively. The oven temperature profile can be found on table 3. The 2,3-BD concentration was determined by calculating the area under the peaks of the isomers of the compound. A dilution concentration of 0.5 g l^{-1}, 1 g l^{-1}, and 2.5 g l^{-1} 2,3-BD was used to determine the concentration curve from the GC-chromatogram.

Table 1. *Gas chromatography method settings*

Column oven temperature (°C)	Injection temperature (°C)	Injection mode	Sampling time (min)	Carrier gas	Flow control mode
65.0	150.0	Split	1.00	Helium	Linear velocity

Pressure (kPa)	Total flow (mL min^{-1})	Column flow (mL min^{-1})	Linear velocity (cm sec^{-1})	Purge flow (mL min^{-1})	Split ratio
19.7	38.5	1.69	50.2	3.0	20.0

Table 2. *Mass spectrophotometry method settings*

Ion source temperature (°C)	Interface temperature (°C)	Solvent cut time (min)	Detector voltage	Start time (min)	End time (min)
200	250	1.5	Relative to the Tuning Result	2.00	33.0

Acquisition mode	Event time (sec)	Scan speed	Start m/z	End m/z
Scan	0.50	333	40.00	200.00

Table 3. *GC-MS oven temperature profile*

Stage	Rate (°C min^{-1})	Final Temperature (°C)	Holding time (min)
0	-	65.0	2.00
1	10.00	70.0	5.00
2	10.00	100.0	5.00
3	60.00	250.0	15.00

2.3.4 Biological activity evaluation via microscopy

To evaluate the activity of the microorganism and determine whether contamination had occurred, samples were observed under a microscope (Zeiss primastar 104799). Approximately

20µl of the sample was placed on top of a glass slide and covered by a slide cover. The microscope was connected to a monitor for ease of group observation. The density, shape and motility of the microorganisms was discussed and evaluated by the two authors and expert supervisors.

2.4 Fermentation methods

2.4.1 Flask scale

Flask scale fermentation occurred inside 100 ml Erlenmeyer flasks with a working volume of 50 ml of the various sterilised substrates, to allow for mixing and aeration during agitation on the incubation plate. For inoculation, 1 ml (1:50 inoculum to substrate ratio) was taken from the stock culture with the inoculum aged 24-28 hours. The flasks were then incubated at 36 °C and shaken at 100 rpm. Samples were taken at the 0, 3, 24, 26, 48 and 50-hour mark using a sterile pipette.

2.4.2 Lab scale

Fermentation at lab scale was done inside a 15-litre bioreactor (Sartorius biostat C-plus) with a working volume of 8-litres of the different substrates. Sterilisation was done via the pre-installed sterilisation programme of the bioreactor. An inoculum of nutrient broth, also known as stock culture, of 160 ml volume (to maintain a 1:50 inoculum to substrate ratio) was prepared to inoculate the bioreactor with an inoculum age of 24-28 hours. Fermentation conditions were: 100 rpm agitation, an aeration flow rate of 20-litres/minute (for cycle 1 aerobic conditions) and a temperature maintained at 36 °C. Similarly, to the samples taken at the flask scale, samples were taken at the 0, 3, 24, 26, 48 and 50-hour mark using the built-in sampling port, which would first be sanitised with steam for a minimum 1 minute duration.

2.4.3 Pilot plant scale

The pilot scale fermentation was performed in a 75-litre bioreactor (AMS Technology, Vorcarburierkessel 75L) with a working volume of 60-litres. The plant facility has a TRL (Technology readiness level) of 4. The substrate was transported to the facility not more than two hours after the washing process as described above and was immediately sterilised in-situ to avoid severe pH inhibition as had been observed to occur in previous tests (see: Section 4.2 Cycle 2). A 1.5-litre dose of inoculum the same as used previously was used for inoculation (maintaining the 1:50 inoculum to substrate ratio in all fermentation scales). Sterilisation was automated, the temperature of the reactor was set to 150°C to ensure that the substrate reached a temperature of 121 °C which was then maintained for 20 minutes. The heating was then turned off and the reactor was allowed to cool. The fermentation was done without aeration, at 36°C and with a stirrer speed of 25% of the max 260rpm (Tschamber – custom manufactured motor). Sampling was done at the 22-hour mark. Due to the complexity of the systems piping and valve layout to the sampling port, more regular sampling could not be undertaken due to the amount of volume lost during the process, 17-litres of volume plus the sample quantity would be lost during each individual sampling.

2.5 Cycles

2.5.1 Cycle 1

The first stage of the research aimed to investigate and document the behaviour of *Bacillus amyloliquefaciens* in respect to the production of 2,3-BD using a conventional substrate (molasses) in a lab scale (8-litre substrate volume in a bioreactor). Investigation of the fermentative process includes determination of the microorganism's growth curve, aerobic/anaerobic demand, product generation curve, fermentation period in which maximum product concentration occurs and generated by-products. A comparison of the results obtained would be made against those found

during the literature research to ensure that the optimum results were being achieved via correct implementation of methodology and technology.

2.5.2 Cycle 2

After documenting the production process of 2,3-BD using a conventional substrate, the feasibility of using WWT effluent from MSW was investigated in cycle 2 and compared to those results achieved in the previous cycle. The experiments conducted were done in parallel in both flask scale and lab scale. Having learnt that there was no noteworthy change in the rate of increase of biomass concentration from the first cycle coupled with the fact that fermentation of 2,3-BD occurred in anaerobic state, it was decided that the lab scale would be trialled fermenting in an anaerobic state from the very beginning. The flask scale on the other hand, was done in aerobic state since it was easier for sampling, with a lesser chance of contamination. The sampling times were decided based on the results of the previous cycle and optimum 2,3-BD concentration per literature review discussed in the “Cycle 1” section of the “Discussions and outlook” chapter and observed in Figure 7.

2.5.3 Cycle 3

Due to the failure of the lab scale from cycle 2 discussed in Section 4.2 cycle 2, cycle 3 re-examines the fermentation process with the exact same setup, with the addition of an initial pH control. Since it had been confirmed that immediate sterilisation was needed to stop the decrease of pH level of the substrate, the WWT effluent was sterilised straight after production. In addition, flask scale testing was conducted in parallel. As the previous flask scale experiment was a success, this cycle was done anaerobically by introducing a paraffin layer into the flask that floats due to difference in density, on top of the inoculated substrate and prevented oxygen from diffusing into the medium.

2.5.4 Cycle 4

With the success of the previous cycle, it was decided to begin the next level of upscaling in a 75-litre bioreactor, with a working volume of 60-litres. All parameters were kept the same as previous cycles dictated were successful. Stirrer speed as mentioned above was set to 25% of the max 260rpm, thus 65rpm. This speed was chosen to ensure similar conditions as found in the 15-litre lab scale bioreactor and thus not subjecting the bacteria to excess shear stress during mixing. No mathematical calculations were done to arrive at this value however as it was based upon the advice of the facilities manager, who was also familiar with the smaller scale bioreactor. Due to the location of this bioreactor away from the main lab location, regular observation of the parameters could not be maintained.

3. Results

3.1 Cycle 1

3.1.1 OD

The biomass concentration curve of *Bacillus amyloliquefaciens* fermentation using molasses as substrate can be seen in Figure 1. It can be noted that growth is apparent inside the 8-litre working volume of the fermentation from the observable increase in OD values. This growth occurs even though dissolved oxygen (DO) levels were at 0% after around 5-hour mark. Analysis of this result was backed up by several literature results and is discussed in this paper in Section 4.1 Cycle 1.

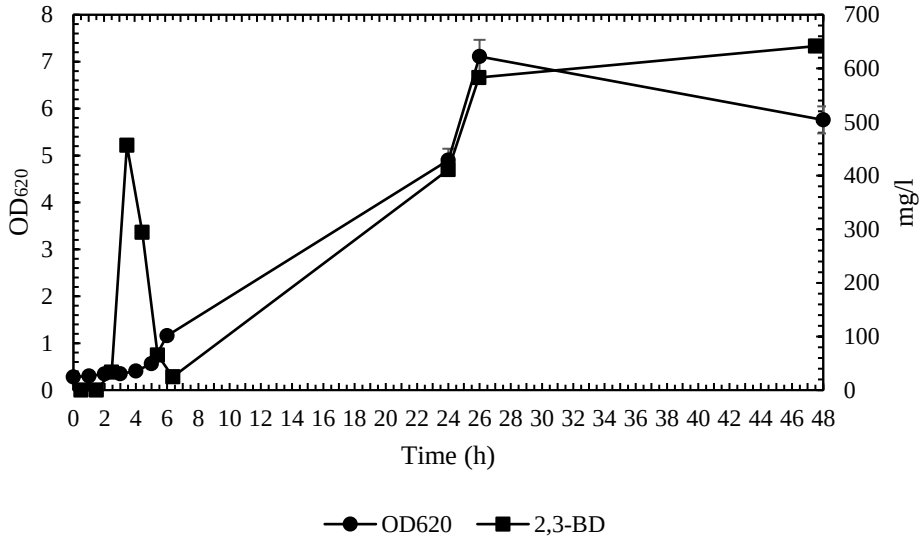


Figure 1. OD620 and 2,3-BD concentration results from cycle 1

3.1.2 GC/MS

The product, 2,3-BD, generation over time can be seen in Figure 1, as can be seen above. It can be observed that the maximum concentration of 2,3-BD was 641,45 mg l^{-1} at the 48-hour

mark. However, the steepest increase occurred at the 24 to 26-hour mark where the 2,3-BD concentration increased from 411,17 mg l^{-1} to 582,88 mg l^{-1}.

3.2 Cycle 2

3.2.1 Flask scale

3.2.1.1 OD and pH

Figure 2 shows the change of the biomass concentrations and pH levels at the flask scale 2,3-BD fermentation using WWT effluent. It can be observed that the biomass concentration experienced a growth curve with maximum biomass concentration at around the 28-hour mark. After that point, the biomass concentration decreased rapidly until the test ended at the 50-hour mark.

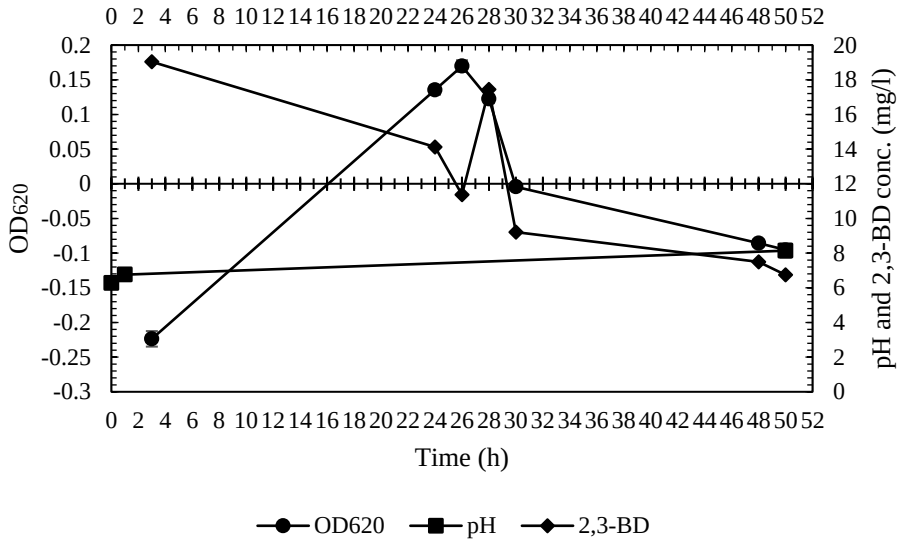


Figure 2. OD620, pH and 2,3-BD concentration results in the flask scale testing of cycle 2

In contrast, the pH level result suggests an alkalinisation occurred with a pH rise of up to 8,14 at the 50-hour mark when the test ended. The missing gap in between the start and the end of the test is due the inability to test the lab scale flasks during fermentation. The risk of contamination

was simply too high for conducting the pH test during the fermentation process. The small increase in the pH level from the two initial data plots was the before and after sterilisation through autoclaving.

3.2.1.2 GC/MS

The change in 2,3-BD concentration over time can be seen in the same chart in Figure 2 above. It can be observed that the overall trend is actually a decrease from the initial 2,3-BD concentration. Despite the increase of 2,3-BD concentration to 17,43 mg l^{-1} at the 28-hour mark, it is still lower than the initial 2,3-BD concentration of 19,04 mg l^{-1}.

3.2.2 Lab scale

3.2.2.1 OD and pH

At the lab scale, the change in biomass concentration and pH levels are plotted in Figure 3. It can be observed that the biomass concentration fluctuates below the 0-level indicating no growth of the microorganisms. The inoculum was uncontaminated and active with a high density which was confirmed momentarily before the inoculation process via microscopy as can be seen in Picture 2. The reason for this can be speculated from the pH levels results. It can be observed that the pH level was constant at around 4. A day before sterilisation of the substrate was conducted, the pH was measured showing an average result of 6,27. As the substrate was poured inside the pilot bioreactor and the sterilisation process completed, the pH level shows a reading of 3,99. After that, almost no change can be observed of the pH level.

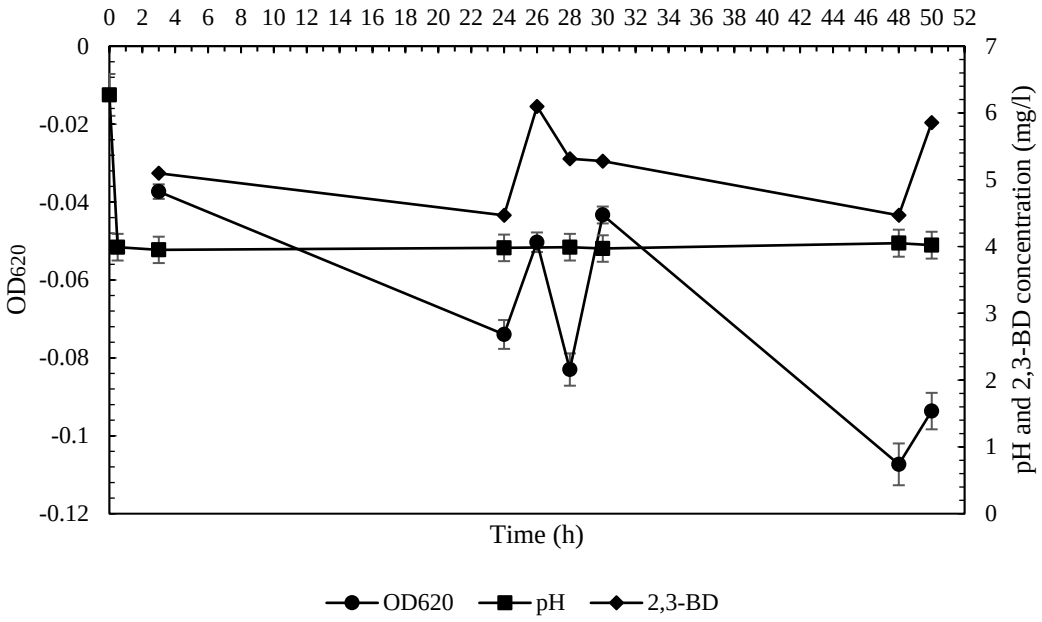
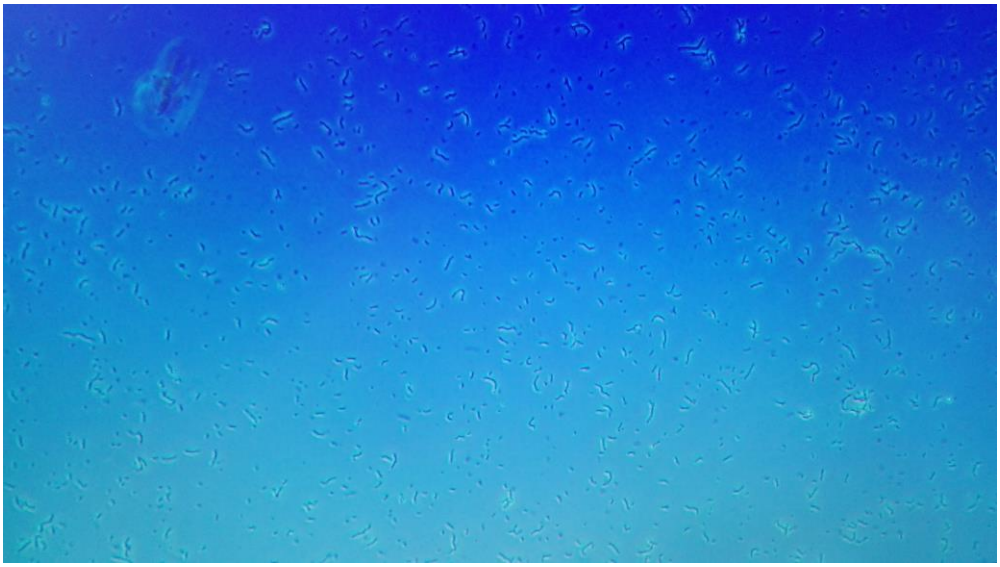


Figure 3. OD620, pH and 2,3-BD concentration results in the lab scale testing of cycle 2



Picture 2. A picture showing a dense and healthy sample of a *Bacillus amyloliquefaciens* culture.

3.2.2.2 GC/MS

At the lab scale, not much change can be observed in the concentration 2,3-BD as can be observed in figure 3. The concentration fluctuates with no clear trend between 4 to 6 mg l^{-1}.

3.3 Cycle 3

3.3.1 Flask scale

3.3.1.1 OD and pH

The change in biomass concentration via OD as well as the change in pH levels for the flask scale testing in cycle three had been plotted on the graph in Figure 4. It can be observed that the OD peaked, as expected, at around the 24-hour mark. However, interestingly the OD increased back up again at around the 48-hour mark after a dip shortly after the 24-hour mark.

In regards to the pH levels, it can be observed that the fermentation started at a pH level of 6,15. Further pH samples were not taken due to the risk of contamination as well as the risk of equipment malfunction because of the paraffin layer on top of the anaerobic flask fermentation. However, it is safe to say that the pH level did not change to inhibition values during the fermentation

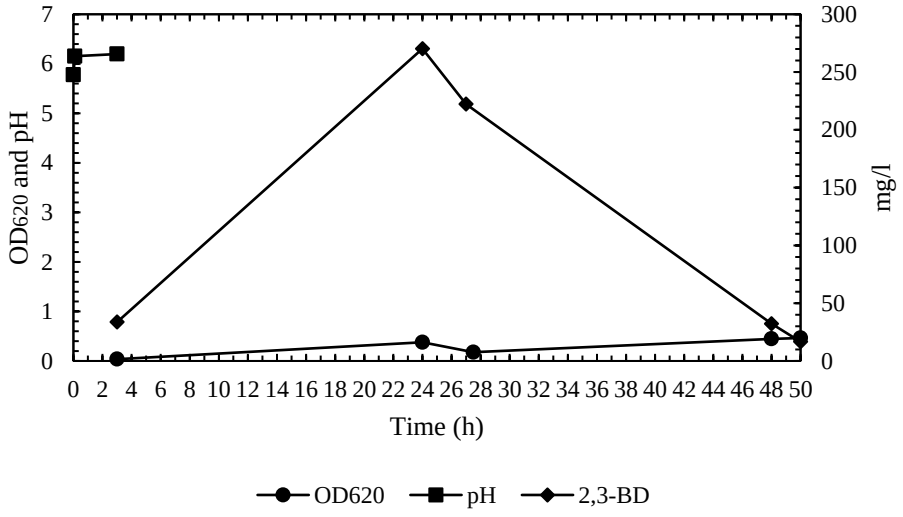


Figure 4. OD620, pH and 2,3-BD concentration results in the flask scale testing of cycle 3

3.3.1.2 GC/MS

A clear peak of 2,3-BD can be seen in the product generation testing as can be observed in figure 4. A concentration 270,22 mg l^{-1} was recorded at the 24-hour mark and decreased to initial concentration levels at the 48-hour mark.

3.3.2 Lab scale

3.3.2.1 OD and pH

Figure 5 shows the change in biomass concentration via OD as well as the change in pH levels of the fermentation at the lab scale of cycle 3. Similarly, to the flask scale of the same cycle, it can be observed that the biomass concentration peaked at around the 24-hour mark. In fact, the biomass concentration kinetic is like that of the flask scale of the same cycle.

From the same graph, the pH level shows an interesting development in which it started at 6,11, decreases as the fermentation occurs until the 24-hour mark, and then seems to stabilise itself further on at the pH level of 4,7.

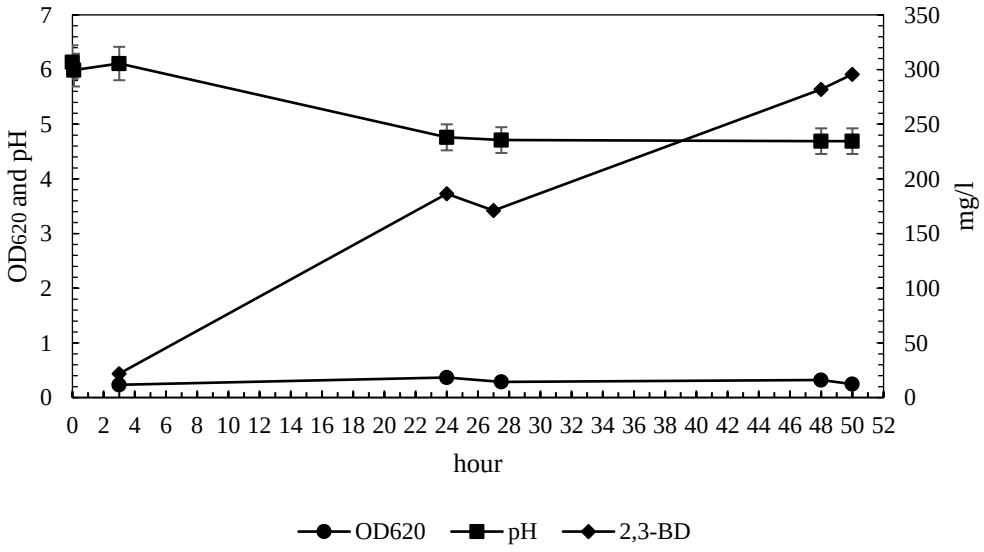


Figure 5. OD620, pH and 2,3-BD concentration results in the lab scale testing of cycle 3

3.3.2.2 GC/MS

The 2,3-BD concentration can be observed to be at the highest at the 50-hour mark according to the chart in Figure 5. A 2,3-BD concentration of 295,66 mg l^{-1} was measured at this point.

3.4 Cycle 4

3.4.1 OD and pH

Little to no change in both OD and pH can be observed in the pilot plant scale as can be seen in Figure 6. It should be noted that a mis-programming of the temperature conditions occurred which resulted in a fermentation temperature of 84°C.

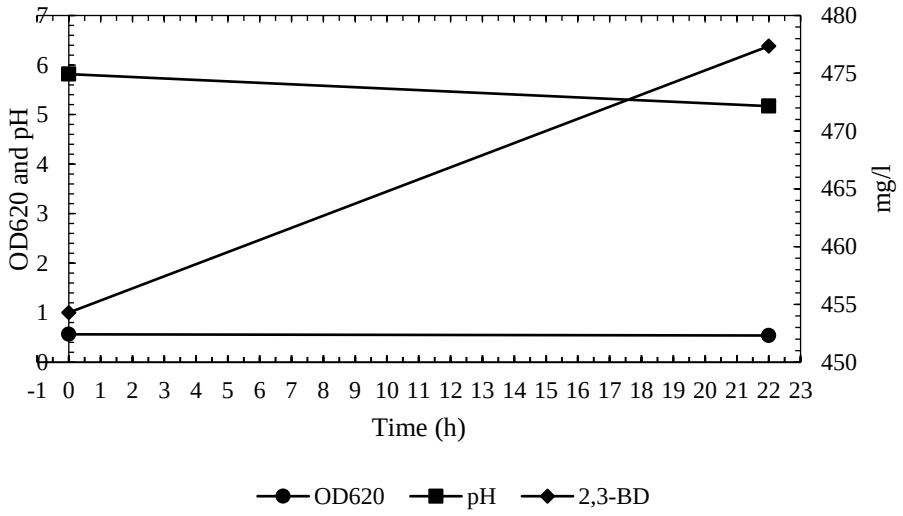


Figure 6. OD620, pH and 2,3-BD concentration results in the pilot plant scale testing of cycle 4

3.4.2 GC/MS

As a result of the aforementioned issue with the temperature, the same can be observed in regards to 2,3-BD production. The small rise in concentration that can be seen in Figure 6 from 454,29 mg l^{-1} to 477,35 mg l^{-1} is miniscule at this scale.

4. Discussions and outlook

4.1 Cycle 1

The decision to use molasses was purposefully considered to minimise problems with the fermentation before using a less-friendly substrate for the *Bacillus amyloliquefaciens*. The tests were conducted with the aim to identify the growth curve as well as the optimum fermentation time to yield the highest 2,3-BD concentration. The understanding of the growth curve of the *Bacillus amyloliquefaciens* was crucial to identify the optimum growth conditions, maximum biomass concentration and ultimately the relation between the product generation and the growth of the microorganism. On the other hand, determination of the optimum fermentation time was essential not only to identify the time point when the highest 2,3-BD concentration was yielded, but to also ease the burden of sampling for the next research cycle. Previous research had identified the reverse transformation of 2,3-BD to acetoin at the decline phase of the fermentation (Zhang, et al. 2011). Therefore, it was important to know the fermentation time in which 2,3-BD concentration peaks, to obtain maximum profit before it reverts to acetoin.

However, it was observed that the 2,3-BD concentration that was generated by *Bacillus amyloliquefaciens* were unexpectedly low; around 600 mg l^{-1}. This lead to the investigation of the bioreactor and it was found the inoculum was already contaminated. Due to competition, the small population of *Bacillus amyloliquefaciens* present in the inoculum could not grow to their maximum potential and produce 2,3-BD at the expected amount of around 25 g l^{-1}. Despite this contamination, the low amount of *Bacillus amyloliquefaciens* still generates 2,3-BD which shows just how robust the fermentation process is.

As observed in Figure 1, the peak of the biomass concentration could be seen at around the 36-hour mark. This is consistent with previous research on the kinetics of 2,3-BD fermentation

(Alam, et al. 1990). Figure 7 taken from Alam, et al. (1990) shows that dissolved oxygen (DO) levels rapidly decline during the early stages of the fermentation. However, the biomass concentration kept increasing showing little difference on the growth rate of *Bacillus amyloliquefaciens* in both aerobic and anaerobic. This led to the outlook that further fermentation processes can be done in anaerobic conditions immediately. The reasoning is that the growth rate of *Bacillus amyloliquefaciens* does not seem to be affected heavily by aeration conditions and the 2,3-BD fermentation itself occurs during anaerobic conditions when DO levels are at 0% which had previously been confirmed (Alam, et al. 1990). This would then either decrease the fermentation time since the *Bacillus amyloliquefaciens* would start fermenting sooner, increase maximum 2,3-BD concentration due to simultaneous growth and fermentation, or both.

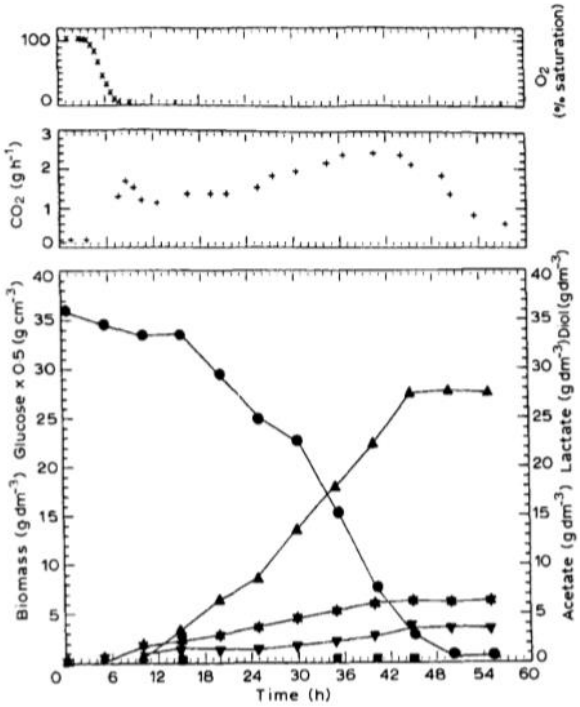


Fig. 2. Time course of 2,3-butanediol fermentation by *Bacillus amyloliquefaciens*, using glucose as main carbohydrate source. Symbols: glucose (●); biomass (★); 2,3-butanediol (▲); acetic acid (▼); lactic acid (■); oxygen as per cent saturation (X); carbon dioxide evolution rate (+).

Figure 7. The kinetics of 2,3-BD fermentation by *Bacillus amyloliquefaciens* taken from the journal article of (Alam, et al. 1990)

4.2 Cycle 2

As can be observed from the result of the flask scale in figure 2, the maximum biomass concentration can be identified at around the 28-hour mark. In fact, filling in the missing data plots could not have been possible due to limited resources and accessibility at Ostfalia, this flask scale test shows similar results to the papers of (Zhang, et al. 2011) as can be seen in Figure 8.

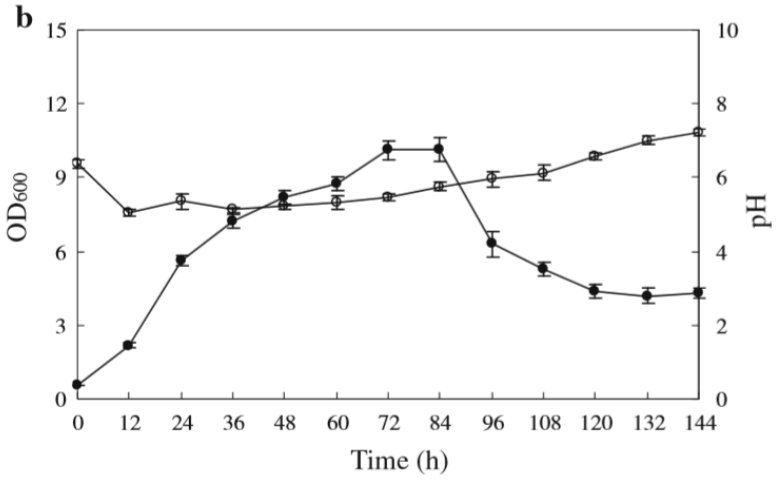


Fig. 4 Time-course date of batch fermentation by *Bacillus subtilis* JNA. **a** Metabolite profiles; **b** Cell growth and pH residual glucose (*square*); acetoin (*filled square*); 2,3-butanediol (*square*); acetic acid (*filled triangle*); lactic acid (*square*); pH (*circle*); OD_{600} (*filled circle*)

Figure 8. pH and OD development in the fermentation by *Bacillus amyloliquefaciens* taken from the journal article of (Zhang, et al. 2011)

Regarding the pH, it was quite surprising to observe the rise in the pH during fermentation. This was contrary to expectations because of the acidic nature of acetic acid, one of the products of the fermentation of *Bacillus amyloliquefaciens*. However, further literature research proves that this is not the most unusual phenomenon. Several studies had shown that the production of acetoin contributes to the alkalinisation of the substrate (Zhang, et al. 2011) (Yang, Rao, et al., Economic conversion of spirit-based distillers' grain to 2,3-butanediol by Bacillus amyloliquefaciens 2015).

Acetoin was produced to counter the acidification of the substrate from acetic acid production (McFall and Montville 1989), and is known to be involved in the generation of energy for the microorganism (Starrenburg and Hugenholtz 1991). So, it was possible that acidification occurred initially during the fermentation process, but was then followed by alkalinisation by acetoin production. However, this could not be confirmed as it was not possible to test the pH during fermentation, again due to the high risk of contamination.

The 2,3-BD concentration did not show successful fermentation either. The decrease in 2,3-BD concentration found in the flask scale could be explained by the reverse transformation to acetoin. In other words, the *Bacillus amyloliquefaciens* converts glucose to acetoin and further to acetic acid and 2,3-BD to store energy, but because the environment became too acidic it then reverse-transformed the 2,3-BD into acetoin. This explains both the decrease in 2,3-BD concentration as well as the increase in the pH level.

The lab scale fermentation test in cycle two experienced severe inhibition due to the extremely low pH levels as can be observed in figure 3. According to Welker & Campbell (1967) the optimal pH range of *Bacillus amyloliquefaciens* is at 5,5 to 6,5, with a maximum activity at 5,9, and an extinction value when outside the pH range of 4-8. The initial pH at the inoculation was at 3,99. Exposed to this, the inoculum was concluded to have died off. This explains the fluctuations of the 2,3-BD and the OD results at around the 0 value since the biomass concentration could not have possibly increased let alone generate any 2,3-BD.

4.3 Cycle 3

Due to the severe inhibition from the pH levels observed at cycle 2, this cycle took extra precautions controlling and monitoring the pH level of the fermentation. Further investigation suggests that the pH fall from cycle two was due to storage of the WWT effluent without

sterilisation. It is suggested that this is most likely due to other bacteria thriving in this environment. A simple test conducted compared the pH level of an autoclaved and a non-autoclaved WWT effluent after 24 hour of storage and it showed that the pH level of the autoclaved WWT effluent did not change whilst the non-autoclaved counterpart fell to a pH level of about 4. Due to this, the WWT effluent was immediately autoclaved after production to maintain a habitable pH level for the *Bacillus amyloliquefaciens*. In addition, flask testing was done in parallel to lab testing to assess the scale difference using the same WWT effluent. The difference in this cycle compared to previously, is that a layer of paraffin was introduced to the flasks to induce anaerobic conditions.

As can be observed in figures 4 and 5, the kinetics of the biomass concentration in both scales behaves similarly, peaking at the 24-hour mark. However, the flask scale testing showed an increase in the biomass concentration towards the end of the fermentation. Further research should be conducted in order to investigate the cause of this phenomenon.

As for the pH, the kinetics of the pH level change in the lab scale fermentation supports previous literature studies. Since acetic acid and 2,3-BD are produced simultaneously during fermentation, acidification of the substrate from the acetic acid is inevitable. However, *Bacillus amyloliquefaciens* has a metabolic pathway that produces acetoin as soon as the glucose is depleted or when the pH level is unfavourable to them. Due to this acetoin production, alkalinisation of the substrate occurs (McFall and Montville 1989) and this was observed in the lab scale fermentation when the pH drop stabilised after the 24-hour mark.

The 2,3-BD concentration in this cycle behaved as expected reaching the peak at round the 24-hour mark. However, the fermentation at the lab scale still experience an increase in the 2,3-BD concentration when the last sample was taken. The reason for this should still be investigated further.

4.4 Cycle 4

After the overnight sterilisation process, when the substrate was being sampled for testing pre-inoculation with a field pH meter, it was noticed that the odour of the substrate was still quite foul and there was absolutely no coagulation of particles as had been observed in all the other sterilisation methods in both the small bioreactor and autoclave. It was discussed that perhaps there was a problem reaching the sterilisation temperature, however due to time constraints it was decided to proceed with the experiment as the substrate was within the desired pH range when tested.

Upon inspection of the operational parameters before sampling at the 22-hour mark, it was found that the internal temperature of the bioreactor substrate was 84°C. Due to mis-programming of the temperature control it is believed that the sterilisation process did not complete fully thus forcing the *Bacillus Amyloliquefaciens* into competition with bacteria already in the substrate. Also, due to a continuing temperature rise during the process, whether any of the *Bacillus amyloliquefaciens* became dominant or even just survived, they would have still been killed as the temperature rose over 50°C.

The results of the GCMS confirmed that none of the *Bacillus amyloliquefaciens* survived long enough to produce 2,3-BD, although a fractional amount was detected, it is believed to have come from the quantity created during cultivation of the inoculation medium.

5. Conclusion

The hypothesis in previous research by Gerlitz (2017), Liebig (2017) and Walpole et al. (2017) that production of 2,3-BD by *Bacillus amyloliquefaciens* using WWT effluent as a substrate is feasible had been proven in this thesis. Although at this stage the quantities of 2,3-BD produced were small, upscaling the production using the batch fermentation method shows no negative influence on the process. The fermentation can be done in both aerobic and anaerobic conditions with the latter being the preferred method due to the low cost and energy consumption. The maximum 2,3-BD concentration achieved so far is at 295,66 mg l^{-1} in the lab scale and further optimisation should look into increasing the yield.

Regarding the process itself, an important feature of the metabolic pathway of *Bacillus amyloliquefaciens* had been identified in that 2,3-BD would be converted back into acetoin once glucose had been depleted for energy gain and to alkalinise the acidic environment. Because of this, a time frame in the fermentation process should be identified in order to yield the maximum 2,3-BD concentration for the extraction from the substrate solution. This thesis narrowed down this time frame between the 24 to 28-hour mark.

The pH level had been identified as an important factor in the fermentation process as well. The *Bacillus amyloliquefaciens* operates best in the pH ranges of 5.5 to 6.5, with an optimum of 5.9 (Welker and Campbell 1967). Sterilisation of the substrate straight after production appears to negate the pH drop that seems to persist during storage of the WWT effluent, however constant monitoring is still recommended for the process.

In conclusion, the paper proved that the novel idea of biotechnologically producing a bio-based chemical using renewable sources is feasible through 2,3-BD production by *Bacillus amyloliquefaciens* using WWT effluent from MSW. The process had been up-scaled and trialled

in a facility with a maximum TRL level of 4. The potential for this process is there, and further research in maximising the yield as well as the down-streaming process should be undertaken.

6. References

Alam, S., F. Capit, W. A. Weigand, and J. Hong. 1990. “Kinetics of 2,3-Butanediol Fermentation by Bacillus amyloliquefaciens: Effect of Initial Substrate Concentration and Aeration .” *Chem. Tech. Biotechnol. Vol. 47* 71-84.

Cossu, Raffaello, and Tiziana Lai. 2012. “Washing of Waste Prior to Landfilling.” *Waste Management Vol. 32* 869-878.

Cossu, Raffaello, Tiziana Lai, and Kostyantyn Pivnenko. 2012. “Waste washing pre-treatment of municipal and special waste.” *Journal of Hazardous Materials 207-208* 75-72.

Feng, Xiaoyu, Terry Walker, William C. Bridges, Charles Thornton, and Karthik Gopalakrishnan. 2014. “Biomass and lipid production of Chlorella protothecoides under heterotrophic cultivation on a mixed waste substrate of brewer fermentation and crude glycerol.” *Bioresource Technology 166C* 17-23.

Fischer, Christian. 2013. *Municipal Waste Management in Germany.* Copenhagen Research Institute: European Topic Centre on Sustainable Consumption and Production (ETC/SCP) under contract with the European Environment Agency (EEA).

Flickinger, M.C. 1980. “Current biological research in conversion of cellulosic carbohydrates into liquid fuels: how far have we come.” *Biotechnol. Bioeng. Vol.22* 27-48.

Fulmer, Ellis I., L. M. Christensen, and A. R. Kendali. 1933. “Production of 2,3-Butylene Glycol by Fermentation.” *Industrial and Engineering Chemistry Vol. 25 No. 7* 798-900.

Garg, S. K., and A. Jain. 1995. “Fermentative Production of 2,3-Butanediol: A Review.” *Bioresource Technology Vol. 51* 103-109.

Gerlitz, Marcus. 2017. *Restabfallwaschung mit biotechnologischer Nutzung des Waschwassers.* Wolfenbüttel, Germany: Ostfalia University of Applied Sciences. Faculty of Environmental Engineering.

Hatti-Kaul, Rajni, Ulrika Törnvall, Linda Gustafsson, and Pål Börjesson. 2007. "Industrial biotechnology for the production of bio-based chemicals -- a cradle-to-grave perspective." *TRENDS in biotechnology Vol.25 No.3* 119-124.

Jain, Sonal, Sheetal Chouhan, Kirti J. Chavan, Pushpendra Singh, and Archana Tiwari. 2016. "Bioethanol production from waste paper sludge as a fermentation substrate by using xylose fermenting yeast." *Minerva Biotecnologica* 201-207.

Ji, Xiao-Jun, He Huang, and Ping-Kai Ouyang. 2011. "Microbial 2,3-Butanediol Production: A state-of-the-art Review." *Biotechnology Advances Vol. 29* 351-364.

Liebig, Jan. 2017. *Restabfallwashung mit biotechnologischer Nutzung des Washwassers.* Wolfenbüttel, Germany: Ostfalia University of Applied Sciences. Faculty of Environmental Engineering.

McFall, S. M., and T. J. Montville. 1989. "pH-Mediated regulation of pyruvate catabolism in Lactobacillus plantarum chemostat cultures." *Journal of Industrial Microbiology Vol. 4* 335-340.

Mozumder, Salatul Islam. 2015. "Optimization of a two-phase fermentation process for the production of polyhydroxybutyrate (PHB) from organic and inorganic (industrial waste) substrate." *Ghent University. Faculty of Bioscience Engineering, Ghent, Belgium.*

Nelles, M., J. Grünes, and G. Morscheck. 2016. "Waste Management in Germany – Development to a Sustainable Circular Economy?" *Procedia Environmental Sciences Vol. 35* 6-14.

Rehm, H. J. 1970. “Modern Industrial Microbiological Fermentations and Their Effects on Technical Developments.” *Angewandte Chemie Vol. 9 Issue. 12* 936-945.

Starrenburg, M. J. C., and J Hugenholtz. 1991. “Citrate fermentation by Lactococcus and Leuconostoc.” *Applications of Environmental Microbiology* 3535-3540.

Umweltbundesamt. 2016. *Landfill.* 05 04. https://www.umweltbundesamt.de/en/topics/waste-resources/waste-disposal/landfill.

Walpole, Robert, Adhitya Prayoga, Tuomas Lindeman, Jannecke Kreutzer, and Tyler Rickabaugh. 2017. *Utilisation of the organic fraction from Municipal Solid Waste via biotechnological pathways.* Wolfenbüttel, Germany: Ostfalia University of Applied Sciences. Faculty of Engineering.

Welker, N. E., and Leon L. Campbell. 1967. “Comparison of the alpha-amylase of Bacillus subtilis and Bacillus amyloliquefaciens.” *Journal of Bacteriology Vol. 94 No. 4* 1131-1135.

Yang, Taowei, Zhiming Rao, Xian Zhang, Meijuan Xu, Zhenghong Xu, and Shang-Tian Yang. 2015. “Economic conversion of spirit-based distillers' grain to 2,3-butanediol by Bacillus amyloliquefaciens.” *Process biochemistry Vol. 50* 20-23.

Yang, Taowei, Zhiming Rao, Xian Zhang, Qing Lin, Haifeng Xia, Zhenghong Xu, and Shangtian Yang. 2011. “Production of 2,3-butanediol from glucose by GRAS microorganism Bacillus amyloliquefaciens .” *Basic Microbiology Vol. 51* 650-658.

Zhang, Xian, Tao-wei Yang, Qing Lin, Mei-juan Xu, Hai-Feng Xia, Zheng-hong Xu, Hua-zhong Li, and Zhi-ming Rao. 2011. “Isolation and identification of an acetoin high production bacterium that can reverse transform 2,3-butanediol to acetoin at the decline phase of fermentation.” *World Journal Microbiol. Biotechnol. Vol. 27* 2785-2790.