CORRECTIONS

The Utilization of Second Generation Feedstocks for the Production of Platform Chemicals by Filamentous Fungi

A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment for the Degree of Masters of Science.

University of the Witwatersrand

School of Molecular and Cell Biology

Faculty of Science

MSc

By

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Introduction

The corrections suggested by the internal and external examiners were very helpful to improve the present dissertation. I have taken into consideration all of their suggestions and reworked the original dissertation. The dissertation is now longer, more detailed and has more logical flow. New figures have been added along with an appendix, which was missing from the previous dissertation.

Internal Examination Corrections

1. Need to indicate the number of independent runs that gave rise to the results presented.
2. Include steps taken to get the reactor up and running, as evidence to mastering his techniques.
3. Provide more results and increase the volume of the thesis.
4. Include more data from the preliminary reactor set up.
5. Include more data involving the effects of major parameters when it comes to bioreactor maintenance; i.e. temperature, dissolved oxygen levels, pH etc.
6. Include more information about retention times i.e. how the HPLC works to determine the different metabolites that were produced.
7. Take out all the short terms, i.e. it's and aren't.
8. Consistency when it comes to giving measurements, i.e. 20L, not 20l.
9. Include a space between the unit and number of measurement, i.e. 5 days, not 5days.
10. Use alternative terms to "I", "We" and "Our".
11. Change the page numbering so that before the introduction and literature review the numbering is in Roman Numerals (i, ii, iii, iv…etc) and afterwards it should be Arabic numerals.
12. Refer the readers to Figures and tables before presenting them, "?". Use the tables and figure numbers in the text to minimize the use of "the above table" etc.
13. Re-organize the section titles and numbering. Esp. Literature review section.

Section Specific Comments:

Contents List

*The list of tables and figures should show the pages of where the latter are located.

List of Tables

List of Figures

Abstract

*Line 8: add the word “on” between ‘focused’ and ‘the’.

*Line 11-12: removed the section after ‘metabolites of interest’. I.e. Removed: ‘such as citric acid and itaconic acid’

Abstract:

The depletion of petroleum and other platform chemical resources are a global concern; therefore alternative substrates must be identified to replace these current sources. Thus allowing research in fungal biotechnology to prosper, as filamentous fungi can utilize second-generation feedstocks or agricultural waste to produce these petroleum derived platform chemicals. This research focuses on the ability of filamentous fungi to use different second-generation feedstocks such as wheat bran and sugar cane bagasse to generate platform chemicals of interest, namely being itaconic acid (IA) and other organic acids of interest, such as citric acid. This study focused on the metabolite producing capabilities of *Aspergillus terreus*, initially in a shake flask fermentation environment and then in an Airlift Bioreactor environment utilizing hydrolyzed wheat bran and sugar cane bagasse as a substrate source to

produce metabolites of interest. The initial shake flask fermentation experiment involved inoculation and incubating *A. terreus* in hydrolyzed wheat bran with additional minerals at 30°C for 5 days at a pH range of between 3-4. The result yielded itaconic acid and citric acid concentrations of 1.01g/l and 6.23g/l at their peaks, respectively. The airlift bioreactor was run for 16 days with a constant pH range between 3-4, at a temperature of 30°C with a dissolved oxygen level of 20g/l. The result of the study yielded a high itaconic acid and citric acid concentration peaking at 59.4 g/l and 59.2 g/l, respectively.

**Note: Abstract was reworked as according to the external examiners requirements.*

Chapter 1

*Section 1.1.2. Can be subdivided and more information on metabolism can be given.

1.1.1 Current Examples of Filamentous Fungi in Industry:

1.1.1.1 Filamentous Fungi:

Filamentous fungi are the microorganisms of choice to produce these platform chemicals due to their ability to secrete vast amount of enzymes that generate high metabolite yield (3) (4) (5), they grow in the form of multicellular filaments called hyphae, which generate connected networks with multiple genetically identical nuclei, which make up the mycelium (3). Filamentous fungi have potential regarding their highly efficient metabolite producing capabilities; humans have only been able to harness a fraction of this potential (6).

1.1.1.2 Fungal Biotechnology in Industry:

Fungal biotechnology is currently utilized in many industries, such as for the production of medical supplies, such as; Penicillin, which is produced by the fungi *Penicillium chrysogenum*. Lovastatin a cholesterol-lowering drug is produced by *Aspergillus terreus* and Cyclosporine an immunosuppressant drug used in organ transplant procedures is produced by *Tolypocladium inflatum* (4). Several other products which filamentous fungi produce are often taken for granted as they are so commonly used and include products of the food industry, such as; cheese produced by Penicillium spp., soy sauce and sake produced by *Aspergillus oryzae* (5). The main reason why filamentous fungi are utilized in industry is due to the high activity of

fungi's enzymatic secretions. These extracellular enzymes allow fungal biomass to degrade plant cell walls and to generate large concentrations of metabolites. *Penicillium simplicissimum* is another fungus of interest; it is currently employed by industry due to its high xylanase activity. It is used in the paper producing process to bleach wood pulp (8). This fungus is commonly known to produce succinate from glucose and studies have been conducted under aerobic and anaerobic conditions to investigate the pathways involved (8). *P. simplicissimum* has been observed to secrete low levels of succinate. Succinic acid is the predominant acid produced under anaerobic conditions. Fungal pellet formation was shown to be important in obtaining maximum succinate production rates, and therefore raised the possibility of fumarate respiration as a biochemical mechanism for succinate production under anaerobic conditions (8). However it is known that succinic acid or succinate is produced as an intermediate during the The Citric Acid cycle (TCA), where citric acid or citrate is the final product. Therefore this fungus is another perfect candidate to evaluate the production of organic acids when utilizing a second-generation feedstock such as lignocellulose substrate obtained from agricultural waste.

1.1.1.3 *Aspergillus Niger* and Citric Acid

Another important fungi utilized in industry is *Aspergillus niger* which became the primary microorganism in the production of citric acid, exceeding production from extracting citric acid from lemons. *A. niger* can metabolize many carbon sources such as sucrose and or glucose to produce vast amounts of citric acid for a fraction of the cost as opposed to using lemons (9) (22). The current industrial process using *Aspergillus niger* and the production of citric acid, which is also a metabolite of interest as listed by the United States Department of Energy, ranges between 200 g/l to 240 g/l, when utilizing glucose/sucrose is utilized as the main substrate source. There are many uses for citric acid in the food and beverage industry, the carbonated beverage industry accounted for 50% of the total citric acid production in 1990 (8) (22). *A. niger* generates citric acid via the TCA in the fungus's metabolic pathway, by taking up glucose into the cell and converting it to the three-carbon acid, pyruvate via the glycolytic pathway, which occurs in the cytosol. Subsequently the newly formed pyruvate molecule is decarboxylated to form acetyl-CoA catalyzed by the mitochondrial pyruvate dehydrogenase complex and another pyruvate molecule is

carboxylated to oxaloacetate in the cytosol by pyruvate carboxylase (8). The oxaloacetate is transported into the mitochondrion of the fungi and combined with acetyl-CoA to form citrate. This product is passed out of the mitochondrion and eventually secreted of the cell. The high yields of citric acid production process are possible because all six carbons of the substrate, glucose or fructose, are conserved in the six-carbon product citric acid, through the glycolytic pathway and the actions of two additional enzymes, pyruvate carboxylase and citrate synthase (8) (22).

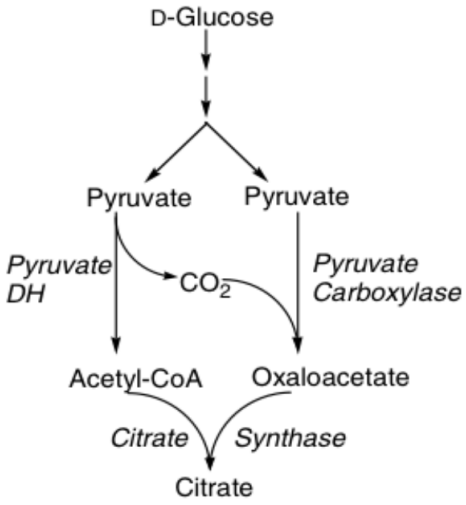

Figure 1. The Simplified Metabolic Pathway Leading to Citric Acid Production in *Aspergillus niger* (8)

Citrate synthase is the end terminal enzyme in the citric acid biosynthetic pathway. Mg^{2+} and ATP inhibit the enzyme from *A. niger*, however citrate the product of citrate synthase is not an inhibitor of the enzyme itself. Other enzymes of the citric acid cycle also have significant effects on the production of citric acid, as aconitase inhibition would theoretically lead to an accumulation of citrate by inhibiting subsequent flux during the citric acid cycle (8) (9). Another enzyme, isocitrate dehydrogenase could have a deleterious effect on citric acid production by decarboxylating isocitrate, which is required to be in equilibrium with citrate via aconitase activity. The $NADP^+$ specific isocitrate dehydrogenase has been purified from *A. niger* and found to be present in both the cytosol and mitochondrion (10). It was published that isocitrate dehydrogenase is inhibited by ATP and citrate via chelation of enzymatic Mg^{2+} (10). It was also noted that intracellular Mg^{2+}concentrations would have little effect to the overall process (8). Citric acid accumulated extracellularly to a final concentration of about 1.0M, therefore an active export system must exist to remove citrate from the

cytosol, where the normal citrate concentration is only 2 – 30mM (8). Citrate export requires low Mn^{2+}concentrations in the range known to be required for efficient citric acid production (11). On the other hand, citrate import required Mn^{2+}both for induction of expression of the citrate importer as well as for its function (9) (11). These results provide an additional explanation of the multiple effects of Mn^{2+}on the physiology of *A. niger* under citric acid production conditions. *Aspergillus niger* is known to produce three major metabolites when undergoing fermentation. These include 2 - carboxylmethyl - 3 - hexyl-maleic acid anhydride, 2 - methylene - 3 - hexyl-butanedioic acid and 2 - methylene - 3 - (6 - hydroxyhexyl)-butanedioic acid (12) (13).

*Section 1.3. More information regarding the South African DOE can be added, i.e. the SA DOE's standing on the issues under discussion. This will then highlight where the study fits both in South African and global contexts.

1.3 Department of Energy and Platform Chemicals:

The department of energy is an organization that exists in many countries and operates in government to monitor aspects regarding energy. Its main responsibilities include; energy conservation, energy-related research, radioactive waste disposal, and domestic energy production. The South African DOE ensures that diverse energy resources are made available, in sustainable quantities and at affordable prices in support of economic growth and poverty alleviation. DOE also sponsors many basic and applied scientific research programs. Platform chemicals as mentioned earlier are building block chemicals, which have a high transformation potential into new families of useful molecules. Most platform chemicals are derived from fossil fuels such as petroleum, of which there are limited resources remaining, therefore biotechnology has been utilized in order to generate these chemicals directly from biomass. Biomass as a renewable feedstock offers the opportunity to replace fossil fuels as a source of energy, materials and chemicals. Sugars, oils and other compounds generated from biomass can be converted into platform chemicals directly or as by-products from fuel products in processes analogous to the petrochemical industry today. Improvements and innovations to existing biological and chemical processing of sugars will provide the opportunity for the production of high-value chemicals and products from biomass and reduced reliance on petrochemical-derived products. The platform chemical of choice that this study focuses on is IA and citric

acid, due to it being a relatively expensive organic acid and due to its wide use in industry respectively, when compared to its counter-parts, such as, lactic acid, succinic acid etc. Renewable energy sources, other than biomass (the energy from plants and plant-derived materials), have not yet been exploited optimally in South Africa.

*Information on current trends with regards to the improvement in fermentative production of similar metabolites can be included; can be in the form of growth parameters and/or genetic engineering. Information can be used from section 4.1.3. And 4.1.4. and chapter 5. This will provide a base for inclusion incorporation of the data suggested under general comments.

1.5 Fungal Growth Parameters:

Optimum fungal growth experimental parameters as published by Nubel and Rabajak include an incubation temperature of 37 – 40° C as well as continuous aeration will allow for optimum fungal metabolism to occur. A low starting pH within the range of 3–5 is required, followed by a lower optimum operating pH within the range of 2.2 – 3.8. A high glucose concentration is required ideally ranging between 10 to 20%. Sufficient nitrogen must be present, as well as a high magnesium sulfate concentration of 0.5% of the total fermentation volume. Low phosphate levels are required to limit the amount of mycelial growth as increased mycelial growth would result in overcrowding and recycling of IA could occur (18). Finally adequate levels of the trace metals, which include zinc, copper, and iron are required (8) (18). IA production by *A. terreus* shares many of the characteristics of citric acid production by *A. niger* due to their ability to proliferate well on a variety of monosaccharides, disaccharides, and polysaccharides (8).

*Note: New section added to the literature review.

1.8 Strain Modification:

Aspergillus terreus has been modified in many ways, including strain selective breeding and genetic modification to improve the yield of its metabolites, such as; IA and Lovastatin. Such alterations to *A. terreus* include; strain improvement via mutagenesis as product inhibition, which plays a vital role in fungi metabolism (25) (26). *A. terreus* also has as a form of self preservation as too much acid production

would change the operating parameters of its environment thus inhibiting normal metabolic functions, therefore there is a need to select an IA tolerant strain to overcome this problem which results in low IA yields (27) (28). Screening six hundred and seventy colonies, Yashiro *et al*. isolated the strain TN-484 in 1995. Industrially 85 g/l of IA was produced using this strain in a 100-kl scale fermenter utilizing simple media consisting of corn steep liquor, small amounts of minerals and glucose (14) (23) (24) (25) (29).

1.8.1 Genetic Transformation:

The general method of genetic transformation of filamentous fungi, begins firstly by removing the permeability barrier presented by the cell wall of the fungus, either by treatment with lithium acetate or by enzyme degradation using crude extracts found in snail gut (29) or by using a *Trichoderma* extract commercially known as Novozym 234 (29) to produce protoplasts. Some filamentous fungi require a mixture of enzyme extracts to produce a sufficient amount of protoplasts. This is followed by the removal of the cell wall by utilizing an osmotic stabilizer such as 0.6 potassium chloride or 1.2M sorbitol, the choice of which stabilizer is used depends on the species of fungus, the two Aspergillus species, *A. niger* and *A. terreus* make use of 1.2M and 0.55M sorbitol respectively as their osmotic stabilizers (29). DNA is then added to the protoplast suspension in the presence of 10-50mM calcium chloride and then followed by the addition of a solution of polyethylene glycerol, which initiates the uptake of the DNA by the protoplasts. The treated protoplasts are then allowed to proliferate of selective medium that selects for only the transformed cells (30). The selectable markers that can be used are genes that complement a nutrition requirement mutation, which allows the growth of the fungus in absence of the required nutritional factor (27).

1.8.2 Limitations regarding Fungal Genetic transformation:

There is a limitation to this methodology as it requires the recipient fungal species to be a mutant strain and this is often undesirable in industrial strains (20). The solution to this drawback is the use of positive selection systems, which is based on supplementation of antimetabolites, for example; oligomycin resistance, prokaryotic antibiotic resistance genes such as kanamycin or G418 resistance (29). Another alternative system utilized the *amdS* gene isolated from *A. nidulans, which* codes for

acetamidase, an enzyme that allows the growth on acetamide as a sole nitrogen or carbon source (7). Several other fungal species such as *A. niger* lack the *amdS* gene, which allows for easier selection of transformants, therefore making it a useful marker for transformation (30). The frequency of transformants is 10-100 stable transformants per μg. A similar technique to transform industrial fungi is to disrupt the gene functioning by homologous recombination using a defective gene or a gene that has been disrupted by a selectable marker, this method produces null mutations that eliminate the chance of undesirable traits developing in industrial strains of filamentous fungi (26) (27).

1.8.3 Future Prospects and other Transformation Methods:

Future prospects involving the heterologous expression of genes in filamentous fungi, where current gene expression systems rely on either powerful inducible or constitutive promoters, and homologous promoters are preferred to the production host as mentioned above to enhance organic acid production (31). There is however a disadvantage involving the constitutive expression of housekeeping gene promoters is that the promoters are functional during growth and therefore unsuitable for over expression of foreign proteins that might be toxic to the host cells. Another major disadvantage of inducible gene promoters, such as the powerful cbh1 gene promoter, is their repression by glucose and other carbon sources therefore they can severely affect the yield of protein secreted. These promoters can be regulated by the induction of carbon and nitrogen compounds and the pH of the growth medium (24).

Experiments have shown a novel metabolically independent expression system that can be regulated by oestrogenic compounds and it has been tested in *Aspergillus*. *A. nidulans* and *A. niger* transformants are highly sensitive with regards to acquiring oestrogen responsive elements or low levels of oestrogenic substances such as diethylstilbestrol, this therefore allows this research to be utilized in the detection of xenoestrogens in food and in the environment (4). Other experiments involving the modification of the traditional method for cultivating filamentous fungi for protein production have been researched. Whereas instead of a submerged fermentation occurring in liquid media, solid-state fermentation, which is supported by high yields of the secreted metabolites, obtained in these systems can be utilized instead (16). Due

to the physical mode of growth and gene regulation of fungi in solid-state fermentation, differ from fungi grown in liquid cultures (4).

Another vital factor in the development of transgenic fungal expression systems is the establishment of effective transformation protocols across fungal genera to add efficiency and flexibility to high throughput screening for evolved proteins and different metabolites of interest (21). Advancement in observation procedures including; microscopy technologies, such as fluorescence resonance energy transfer and fluorescent life time imaging, allows scientists to visualize metabolic pathways and protein–protein interactions in living systems therefore allowing greater understanding with regards to these areas, as fluorescent imaging, coupled with molecular biology, bioinformatics, biochemistry, genomics and proteomics, will redesign the concept of microbial metabolite production (21).

Another transformation method, which is being used more commonly in experiments involving the genetic transformation of filamentous fungi, is biolistic bombardment and inclusion of the seven barrels Hepta adaptor system has noticeably increased the number of transformations achieved (32). *Agrobacterium*-mediated transformation method has also gained a lot of interest, and claims of up to 100–1000 times greater efficiency of the T-DNA transfer and chromosomal integration, compared with conventional methods mentioned before, as one is aware that the T-DNA of the bacterium *Agrobacterium tumefaciens* can be transferred to plants, yeasts, fungi and human cells (26). We are entering an era of accelerating development of novel fungal fermentations and transformations due to the exponential increase and development of information and tools to exploit nature. Therefore there are many methods our study can be expanded with regards to higher yield of organic acids from agricultural waste.

*Changed the following according to the examiners requirements:

*Page 10, Section 1.1.1: If one were to => If one was to.

*Original “...if one were to...”

*New “...if one was to...”

*Page 11, Second Paragraph:

*Original "...Granted as they so commonly..."

*New "...granted as they are commonly..."

*Page 11, Second Paragraph:

*Original "Fungis' "

*New "fungi's"

*Page 11, Second Paragraph:

*Original "to secreted"

*New "to secrete"

*Page 11-12: The current industrial...Department of Energy - Reword to convey information.

*Original "The current industrial process involving *Aspergillus niger* and the production of citric acid, which is also a metabolite of interest as listed by the United States Department of Energy."

*New "The current industrial process using *Aspergillus niger* and the production of citric acid, which is also a metabolite of interest as listed by the United States Department of Energy, ranges between 200 g/l to 240 g/l, when utilizing glucose/sucrose is utilized as the main substrate source."

*Page 12: Industrial production…

*Removed "in concentrations of"

*Page 17, Section 1.7 Last Sentence:

*Moved the sentence to methodology. “The *Aspergillus terreus* utilized in this study was ordered and obtained from The *Agricultural Research Council* of South Africa in Pretoria.”

*Page 17, Section 1.8:

*Removed “is so that they are able to”

*Page 18, AIM: Rephrased the paragraph to be more precise.

*Original “The objective of the study is to produce itaconic acid (IA) and citric acid (CA) in levels of excess of 60g/l from sugar cane bagasse and or wheat bran, which has been chemically digested to produce a hydrolysate, which *A. terreus* will utilize, the fermentation and itaconic acid production will take place in an Airlift bioreactor, the pH and dissolved oxygen parameters will be constantly monitored using a DataTaker DT50 machine and samples containing itaconic acid will be assayed using High Performance Liquid Chromatography.”

*New “The objective of the study is to produce itaconic acid (IA) and citric acid (CA) in levels of excess of 60g/l from chemically digested sugar cane bagasse and or wheat bran hydrolysate, using *A. terreus*, the fermentation and itaconic acid production will take place in an Airlift bioreactor, the pH and dissolved oxygen parameters will be constantly monitored using a DataTaker DT50 machine and samples will be assayed using High Performance Liquid Chromatography.”

*Page 18, Objective 1: Reworded the beginning of the sentence.

*Original “The successfully hydrolyze wheat bran and sugar cane bagasse via chemical digestion to provide a nutrient source for the fungus to utilize.”
*New “To successfully hydrolyze wheat bran and sugar cane bagasse and provide a nutrient source for the fungus to utilize.”

Chapter 2

1. Included the names of the manufacturers of the equipment and their locations.
2. Used past tense when presenting the methods.
3. Example Page 22 Section 2.6
    a. Avoided the inclusion of results in the methodology.
    b. Included the above information in the results to show that you, that I had a difficult time in performing the experiments.

Chapter 3

1. Included the x-axis on the Figure. Page 27, Figure 5.

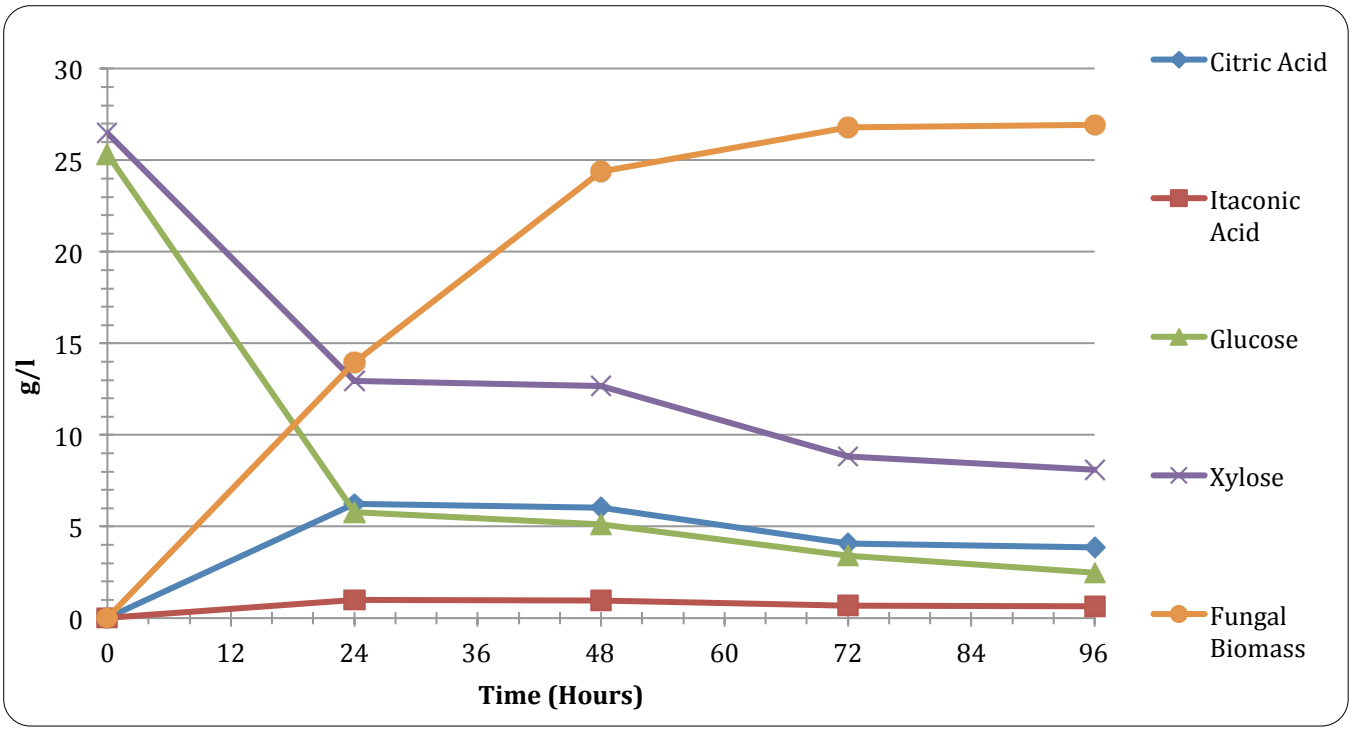

Figure 6. The Utilization of Agricultural Waste as Substrates for Organic Acid Production by *A. terreus*

*Note Figure 5. Became Figure 6. Due to the added Figure 2.

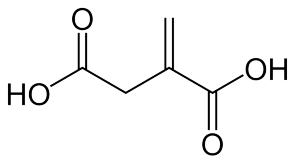

Figure 2. Chemical structure of Itaconic Acid

2. Reworked the methods for 3.3 and 3.4.

   *Original

3.3 Airlift Bioreactor Design and Assembly Strategy

The Airlift Bioreactor parts and machinery was obtained from Prof. Vince Gray's lab in OLS, it consists of a 2 liter fermentation chamber connected to another 5 liter collection chamber via master flex tubing and the nutrients are recycled into a 10 liter feedstock barrel also via master flex tubing. The airlift bioreactor consists of three separate chambers, one being a 5L fermentation chamber where the airlift apparatus is housed along with a wire gauze chamber, created from flexible plastic netting with 1-2mm spaces, this was placed in the center of the fermentation chamber and the fungal pellets were seeded directly into this chamber to prevent the pellets from depositing on the oxygen feeding tubes and nutrient entry point, therefore preventing blockage of oxygen flow, media agitation and nutrients from entering the fermentation chamber and also, most importantly to prevent the fungi from flowing into the neighboring product collection chamber which was a 10L flask. The reason for the separate collection and nutrient supply chambers is so that the product being produced won't be mixed together with the incoming new nutrients until it has been pumped back into the fermentation chamber to avoid contamination by fungal enzymes still present in the fermentation product.

3.4 Airlift Bioreactor Fermentation

The entire bioreactor will be set up to run independently and the pH will be maintained to be between 3-4 and dissolved oxygen levels will be monitored using a DataTaker DT50 machine, in order to maintain it at 20g/l, the temperature of the fermentation chamber was kept constant via circulating 35-40°C water in the surrounding structure of the fermentation chamber to maintain the temperature within the fermentation chamber at 30°C. Master-flexes were set up to recycle the nutrients at a rate of 15ml/hour. The reason a 2L airlift bioreactor was used for this study is that it's the first step to scaling up from the initial shake flask fermentation experiment, it's function was to have a continuous supply of oxygen and nutrients to create the ideal environment for the fungus to achieve maximum metabolite (IA/CA) production. Three samples were taken daily from the product recovery chamber and were frozen

immediately in the -20°C freezer. The experiment was carried out for 16 days, once fungal biomass had ceased to increase and had reached equilibrium.

*New

3.3 Airlift Bioreactor Design and Assembly Strategy

The Airlift Bioreactor parts and machinery was obtained from Prof. Vince, it consists of a 2 liter fermentation chamber made up of Perspex connected to a Pyrex 5 liter collection Erlenmeyer Flask via Watson Masterflex Tubing and the nutrients are pumped via a Cole Palmer Masterflex L/S and recycled into a 10 liter Pyrex vat feedstock barrel also connected via Watson Masterflex Tubing. Fermentation hydrolysate was pumped from the 10 liter Pyrex feedstock vat using a Cole Palmer Masterflex L/S operating at 15 ml/h to the airlift fermentation chamber, which houses the airlift apparatus along with a wire gauze chamber, created from flexible plastic netting with 1-2mm spaces, this was placed in the center of the fermentation chamber and the fungal pellets were seeded directly into this chamber to prevent the pellets from depositing on the oxygen feeding tubes and nutrient entry point, therefore preventing blockage of oxygen flow, media agitation and nutrients from entering the fermentation chamber and also, most importantly to prevent the fungi from flowing into the neighboring product collection chamber. Within the fermentation chamber the fungi utilize the nutrients within the hydrolysate and produce itaconic acid, the product was then pumped to the collection vat also using a Cole Palmer Masterflex L/S operating at 15 ml/h. The reason for the separate collection and nutrient supply chambers is so that the product being produced won't be mixed together with the incoming fresh nutrients to avoid contamination by fungal enzymes still present in the fermentation product.

3.4 Airlift Bioreactor Fermentation

The entire bioreactor was set up to run independently and the pH will be monitored by a Eutech Instruments Alpha-pH800 pH/ORP controller and was maintained between pH 3-4. Dissolved oxygen levels was pumped into the fermentation chamber using a KNF LAB Laboport Air Pump and the subsequent oxygen levels were monitored using a DataTaker DT50 machine, in order to maintain it at 20 g/l, the temperature of the fermentation chamber was kept constant via A Mannheim boehringer Liquitherm FT Water Bath circulating 35-40 °C water in the surrounding structure of the fermentation chamber to maintain the temperature within the fermentation chamber at

30°C. Cole Palmer Master-flexes were set up to recycle the nutrients at a rate of 15 ml/h. The reason a 2 l airlift bioreactor was used for this study is that it's the first step to scaling up from the initial shake flask fermentation experiment, it's function was to have a continuous supply of oxygen and nutrients to create the ideal environment for the fungus to achieve maximum metabolite (IA/CA) production. Three samples were taken daily from the product recovery chamber and were frozen immediately in the -20°C freezer. The experiment was carried out for 16 days, once fungal biomass had ceased to increase and had reached equilibrium.

3. The Original Table 1 was moved to the appendix,

4. Included more information on reactor set up.
   *Done in Methodology.

Chapter 4

Discussion was completely reworked as per the requirements of the external examiner; please see below for changes made.

Chapter 5

Chapter 5 was also completely reworked as per the requirements of the external examiner; please see below for changes made.

References

The References section was also completely reworked as per the requests from both examiners; it has been changed from Alphabetically ordered to Arranged due to the order of appearance in the text.

External Examination Corrections

1. Literature Review was reworked. I.e. To create more of a logical flow to the sections; inserted more subheadings to allow the literature review to flow better and work on the references.
   *Original

Chapter 1:

1.1 Literature Review:

1.1.2 Global Dilemma:

Worldwide the human population is increasing at an astonishing rate, this therefore creates a high demand for energy that we as humans have become accustomed to and require to maintain our current standard of living. Thereby generating a thirst for new energy resources with the ability to supply power to newly emerging markets, from the fast developing Asian super powers in the form of The Peoples Republic of China and India.

This has left many industries with a huge dilemma, being; how can this planet continue to support the increasing number of inhabitants with a diminishing amount of arable land available. To make the situation worse is that the many advances in current biotechnology research are directed at the production of biofuels. This is to support the growing demand for fuel, especially considering the global recession and the ever-dwindling fossil fuel reserves (4). Filamentous fungi play an important role in the production of biofuels, as they are able to generate high levels of enzymes that are used in hydrolysis of cellulosic biomass (4). However a recent study conducted in Germany stated that if one were to utilize the entire agricultural land available for food production and alter it to produce crops for biofuel production it would only cover 7% of the gas bill, due to the low energy generated from biofuel combustion (16). Alternatively the metabolite producing capabilities of filamentous fungi can be put to better use.

1.1.2 Current Examples of Filamentous Fungi in Industry:

Filamentous fungi are the microorganisms of choice to produce these platform chemicals due to their ability to secrete vast amount of enzymes that generate high metabolite yield (17)(28)(33), filamentous fungi grow in the form of multicellular filaments called hyphae, which generate connected networks with multiple genetically

identical nuclei, which make up the mycelium (17). Filamentous fungi have endless potential regarding their highly efficient metabolite producing capabilities; humans have only been able to harness a fraction of this potential (19). Fungal biotechnology is currently utilized in many aspects of industries, such as for the production of medical supplies, such as; Alexander Fleming's famous antibiotic, Penicillin, which is produced by the fungi *Penicillium chrysogenum*. Lovastatin a cholesterol-lowering drug is produced by *Aspergillus terreus* and also Cyclosporine an immunosuppressant drug used in organ transplant procedures is produced by *Tolypocladium inflatum* (28).

Several other products which filamentous fungi produce are often taken for granted as they as so commonly used. These include products of the food industry, such as; cheese produced by Penicillium spp., soy sauce and sake produced by *Aspergillus oryzae* (5). The main reason why filamentous fungi are utilized in industry is again due to the activity of fungi's' enzymatic excretion capabilities. These extracellular enzymes allow fungal biomass to degrade plant cell walls and to generate large concentrations of metabolites. *Penicillium simplicissimum* is another fungus of interest; it is currently employed by industry due to its highly efficient xylanase activity. It's used in the paper producing process to bleach wood pulp (18). This fungus is commonly known to produce succinate from glucose and studies have been conducted under aerobic and anaerobic conditions to investigate the pathways involved (18). *P. simplicissimum* is observed to secreted low levels of succinate. Succinic acid is the predominant acid produced under anaerobic conditions. Fungal pellet formation was shown to be important in obtaining maximum succinate production rates, and therefore raised the possibility of fumarate respiration as a biochemical mechanism for succinate production under anaerobic conditions (18). However it is known that succinic acid or succinate is produced as an intermediate during the 'The Citric Acid cycle' TCA, where citric acid or citrate is the final product, therefore this fungus is another perfect candidate to evaluate the production of organic acids when utilizing a second generation feedstock such as, lignocellulose substrate obtained from agricultural waste. Another important fungi utilized in industry is *Aspergillus niger* which became the primary microorganism in the production of citric acid, by taking over the burden from extracting citric acid from lemons, *A. niger* can metabolize many carbon sources such as sucrose and or glucose to produce vast amounts of citric acid for a fraction of the cost as opposed to using

lemons (8). The current industrial process involving *Aspergillus niger* and the production of citric acid, which is also a metabolite of interest as listed by the United States Department of Energy. Industrial production of citric acid by filamentous fungi ranges in concentrations of between 200g/L to 240g/L, when utilizing glucose/sucrose is utilized as the main substrate source. *A. niger* generates citric acid via the TCA in the fungus's metabolic pathway, by taking up glucose into the cell and converting it to the three-carbon acid, pyruvate via the glycolytic pathway, which occurs in the cytosol. Subsequently the newly formed pyruvate molecule is decarboxylated to form acetyl-CoA catalyzed by the mitochondrial pyruvate dehydrogenase complex and another pyruvate molecule is carboxylated to oxaloacetate in the cytosol by pyruvate carboxylase (18). The then formed oxaloacetate must be transported into the mitochondrion of the fungi and combined with acetyl-CoA to form citrate. This product is passed out of the mitochondrion and eventually excreted out of the cell. The high yields of citric acid production process are possible because all six carbons of the substrate, glucose or fructose, are conserved in the six-carbon product citric acid, through the glycolytic pathway and the actions of two additional enzymes, pyruvate carboxylase and citrate synthase (18).

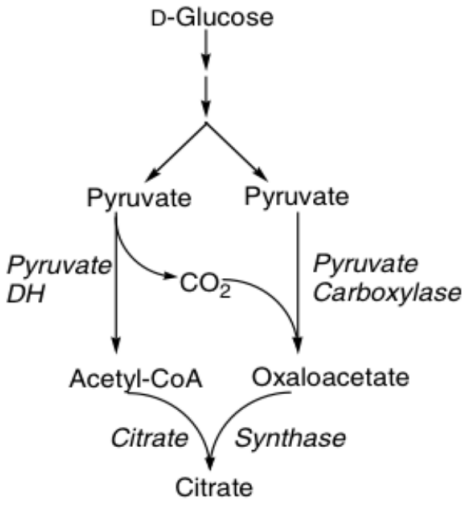

Figure 1. The Simplified Metabolic Pathway Leading to Citric Acid Production in *Aspergillus niger* (18)

Citrate synthase is the terminal enzyme in the citric acid biosynthetic pathway. Mg^{2+} and ATP inhibit the enzyme from *A. niger*, however citrate the product of citrate synthase is not an inhibitor of the enzyme itself. Other enzymes of the citric acid cycle also have significant effects on the production of citric acid, as aconitase inhibition

would theoretically lead to an accumulation of citrate by inhibiting subsequent flux during the citric acid cycle (8)(18). Another enzyme isocitrate dehydrogenase could have a deleterious effect on citric acid production by decarboxylating isocitrate, which is required to be in equilibrium with citrate via aconitase activity. The $NADP^+$ specific isocitrate dehydrogenase has been purified from *A. niger* and found to be present in both the cytosol and mitochondrion (20). It was published that isocitrate dehydrogenase is inhibited by ATP and citrate via chelation of enzymatic Mg^{2+} (20). It was also noted that intracellular Mg^{2+}concentrations would have little effect to the overall process (18). Citric acid accumulated extracellularly to a final concentration of about 1.0M, therefore an active export system must exist to remove citrate from the cytosol, where the normal citrate concentration is only 2 – 30mM (18). Citrate export requires low Mn^{2+}concentrations in the range known to be required for efficient citric acid production (22). On the other hand, citrate import required Mn^{2+}both for induction of expression of the citrate importer as well as for its function (8)(22). These results provide an additional explanation of the multiple effects of Mn^{2+}on the physiology of *A. niger* under citric acid production conditions. There are many uses for citric acid in the food and beverage industry, the carbonated beverage industry accounted for 50% of the total citric acid production in 1990 (18).

1.2 Introduction to Itaconic Acid:

Due to increasing interest in sustainable development within the biotechnological field, industries are making many attempts to replace petrochemical-based monomers with organic substitutes. IA for example is one of the promising substances within the group of organic acids known as platform chemicals, which can be utilized to derive many other useful chemicals from. IA is a white crystalline unsaturated dicarbonic acid with one carboxyl group conjugated to the methylene group, it costs around US$ 2/kg (25). Current organic acid industrial processes involving filamentous fungi currently utilize substrates such as pure glucose, xylose, starch and sucrose etc. These chemicals are relatively expensive, as stated in a review by Okabe, M. in 2009 the carbon source accounts for around 25% of the production costs and most importantly these sources are depleting due to the current circumstances the world faces caused by exponential growth of the human population (14), where more food, fuel and importantly space is required to grow these nutrient sources.

This study is focused on seeking out an alternative source of renewable substrates that can be utilized to maintain the current luxuries we have available, with regards to food and the textile industry. This study mainly focuses on the ability of *A. terreus* to utilize wheat bran and sugar cane bagasse as nutrient sources to generate platform chemicals of interest; i.e. organic acids such as; gluconic acid, succinic acid, fumaric acid, citric acid, lactic acid, L-malic acid and most importantly Itaconic acid. IA produced by *Aspergillus terreus* has been classified by the United States Department of Energy as a platform chemical that has the potential to give rise to many other useful chemicals, such as itaconic diamide, 3 - Methylpyrrollidine and 3 - Methyl THF (18).

1.3 Department of Energy and Platform Chemicals:

The department of energy is an organization that exists in many countries and operates in government to monitor aspects regarding energy and safety of handling nuclear material. Its main responsibilities include; energy conservation, energy-related research, radioactive waste disposal, and domestic energy production. DOE also sponsors many basic and applied scientific research programs. Platform chemicals as mentioned earlier are building block chemicals, which have a high transformation potential into new families of useful molecules. Most platform chemicals are derived from fossil fuels such as petroleum, of which there are limited resources remaining, therefore biotechnology has been utilized in order to generate these chemicals directly from biomass. Biomass as a renewable feedstock offers the opportunity to replace fossil fuels as a source of energy, materials and chemicals. Sugars, oils and other compounds generated from biomass can be converted into platform chemicals directly or as by-products from fuel products in processes analogous to the petrochemical industry today. Improvements and innovations to existing biological and chemical processing of sugars will provide the opportunity for the production of high-value chemicals and products from biomass and reduced reliance on petrochemical-derived products. The platform chemical of choice that this study focuses on is IA and citric acid, due to it being a relatively expensive organic acid and due to its wide use in industry respectively, when compared to its counter-parts, such as, lactic acid, succinic acid etc.

1.4 Substrates Utilized:

The main issue regarding feedstock choice is that all natural resources are being depleting, such primary choice or first generation feedstocks, being petroleum from

which platform chemicals can be derived from are running out. The global human population is increasing thus the demand for energy is always on the rise. To solve this problem one must resolve to other forms of feedstocks, i.e. renewable and abundant second-generation feedstocks. Such feedstocks that have the potential to be utilized in industry to generate platform chemicals include; cellulose powder and milled newspaper were used as a substrate to produce lactic acid by *L. delbruckii* in the presence of cellulases extracted from *Trichoderma reesei* (32)(35). The highest conversion occurred at pH 5 after 120 hours of fermentation and for cellulose (100g/l) and newspaper (50g/l) were 52 and 23g/l respectively (32). Corn stover and sugar cane bagasse has been utilized to generate citric acid via fermentation by *Aspergillus niger* (18) and Jatropha seed cake has been fermented using *Aspergillus terreus* to generate IA, with a maximum yield of 24.45g/l after 120 hours of fermentation (10).

1.5 Fungal Growth Parameters:

Optimum fungal growth experimental parameters as published by Nubel and Rabajak states that an incubation temperature of 37 – 40° C as well as continuous aeration must be maintained for optimum fungal metabolism to occur. A low starting pH within the range of 3–5 is required, followed by a lower operating pH within the range of 2.2 – 3.8. A high glucose concentration is required ideally ranging between 10 to 20%, also sufficient nitrogen must be present, as well as a high magnesium sulfate concentration of 0.5% of the total fermentation volume. Low phosphate is also required to limit the amount of mycelial growth as increased mycelial growth would result in overcrowding and recycling of IA could occur (1), finally adequate levels of the trace metals, which include zinc, copper, and iron is required (1)(18). Since IA production by *A. terreus* shares many of the characteristics of citric acid production by *A. niger*, due to their ability to proliferate well on a variety of monosaccharides, disaccharides, and polysaccharides (18).

Aspergillus niger is known to produce three major metabolites when undergoing fermentation these include; 2 - carboxylmethyl - 3 - hexyl-maleic acid anhydride, 2 - methylene - 3 - hexyl-butanedioic acid and 2 - methylene - 3 - (6 - hydroxyhexyl)-butanedioic acid (3)(12).

1.6 *Aspergillus terreus* and Itaconic Acid:

Examples of other metabolites produced by the filamentous fungal species *Aspergillus terreus,* include; Asterriquinone, which is an antitumor metabolite. *A. terreus* is more

commonly known to produce terrain, which is another fungal metabolite that inhibits the epidermal proliferation of skin equivalents, however the metabolite of interest this study is focusing on is IA from *A. terreus*, which is utilized in the polymer industry where it is an important ingredient in the manufacturing of synthetic fibers, coatings, adhesives, thickeners and binders (9) (12)(18)(34).The first reported source of IA was by the fungus *Aspergillus itaconicus*, which hence coined the name itaconic acid. Following this ground breaking discovery, it was observed that *A. terreus* also produced IA and in higher concentrations (7)(9). Lockwood and Reeves (1945) experimented with more than 300 isolates of *A. terreus* and came to the conclusion that eleven species were efficient producers of IA, when utilizing glucose as a substrate source with a resulting yield of 45% (18). Current work regarding fermentation parameters and the biochemistry of IA production has been performed utilizing strain NRRL 1960 of *A. terreus*. Thereby resulting in an efficient process for the fermentation of sucrose in molasses to IA been patented in 1962 by Nubel and Rabajak with a reported yield 70% 18).

More than 80,000 tons of IA is produced worldwide each year and are sold at a price of around US$ 2/kg (2)(21)(25). The IA production yielded from liquid sucrose is higher than 80 g/l. The widespread use of IA in synthetic resins, synthetic fibers, plastics, rubbers, surfactants, and oil additives has resulted in an increased demand for this product. However, at present, the IA production capacity exceeds the demand because this product has a restricted range of applications. Studies have been actively conducted in different biomedical fields such as; dental, ophthalmic, and drug delivery. The reason behind the research is to extend the range of applications of itaconic acid. Recently, many researchers have attempted to replace the carbon source used for microbial production of IA with cheaper alternative substrates (25). However, there is still a need for new biotechnology innovations that would help to reduce the production costs, such as innovative process development and strain improvement to allow the use of a low-quality carbon source (25).

1.7 Strain Modification:

Aspergillus terreus has been modified in many ways to improve the yield of its metabolites, such as; IA and Lovastatin. Such alterations to *A. terreus* include; strain improvement via mutagenesis as product inhibition, which plays a vital role in fungi metabolism (6)(13). *A. terreus* also has as a form of self preservation as too much acid

production would change the operating parameters of its environment thus inhibiting normal metabolic functions, therefore there is a need to select an IA tolerant strain to overcome this problem which results in low IA yields (15)(26). Screening six hundred and seventy colonies, Yashiro *et al*. isolated the strain TN-484 in 1995. Industrially 85g/l of IA was produced using this strain in a 100-kl scale fermenter utilizing simple media consisting of corn steep liquor, small amounts of minerals and glucose (6)(23)(25)(29)(30). The *Aspergillus terreus* utilized in this study was ordered and obtained from The *Agricultural Research Council* of South Africa in Pretoria.

1.8 High Carbohydrate Content leading to High Acid Production:

Due to the high carbohydrate content made available to the fungus in artificial growth medium the filamentous fungi are able to produce these organic acids at astonishing rates. Naturally these filamentous fungi proliferate in soils, where they would not encounter high concentrations of free sugars; therefore they have evolved a tight regulation of organic acid production (12)(18)(30). Fungi produce organic acids is so that they are able to outcompete and inhibit the growth of competitors by lowering the pH of the surrounding environment thereby restricting growth of competitors. Also the chelating capabilities of citric acid coupled with the increasing solubility of most metal compounds at acidic pH would allow *Aspergillus niger* to proliferate in environments containing metals present in an insoluble state or in low concentrations. The resulting low pH also deters rapidly growing bacteria and many fungi species, which can't grow below pH 3 (18). In the case of *Aspergillus terreus* IA plays a similar role in inhibiting competitors for nutrients (18).

*New

Chapter 1: Literature Review

1.1 Energy Requirements and Fungi in Industry

1.1.3 Global Dilemma:

Worldwide the human population is increasing at an astonishing rate, this therefore creates a high demand for energy that we as humans have become accustomed to and require to maintain our current standard of living. This generates a thirst for new energy resources to supply power to emerging markets, from the fast developing Asian super powers in the form of The Peoples Republic of China and India.

This has left many industries with a dilemma; how can this planet continue to support the increasing number of inhabitants with a diminishing amount of arable land available. To make the situation worse is that the many advances in current biotechnology research are directed at the production of biofuels. This is to support the growing demand for fuel, especially considering the global recession and the ever-dwindling fossil fuel reserves (1). Filamentous fungi play an important role in the production of biofuels, as they are able to generate high levels of enzymes that are used in hydrolysis of cellulosic biomass (1). However a recent study conducted in Germany stated that if one was to utilize the entire agricultural land available for food production and alter it to produce crops for biofuel production it would only cover 7% of the gas bill, due to the low energy generated from biofuel combustion (2). Alternatively the metabolite producing capabilities of filamentous fungi can be put to better use.

1.1.4 Current Examples of Filamentous Fungi in Industry:

1.1.4.1 Filamentous Fungi:

Filamentous fungi are the microorganisms of choice to produce these platform chemicals due to their ability to secrete vast amount of enzymes that generate high metabolite yield (3) (4) (5), they grow in the form of multicellular filaments called hyphae, which generate connected networks with multiple genetically identical nuclei, which make up the mycelium (3). Filamentous fungi have potential regarding their highly efficient metabolite producing capabilities; humans have only been able to harness a fraction of this potential (6).

1.1.4.2 Fungal Biotechnology in Industry:

Fungal biotechnology is currently utilized in many industries, such as for the production of medical supplies, such as; Penicillin, which is produced by the fungi *Penicillium chrysogenum*. Lovastatin a cholesterol-lowering drug is produced by *Aspergillus terreus* and Cyclosporine an immunosuppressant drug used in organ transplant procedures is produced by *Tolypocladium inflatum* (4). Several other products which filamentous fungi produce are often taken for granted as they are so commonly used and include products of the food industry, such as; cheese produced by Penicillium spp., soy sauce and sake produced by *Aspergillus oryzae* (5). The main reason why filamentous fungi are utilized in industry is due to the high activity of

fungi's enzymatic secretions. These extracellular enzymes allow fungal biomass to degrade plant cell walls and to generate large concentrations of metabolites. *Penicillium simplicissimum* is another fungus of interest; it is currently employed by industry due to its high xylanase activity. It is used in the paper producing process to bleach wood pulp (8). This fungus is commonly known to produce succinate from glucose and studies have been conducted under aerobic and anaerobic conditions to investigate the pathways involved (8). *P. simplicissimum* has been observed to secrete low levels of succinate. Succinic acid is the predominant acid produced under anaerobic conditions. Fungal pellet formation was shown to be important in obtaining maximum succinate production rates, and therefore raised the possibility of fumarate respiration as a biochemical mechanism for succinate production under anaerobic conditions (8). However it is known that succinic acid or succinate is produced as an intermediate during the The Citric Acid cycle (TCA), where citric acid or citrate is the final product. Therefore this fungus is another perfect candidate to evaluate the production of organic acids when utilizing a second-generation feedstock such as lignocellulose substrate obtained from agricultural waste.

1.1.4.3 *Aspergillus Niger* and Citric Acid

Another important fungi utilized in industry is *Aspergillus niger* which became the primary microorganism in the production of citric acid, exceeding production from extracting citric acid from lemons. *A. niger* can metabolize many carbon sources such as sucrose and or glucose to produce vast amounts of citric acid for a fraction of the cost as opposed to using lemons (9) (22). The current industrial process using *Aspergillus niger* and the production of citric acid, which is also a metabolite of interest as listed by the United States Department of Energy, ranges between 200 g/l to 240 g/l, when utilizing glucose/sucrose is utilized as the main substrate source. There are many uses for citric acid in the food and beverage industry, the carbonated beverage industry accounted for 50% of the total citric acid production in 1990 (8) (22). *A. niger* generates citric acid via the TCA in the fungus's metabolic pathway, by taking up glucose into the cell and converting it to the three-carbon acid, pyruvate via the glycolytic pathway, which occurs in the cytosol. Subsequently the newly formed pyruvate molecule is decarboxylated to form acetyl-CoA catalyzed by the mitochondrial pyruvate dehydrogenase complex and another pyruvate molecule is

carboxylated to oxaloacetate in the cytosol by pyruvate carboxylase (8). The oxaloacetate is transported into the mitochondrion of the fungi and combined with acetyl-CoA to form citrate. This product is passed out of the mitochondrion and eventually secreted of the cell. The high yields of citric acid production process are possible because all six carbons of the substrate, glucose or fructose, are conserved in the six-carbon product citric acid, through the glycolytic pathway and the actions of two additional enzymes, pyruvate carboxylase and citrate synthase (8) (22).

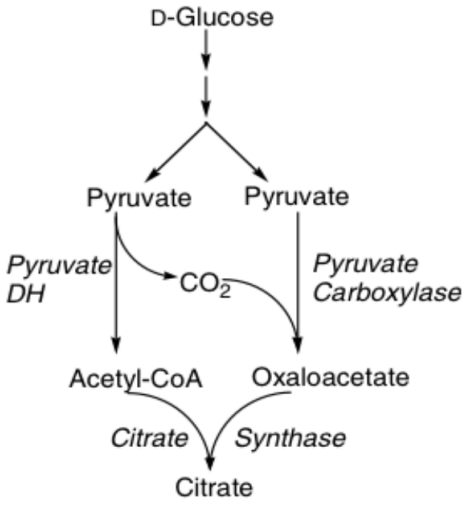

Figure 1. The Simplified Metabolic Pathway Leading to Citric Acid Production in *Aspergillus niger* (8)

Citrate synthase is the end terminal enzyme in the citric acid biosynthetic pathway. Mg^{2+} and ATP inhibit the enzyme from *A. niger*, however citrate the product of citrate synthase is not an inhibitor of the enzyme itself. Other enzymes of the citric acid cycle also have significant effects on the production of citric acid, as aconitase inhibition would theoretically lead to an accumulation of citrate by inhibiting subsequent flux during the citric acid cycle (8) (9). Another enzyme, isocitrate dehydrogenase could have a deleterious effect on citric acid production by decarboxylating isocitrate, which is required to be in equilibrium with citrate via aconitase activity. The $NADP^+$ specific isocitrate dehydrogenase has been purified from *A. niger* and found to be present in both the cytosol and mitochondrion (10). It was published that isocitrate dehydrogenase is inhibited by ATP and citrate via chelation of enzymatic Mg^{2+} (10). It was also noted that intracellular Mg^{2+}concentrations would have little effect to the overall process (8). Citric acid accumulated extracellularly to a final concentration of about 1.0M, therefore an active export system must exist to remove citrate from the

cytosol, where the normal citrate concentration is only 2 – 30mM (8). Citrate export requires low Mn^{2+}concentrations in the range known to be required for efficient citric acid production (11). On the other hand, citrate import required Mn^{2+}both for induction of expression of the citrate importer as well as for its function (9) (11). These results provide an additional explanation of the multiple effects of Mn^{2+}on the physiology of *A. niger* under citric acid production conditions. *Aspergillus niger* is known to produce three major metabolites when undergoing fermentation. These include 2 - carboxylmethyl - 3 - hexyl-maleic acid anhydride, 2 - methylene - 3 - hexyl-butanedioic acid and 2 - methylene - 3 - (6 - hydroxyhexyl)-butanedioic acid (12) (13).

1.2 Introduction to Itaconic Acid:

Due to increasing interest in sustainable development within the biotechnological field, industries are making many attempts to replace petrochemical-based monomers with organic substitutes. IA ($C_5H_6O_4$) for example is one of the promising substances within the group of organic acids known as platform chemicals, which can be utilized to derive many other useful chemicals from. IA is a white crystalline unsaturated dicarbonic acid with one carboxyl group conjugated to the methylene group, it costs around US$ 2/kg (14). Current organic acid industrial processes involving filamentous fungi currently utilize substrates such as pure glucose, xylose, starch and sucrose etc. These chemicals are relatively expensive. As stated in a review by Okabe, M. in 2009 the carbon source accounts for around 25% of the production costs and most importantly these sources are dwindling.

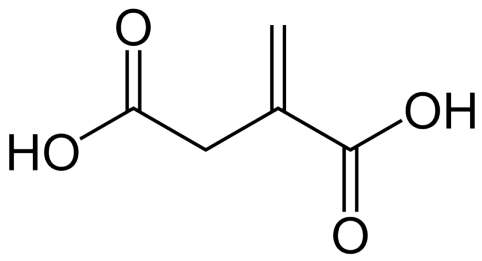

Figure 2. Chemical structure of Itaconic Acid

This study is focused on seeking out an alternative source of renewable substrates that can be utilized to maintain the current luxuries we have available, with regards to food and the textile industry. This study mainly focuses on the ability of *A. terreus* to utilize wheat bran and sugar cane bagasse as nutrient sources to generate platform chemicals of interest; i.e. organic acids such as; gluconic acid, succinic acid, fumaric acid, citric

acid, lactic acid, L-malic acid and most importantly IA. IA produced by *Aspergillus terreus* has been classified by the United States Department of Energy as a platform chemical that has the potential to give rise to many other useful chemicals, such as itaconic diamide, 3 - Methylpyrrollidine and 3 - Methyl THF (8).

1.3 Department of Energy and Platform Chemicals:

The department of energy is an organization that exists in many countries and operates in government to monitor aspects regarding energy. Its main responsibilities include; energy conservation, energy-related research, radioactive waste disposal, and domestic energy production. The South African DOE ensures that diverse energy resources are made available, in sustainable quantities and at affordable prices in support of economic growth and poverty alleviation. DOE also sponsors many basic and applied scientific research programs. Platform chemicals as mentioned earlier are building block chemicals, which have a high transformation potential into new families of useful molecules. Most platform chemicals are derived from fossil fuels such as petroleum, of which there are limited resources remaining, therefore biotechnology has been utilized in order to generate these chemicals directly from biomass. Biomass as a renewable feedstock offers the opportunity to replace fossil fuels as a source of energy, materials and chemicals. Sugars, oils and other compounds generated from biomass can be converted into platform chemicals directly or as by-products from fuel products in processes analogous to the petrochemical industry today. Improvements and innovations to existing biological and chemical processing of sugars will provide the opportunity for the production of high-value chemicals and products from biomass and reduced reliance on petrochemical-derived products. The platform chemical of choice that this study focuses on is IA and citric acid, due to it being a relatively expensive organic acid and due to its wide use in industry respectively, when compared to its counter-parts, such as, lactic acid, succinic acid etc. Renewable energy sources, other than biomass (the energy from plants and plant-derived materials), have not yet been exploited optimally in South Africa.

1.4 Substrates Utilized:

Renewable and abundant second-generation feedstocks, such as those that have the potential to be utilized in industry to generate platform chemicals include; cellulose powder and milled newspaper. These were used as a substrate source to produce lactic

acid in a study by *L. delbruckii* in the presence of cellulases extracted from *Trichoderma reesei* (15) (16). The highest conversion occurred at pH 5 after 120 hours of fermentation and for cellulose (100 g/l) and newspaper (50 g/l) were 52 and 23 g/l respectively (15). Corn stover and sugar cane bagasse has been utilized to generate citric acid via fermentation by *Aspergillus niger* (8) and Jatropha seed cake has been fermented using *Aspergillus terreus* to generate IA, with a maximum yield of 24.5 g/l after 120 hours of fermentation (17). However due to their availability, sugar cane bagasse and wheat bran were chosen to be used for this study.

1.5 Fungal Growth Parameters:

Optimum fungal growth experimental parameters as published by Nubel and Rabajak include an incubation temperature of 37 – 40° C as well as continuous aeration will allow for optimum fungal metabolism to occur. A low starting pH within the range of 3–5 is required, followed by a lower optimum operating pH within the range of 2.2 – 3.8. A high glucose concentration is required ideally ranging between 10 to 20%. Sufficient nitrogen must be present, as well as a high magnesium sulfate concentration of 0.5% of the total fermentation volume. Low phosphate levels are required to limit the amount of mycelial growth as increased mycelial growth would result in overcrowding and recycling of IA could occur (18). Finally adequate levels of the trace metals, which include zinc, copper, and iron are required (8) (18). IA production by *A. terreus* shares many of the characteristics of citric acid production by *A. niger* due to their ability to proliferate well on a variety of monosaccharides, disaccharides, and polysaccharides (8).

1.6 *Aspergillus terreus* and Itaconic Acid:

Examples of other metabolites produced by the filamentous fungal species *Aspergillus terreus,* include; Asterriquinone, which is an antitumor metabolite. *A. terreus* is more commonly known to produce terrain, which is another fungal metabolite that inhibits the epidermal proliferation of skin equivalents, which is an in vitro skin model using to conduct experiments on processes involving the skin, such as wound healing and keratinocyte migration. The metabolite of interest this study is focusing on is IA from *A. terreus*. This is utilized in the polymer industry where it is an important ingredient in the manufacturing of synthetic fibers, coatings, adhesives, thickeners and binders (8) (13) (19) (20) (23). The first reported production synthesis of IA was by the fungus *Aspergillus itaconicus*, which hence coined the name itaconic acid.

Following this discovery, it was observed that *A. terreus* also produced IA and in higher concentrations (7) (9) (23). Lockwood and Reeves (1945) experimented with more than 300 isolates of *A. terreus* and came to the conclusion that eleven species were efficient producers of IA, when utilizing glucose as a substrate source with a resulting yield of 45% (8). Current work regarding fermentation parameters and the biochemistry of IA production has been performed utilizing strain NRRL 1960 of *A. terreus*.

More than 80,000 tons of IA is produced worldwide each year and are sold at a price of around US$ 2/kg (2) (14) (21). The IA production yielded from liquid sucrose is higher than 80 g/l. The widespread use of IA in synthetic resins, synthetic fibers, plastics, rubbers, surfactants, and oil additives has resulted in an increased demand for this product. However, at present, the IA production capacity exceeds the demand because this product has a restricted range of applications. Studies have been actively conducted in different biomedical fields such as; dental, ophthalmic, and drug delivery. The reason behind the research is to extend the range of applications of itaconic acid. Recently, many researchers have attempted to replace the carbon source used for microbial production of IA with cheaper alternative substrates (33). However, there is still a need for new biotechnology innovations that would help to reduce the production costs, such as innovative process development and strain improvement to allow the use of a low-quality carbon source (33).

1.7 High Carbohydrate Content leading to High Acid Production:

Due to the high carbohydrate content made available to the fungus in artificial growth medium the filamentous fungi are able to produce these organic acids at high rates. Naturally these filamentous fungi proliferate in soils, where they would not encounter high concentrations of free sugars; therefore they have evolved a tight regulation of organic acid production (8) (13) (24). Fungi produce organic acids to outcompete and inhibit the growth of competitors by lowering the pH of the surrounding environment thereby restricting growth of competitors. In addition the chelating capabilities of citric acid coupled with the increasing solubility of most metal compounds at acidic pH would allow *Aspergillus niger* to proliferate in environments containing metals present in an insoluble state or in low concentrations. The resultant low pH also deters rapidly growing bacteria and many fungi species, which can't grow below pH 3 (8). In

the case of *Aspergillus terreus* IA plays a similar role in inhibiting competitors for nutrients (8).

1.8 Strain Modification:

Aspergillus terreus has been modified in many ways, including strain selective breeding and genetic modification to improve the yield of its metabolites, such as; IA and Lovastatin. Such alterations to *A. terreus* include; strain improvement via mutagenesis as product inhibition, which plays a vital role in fungi metabolism (25) (26). *A. terreus* also has as a form of self preservation as too much acid production would change the operating parameters of its environment thus inhibiting normal metabolic functions, therefore there is a need to select an IA tolerant strain to overcome this problem which results in low IA yields (27) (28). Screening six hundred and seventy colonies, Yashiro *et al*. isolated the strain TN-484 in 1995. Industrially 85 g/l of IA was produced using this strain in a 100-kl scale fermenter utilizing simple media consisting of corn steep liquor, small amounts of minerals and glucose (14) (23) (24) (25) (29).

1.8.1 Genetic Transformation:

The general method of genetic transformation of filamentous fungi, begins firstly by removing the permeability barrier presented by the cell wall of the fungus, either by treatment with lithium acetate or by enzyme degradation using crude extracts found in snail gut (29) or by using a *Trichoderma* extract commercially known as Novozym 234 (29) to produce protoplasts. Some filamentous fungi require a mixture of enzyme extracts to produce a sufficient amount of protoplasts. This is followed by the removal of the cell wall by utilizing an osmotic stabilizer such as 0.6 potassium chloride or 1.2M sorbitol, the choice of which stabilizer is used depends on the species of fungus, the two Aspergillus species, *A. niger* and *A. terreus* make use of 1.2M and 0.55M sorbitol respectively as their osmotic stabilizers (29). DNA is then added to the protoplast suspension in the presence of 10-50mM calcium chloride and then followed by the addition of a solution of polyethylene glycerol, which initiates the uptake of the DNA by the protoplasts. The treated protoplasts are then allowed to proliferate of selective medium that selects for only the transformed cells (30). The selectable markers that can be used are genes that complement a nutrition requirement mutation, which allows the growth of the fungus in absence of the required nutritional factor (27).

1.8.2 Limitations regarding Fungal Genetic transformation:

There is a limitation to this methodology as it requires the recipient fungal species to be a mutant strain and this is often undesirable in industrial strains (20). The solution to this drawback is the use of positive selection systems, which is based on supplementation of antimetabolites, for example; oligomycin resistance, prokaryotic antibiotic resistance genes such as kanamycin or G418 resistance (29). Another alternative system utilized the *amdS* gene isolated from *A. nidulans, which* codes for acetamidase, an enzyme that allows the growth on acetamide as a sole nitrogen or carbon source (7). Several other fungal species such as *A. niger* lack the *amdS* gene, which allows for easier selection of transformants, therefore making it a useful marker for transformation (30). The frequency of transformants is 10-100 stable transformants per μg. A similar technique to transform industrial fungi is to disrupt the gene functioning by homologous recombination using a defective gene or a gene that has been disrupted by a selectable marker, this method produces null mutations that eliminate the chance of undesirable traits developing in industrial strains of filamentous fungi (26) (27).

1.8.3 Future Prospects and other Transformation Methods:

Future prospects involving the heterologous expression of genes in filamentous fungi, where current gene expression systems rely on either powerful inducible or constitutive promoters, and homologous promoters are preferred to the production host as mentioned above to enhance organic acid production (31). There is however a disadvantage involving the constitutive expression of housekeeping gene promoters is that the promoters are functional during growth and therefore unsuitable for over expression of foreign proteins that might be toxic to the host cells. Another major disadvantage of inducible gene promoters, such as the powerful cbh1 gene promoter, is their repression by glucose and other carbon sources therefore they can severely affect the yield of protein secreted. These promoters can be regulated by the induction of carbon and nitrogen compounds and the pH of the growth medium (24).

Experiments have shown a novel metabolically independent expression system that can be regulated by oestrogenic compounds and it has been tested in *Aspergillus*. *A. nidulans* and *A. niger* transformants are highly sensitive with regards to acquiring oestrogen responsive elements or low levels of oestrogenic substances such as diethylstilbestrol, this therefore allows this research to be utilized in the detection of

xenoestrogens in food and in the environment (4). Other experiments involving the modification of the traditional method for cultivating filamentous fungi for protein production have been researched. Whereas instead of a submerged fermentation occurring in liquid media, solid-state fermentation, which is supported by high yields of the secreted metabolites, obtained in these systems can be utilized instead (16). Due to the physical mode of growth and gene regulation of fungi in solid-state fermentation, differ from fungi grown in liquid cultures (4).

Another vital factor in the development of transgenic fungal expression systems is the establishment of effective transformation protocols across fungal genera to add efficiency and flexibility to high throughput screening for evolved proteins and different metabolites of interest (21). Advancement in observation procedures including; microscopy technologies, such as fluorescence resonance energy transfer and fluorescent life time imaging, allows scientists to visualize metabolic pathways and protein–protein interactions in living systems therefore allowing greater understanding with regards to these areas, as fluorescent imaging, coupled with molecular biology, bioinformatics, biochemistry, genomics and proteomics, will redesign the concept of microbial metabolite production (21).

Another transformation method, which is being used more commonly in experiments involving the genetic transformation of filamentous fungi, is biolistic bombardment and inclusion of the seven barrels Hepta adaptor system has noticeably increased the number of transformations achieved (32). *Agrobacterium*-mediated transformation method has also gained a lot of interest, and claims of up to 100–1000 times greater efficiency of the T-DNA transfer and chromosomal integration, compared with conventional methods mentioned before, as one is aware that the T-DNA of the bacterium *Agrobacterium tumefaciens* can be transferred to plants, yeasts, fungi and human cells (26). We are entering an era of accelerating development of novel fungal fermentations and transformations due to the exponential increase and development of information and tools to exploit nature. Therefore there are many methods our study can be expanded with regards to higher yield of organic acids from agricultural waste.

1.9 Analysis Using High-Performance Liquid Chromatography:

The HPLC utilized a column that holds chromatographic packing material, i.e. the stationary phase, in our case being an ion exchange column is used, which operates on

the basis of selective exchange of ions in the sample with counter-ions in the stationary phase (35). Ion exchange is performed with columns containing charge-bearing functional groups attached to a polymer matrix. The functional ions are permanently bonded to the column and each has a counter-ion attached. The sample will then be retained via replacing the counter-ions of the stationary phase with its own ions. The sample is eluted from the column by changing the properties of the mobile phase do that the mobile phase will now displace the sample ions from the stationary phase. A pump is utilized to maintain the pressure of between 40.2 – 41.2 bar and a flow rate of 0.8 ml/min for the mobile phase, which was 0.001 M H_2SO_4, which was pumped through the column, and a Refractive Index Detector of 8.85 nRIU was used to show the retention times of the molecules (34). The concept behind the HPLC is to compare the retention time of each of the molecules within the sample and compare it to that of the calibrated standards of citric acid and itaconic acid, the retention time varies depending on the interactions between the stationary phase, the molecules being analyzed, and the solvent used. As the samples which have stronger interactions with the stationary phase than with the mobile phase will elute from the column less quickly, and thus have a longer retention time.

2. Reworked the Aims and Objectives.
   *Original

Aim:

The objective of the study is to produce itaconic acid (IA) and citric acid (CA) in levels of excess of 60g/l from sugar cane bagasse and or wheat bran, which has been chemically digested to produce a hydrolysate, which *A. terreus* will utilize, the fermentation and itaconic acid production will take place in an Airlift bioreactor, the pH and dissolved oxygen parameters will be constantly monitored using a DataTaker DT50 machine and samples containing itaconic acid will be assayed using High Performance Liquid Chromatography.

Objectives:

1. The successfully hydrolyze wheat bran and sugar cane bagasse via chemical digestion to provide a nutrient source for the fungus to utilize.

2. To successfully set up and operate a 5L airlift bioreactor that recycles the nutrients.
3. To utilize this bioreactor to produce metabolites of interest, namely; IA and CA in concentrations in excess of 60g/l.

*New

Aim:

The objective of the study is to produce itaconic acid (IA) and citric acid (CA) in levels of excess of 60g/l from chemically digested sugar cane bagasse and or wheat bran hydrolysate, using *A. terreus*, the fermentation and itaconic acid production will take place in an Airlift bioreactor, the pH and dissolved oxygen parameters will be constantly monitored using a DataTaker DT50 machine and samples will be assayed using High Performance Liquid Chromatography.

Objectives:

1. To successfully hydrolyze wheat bran and sugar cane bagasse and provide a nutrient source for the fungus to utilize.
2. To successfully set up and operate a 5 l airlift bioreactor that recycles the nutrients.
3. To utilize this bioreactor to produce metabolites of interest, namely; IA and CA in concentrations in excess of 60 g/l.

3. The materials and methods were reworked, as logical flow is essential. Also listed the equipment used etc.

   *Original

Chapter 2:

Materials and Methods:

2.1 Bioreactor Nutrient Medium Formulation:

The methodology utilized for this study would initially begin with the preparation of the growth medium or hydrolysate. Wheat bran a form of agricultural waste was obtained from Prof. Gray's biotechnology lab in the Oppenheimer Life Sciences building at the University of the Witwatersrand's East Campus and used it as

the raw materials due to its abundance and accessibility and in the case of other agricultural waste the inability of humans to utilize it as a nutrient source. Wheat bran like sugar cane bagasse consists mainly of lignocellulose, which is made up of hemicellulose, cellulose and lignin. Other forms of agricultural waste that contain lignocellulose include; wood residues such as sawmill and paper mill waste, municipal paper waste, agricultural residues including corn stover and dedicated energy crops which are mostly composed of fast growing tall, woody grasses. All of these above mentioned substrate sources are of very poor quality with regards to nutritional value, and the current method of utilization by combustion to generate energy. In current industrial production of organic acids, the energy generated via combustion of these lignocellulose sources are used to heat the distilling vats.

Lignocellulose is a rigid structure and most fungi aren't able to gain access to the sugars trapped within the lignin outer lining, therefore creating a requirement of chemical pretreatment to release the encapsulated sugars. Chemical digestion of the wheat bran and sugar cane bagasse involves, pretreatment of biomass using concentrated acid such as Sulphuric acid the ratio of approximately 2:1 (w/w) with regards to acid and lignocellulose pulp. Thereby for the first batch of hydrolysate, 336ml of 70% Sulphuric acid was added to 200g of bran within a 5 litre Schott bottle and mixture was then rolled for 24 hours at 30ºC using a bench top shaking incubator from the Chemical Engineering Department in the Richard Ward Building on The University of the Witwatersrand's East Campus. Following this the oak coloured wheat bran became a black tar-like mixture after 24 hours. 2400 ml of water was then added to wash the mixture and allow for hydrolysis to occur, where the glycosidic bonds between the lignocelluloses' polysaccharides are to be cleaved, this resulting mixture was then transferred and incubated at 95 ºC for 3 hours in the same bench top incubator with no agitation. The 5-litre Schott bottle was partially opened to allow for the newly formed hydrogen gas to escape as hydrolysis takes place. Calcium hydroxide ($CaOH_2$) was then added to achieve neutralization of the suspension resulting in a pH ranging between of 5.5–6.5 is reached, sulfate removal occurs simultaneously due to the addition of calcium hydroxide. At this point the fluid thickened to a cement-like liquid. This slurry was then centrifuged for 20 min. at 6000 rpm to achieve an RCF of approximately 16000m/s^2 using a Beckman J2-21 centrifuge to separate the $CaSO^4$ and other solids (lignin) from the liquid containing the sugars

released from the biomass. The glucose and xylose concentration was analyzed using Prestige Smart System Test Strips provided by Dr. Karl Rumbold and the result collated with that in literature being 25.35 g/l for glucose and 26.84 g/l for xylose released from de-starched bran. The supernatant containing the sugars was then removed, autoclaved at 121° C and stored at -20 ºC, to inhibit the proliferation of any microbes which would deplete the glucose and xylose released from the wheat bran, thereby the hydrolysate was frozen until it was used for fermentation.

The second-generation feedstock utilized in the Airlift Bioreactor experiment setup is sugar cane bagasse, which was kindly donated by the Illovo Sugar Company based in KwaZulu Natal. The lignocellulose source was then chemically digested in the same manner as wheat bran the only difference being that the hydrolysate production will be up scaled, i.e. 840ml of 70% Sulphuric acid was added to 500g of sugar cane bagasse within a 10 litre Schott bottle and mixture was then rolled for 24 hours at 30ºC using a bench top shaking incubator from the Chemical Engineering Department in the Richard Ward Building on The University of the Witwatersrand's East Campus. The oak coloured bagasse also became a black tar-like mixture after 24 hours. 4800 ml of water was then added to wash the mixture and allow for hydrolysis to occur, this occurred in a 20L beaker, where the glycosidic bonds between the lignocelluloses' polysaccharides are to be cleaved, this resulting mixture was then transferred and incubated at 95 ºC for 3 hours in the same bench top incubator with no agitation. Calcium hydroxide ($CaOH_2$) was not used to neutralize the suspension, instead 1.76l Ammonium Nitrate was added to achieve neutralization of the suspension resulting in a pH ranging between of 5.5–6.5 being reached. The mixture was then filtered overnight to remove all the unwanted debris left over from the sugar cane bagasse.

2.2 Fungal Strain Pre-Experiment Preparation:

Aspergillus terreus was ordered and obtained from The *Agricultural Research Council* of South Africa in Pretoria. The fungus was then sub-cultured onto 15% MEA (Malt Extract Agar) and allowed to proliferate in the 30° C incubation room for 24 hours, thereby generating 30 plates with 10 plates of the single fungal species, which was then refrigerated at 4° C.

2.3 Additional Minerals:

The additional supplement minerals were made up to concentrations in de-mineralized water, these include; 8 g/l Ammonium chloride, 0.3 g/l magnesium chloride hexahydrate, 40 mg/l EDTA (Disodium salt), 2 mg/l zinc sulphate heptahydrate, 1 mg/l calcium chloride dihydrate, 0.2 mg/l sodium molybdate dihydrate, 0.4 mg/l cobalt (II) chloride hexahydrate, 2 mg/l copper (II) sulphate pentahydrate, 0.5 g/l ammonium sulphate, 15 mg/l ferrous sulphate heptahydrate and 1 mg/l manganese (II) chloride tetrahydrate. The ph was adjusted using di potassium hydrogen phosphate and di sodium hydrogen phosphate anhydrous, The above minerals and ions were made up separately and autoclaved, except for the EDTA and the ferrous sulphate heptahydrate which were sterile filtered due to precipitation forming after being autoclaved. These additional minerals helped provide a broad-spectrum growth medium when coupled with the hydrolysate, which housed the sugars that would be metabolized to form the organic acids.

2.4 Inoculation:

The fungus was allowed to sporulate, then utilizing distilled water and sterile glass beads the spores were washed and counted using a hemocytometer, then approximately 10^6 spores of each species were seeded into triplicate 1000 ml pre-autoclaved Erlenmeyer flasks containing the 150 ml minerals and 100 ml of the hydrolysate, thereby making up a total volume of 250 ml within each Erlenmeyer flask. Then shake flask fermentation at 250 rpm was implemented in the 30° C incubation room, as the flasks would be constantly shaken to ensure that continuous aeration occurs which is vital to achieve maximum metabolite production.

2.5 Shake Flask Fermentation:

The experiment was carried out over a period of 5 days during which rapid sampling was performed at 0H, 24H, 48H, 72H and 96H, where 10 ml of each sample was removed and centrifuged at 25G's and the supernatant was then sterile filtered using 45μm filters, to discard of any remaining fungal hyphae. The samples were then frozen utilizing dry ice to quench the metabolism. The pellet obtained from centrifugation was then placed into the drying oven in the Biology Building room 223 for 24 hours at 72° C after which it was weighted out and used to calculate the amount of fungal biomass. After 96 hours the experiment was terminated due to the fungal biomass reaching equilibrium. Wet pellets were stored on ice and used for the bioreactor part of the study.

2.6 Bioreactor set-up and preparation:

The Airlift Bioreactor was obtained from Prof. Vince Gray's lab in OLS, it consists of a 2 liter fermentation chamber connected to another 5 liter collection chamber and the nutrients are recycled into a 10 liter feedstock barrel. The entire bioreactor will be set up to run independently and the pH will be maintained to be between 3-4 and dissolved oxygen levels will be monitored using a DataTaker DT50 machine, in order to maintain it at 20g/l. A wire gauze chamber with 1-2mm spaces was placed in the center of the fermentation chamber and the pellets were seeded directly into this chamber to prevent the pellets from depositing on the oxygen feeding tubes and nutrient entry point, therefore preventing blockage of oxygen flow, media agitation and nutrients from entering the fermentation chamber and also, most importantly to prevent the fungi from flowing into the neighboring product collection chamber, as problems were encountered beforehand with the first attempt to run this bioreactor where the fungi mycelium grew over the exit outlet connecting the pipe from the fermentation chamber to product collection enclosure and therefore created a water proof seal, and with the pump rate continuously pumping fresh and recycled nutrients into the fermentation chamber all of the fluid had no place to escape the fermentation chamber therefore it over flowed and exploded out the top of the bioreactor damaging the airlift bioreactor's lid, however with the wire gauze in place it prevented this form happening in later experiments.

*Note: Figure was removed to save space.

2.6.1 Maintenance of optimum experiment parameters:

Temperatures of the bioreactor was be maintained by passing warm water around the fermentation chamber from the 60 degrees water bath, therefore heating the fermentation chamber to 30 degrees Celsius. The pH will be adjusted using nitric acid, and NaOH whilst the oxygen levels can be adjusted accordingly on the Airlift Bioreactor chamber itself. The oxygen input levels can also be controlled by agitation of the media. Due to it being and airlift bioreactor shear forces would be at a minimum, thus allowing for optimum proliferation of the submerged pellets.

2.7 Product recovery:

The itaconic acid produced in the fermentation chamber would be extracted in the collection chamber in 10 ml samples, then the amounts of itaconic acid produced will then be assayed using High Performance Liquid Chromatography and finally the

samples will then underwent rapid dehydration in the drying oven in the 3rd year lab. To extract the itaconic acid in powder form.

2.8 Analysis:

The frozen fungal product samples were then defrosted at room temperature and analyzed utilizing High-Performance Liquid chromatography, which is an advanced form of column chromatography where the Bio-Rad 125-0115 column, with a length of 150mm, a diameter of 7.8mm and a particle size of 9mm, which was provided by Dr. Karl Rumbold. This column was used to separate, identify, and quantify the compounds present within the samples taken from the fermentation experiments. The HPLC utilized a column that holds chromatographic packing material, i.e. the stationary phase, in our case being an ion exchange column is used, which operates on the basis of selective exchange of ions in the sample with counter-ions in the stationary phase. Ion exchange is performed with columns containing charge-bearing functional groups attached to a polymer matrix. The functional ions are permanently bonded to the column and each has a counter-ion attached. The sample will then be retained via replacing the counter-ions of the stationary phase with its own ions. The sample is eluted from the column by changing the properties of the mobile phase do that the mobile phase will now displace the sample ions from the stationary phase. A pump is utilized to maintain the pressure of between 40.2 – 41.2 bar and a flow rate of 0.8 ml/min for the mobile phase, which was 0.001 M H_2SO_4, which was pumped through the column, and a Refractive Index Detector of 8.85 nRIU was used to show the retention times of the molecules. The concept behind the HPLC is to compare the retention time of each of the molecules within the sample and compare it to that of the calibrated standards of citric acid and itaconic acid, the retention time varies depending on the interactions between the stationary phase, the molecules being analyzed, and the solvent used. As the samples which have stronger interactions with the stationary phase than with the mobile phase will elute from the column less quickly, and thus have a longer retention time.

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Chemical pretreatment of the lignin is required to release the encapsulated sugars. Chemical digestion of the wheat bran and sugar cane bagasse involves, pretreatment of biomass using concentrated acid such as sulphuric acid in a ratio of approximately 2:1 (w/w) with regards to acid and lignocellulose pulp. For the first batch of hydrolysate, 336 ml of 70% sulphuric acid was added to 200 g of bran within a 5 l Schott bottle and the mixture was then rolled for 24 hours at 30ºC using a bench top shaking. Following this the oak coloured wheat bran became a black tar-like mixture after 24 hours. 2400 ml of water was then added to wash the mixture and allow for hydrolysis to occur, where the glycosidic bonds between the lignocelluloses' polysaccharides were cleaved. This resulting mixture was then transferred and incubated at 95 ºC for 3 hours in the same bench top incubator with no agitation. The 5 l Schott bottle was partially opened to allow for the newly formed hydrogen gas to escape as hydrolysis takes place. Calcium hydroxide ($CaOH_2$) was then added to achieve neutralization of the suspension resulting in a pH ranging between of 5.5–6.5 is reached and sulfate removal occurs simultaneously due to the addition of calcium hydroxide. At this point the fluid thickened to a wet cement-like liquid. This slurry was then centrifuged for 20 min. at 6000 RPM to achieve an RCF of approximately 16000 m/s^2 using a Beckman J2-21 centrifuge to separate the $CaSO_4$ and other solids (lignin) from the liquid containing the sugars released from the biomass. The glucose and xylose concentration

were analyzed using Prestige Smart System Test Strips and the result collated with that in literature being 25.35 g/l glucose and 26.84 g/l xylose were released from the chemically digested wheat bran. The supernatant containing the sugars was then removed, autoclaved at 121° C and stored at -20 ºC to inhibit the proliferation of any microbes which would deplete the glucose and xylose released from the wheat bran. Thereafter the hydrolysate was frozen until it was used for fermentation.

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The additional supplement minerals were made up to concentrations in de-mineralized water, these include; 8 g/l Ammonium chloride, 0.3 g/l magnesium chloride hexahydrate, 40 mg/l EDTA (Disodium salt), 2 mg/l zinc sulphate heptahydrate, 1 mg/l calcium chloride dihydrate, 0.2 mg/l sodium molybdate dihydrate, 0.4 mg/l cobalt (II) chloride hexahydrate, 2 mg/l copper (II) sulphate pentahydrate, 0.5 g/l ammonium sulphate, 15 mg/l ferrous sulphate heptahydrate and 1 mg/l manganese (II) chloride tetrahydrate. The ph was adjusted using di potassium hydrogen phosphate and

anhydrous di sodium hydrogen phosphate, The above minerals and ions were made up separately and autoclaved, except for the EDTA and the ferrous sulphate heptahydrate which were sterile filtered due to precipitation forming after being autoclaved. These additional minerals helped provide a broad-spectrum growth medium when coupled with the hydrolysate, which housed the sugars that would be metabolized to form the organic acids.

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The fungus was allowed to sporulate, then utilizing distilled water and sterile glass beads the spores were washed and counted using a hemocytometer, then approximately 10^6 spores of were seeded into triplicate 1000 ml pre-autoclaved Erlenmeyer flasks containing the 150 ml minerals and 100 ml of the hydrolysate, thereby making up a total volume of 250 ml within each Erlenmeyer flask. Following this shake flask fermentation at 250 RPM was implemented in the 30° C incubation room, as the flasks would be constantly shaken to ensure that continuous aeration occurs which is vital to achieve maximum metabolite production.

2.5 Shake Flask Fermentation:

Shake flask fermentation was carried out over a period of 5 days during which rapid sampling was performed at 0 HRS, 24 HRS, 48 HRS, 72 HRS and 96 HRS, where 10 ml of each sample was removed and centrifuged at 5000 RPM and the supernatant was then sterile filtered using 45 μm filters, to discard of any remaining fungal hyphae. The samples were then frozen utilizing dry ice to quench the metabolism. The pellet obtained from centrifugation was then placed into the drying oven for 24 HRS at 72 °C after which it was weighed out and used to calculate the amount of fungal biomass. After 96 HRS the experiment was terminated due to the fungal biomass reaching equilibrium. Wet pellets were stored on ice and used for the bioreactor part of the study.

2.6 Bioreactor set-up and preparation:

The Airlift Bioreactor Fermentation Chamber was obtained from Prof. Vince Gray, it consists of a 2 l fermentation chamber made of Perspex interconnected via Watson Masterflex Tubing to another 5 l Pyrex collection chamber and the nutrients are recycled into a Pyrex 10 l feedstock vat. A Mannheim boehringer Liquitherm FT Water Bath was also connected to circulate warm water around the fermentation chamber to maintain the temperature. The entire bioreactor was set up to run

independently and the pH in the fermentation chamber was maintained between 3-4 and was monitored using a Eutech Instruments Alpha-pH800 pH/ORP controller and oxygen was pumped into the fermentation system using a KNF LAB Laboport Air Pump and the subsequent dissolved oxygen levels were monitored using a DataTaker DT50 machine, in order to maintain it at 20 g/l. Liquid was pumped from the feedstock vat to the fermentation chamber and then to the collection vat via a Cole Palmer Masterflex L/S operating at 15 ml/h. A wire gauze chamber with 1-2 mm spaces was placed in the center of the fermentation chamber and the pellets were seeded directly into this chamber. This was to prevent the pellets from depositing on the oxygen feeding tubes and nutrient entry point. Therefore preventing blockage of oxygen flow, media agitation and nutrients from entering the fermentation chamber. This was also to prevent the fungi from flowing into the neighboring product collection chamber, as problems were encountered beforehand with the first attempt to run this bioreactor. The fungi mycelium grew over the exit outlet connecting the fermentation chamber to product collection chamber creating a water proof seal, and with the pump rate continuously pumping fresh and recycled nutrients into the fermentation chamber all of the fluid had no place to exit the fermentation chamber. Therefore it over flowed and exploded out the top of the bioreactor damaging the airlift bioreactor's lid, however with the wire gauze in place it prevented this form happening in later experiments.

*Note: Figure was removed to save space.

2.6.1 Maintenance of optimum experiment parameters:

Temperatures of the bioreactor was be maintained by passing warm water from the Mannheim boehringer Liquitherm FT Water Bath around the fermentation chamber from the 60 degrees water bath, therefore heating the fermentation chamber to 30 °C. The pH was adjusted during the hydrolysis phase whilst the oxygen levels were adjusted accordingly on the Airlift Bioreactor chamber itself. The oxygen input levels also controlled the agitation of the media. Due to it being an airlift bioreactor shear forces would be at a minimum, thus allowing for optimum proliferation of the submerged pellets.

2.7 Product recovery:

The itaconic acid produced in the fermentation chamber was extracted in the collection chamber in 10 ml sample vials and the quantity of itaconic acid produced were assayed using High Performance Liquid Chromatography.

2.8 Analysis:

The frozen fungal product samples were then defrosted at room temperature and analyzed utilizing HPLC, which is an advanced form of column chromatography with the Bio-Rad 125-0115 column, a length of 150 mm, a diameter of 7.8 mm and a particle size of 9 mm. This column was used to separate, identify, and quantify the compounds present within the samples taken from the fermentation experiments.

4. Rechecked the units used when presenting the results of the experiments, Table 1 was moved to the appendix.
5. Discussion was completely reworked, to create more Logical flow. *Original

Chapter 4

4.1 Discussion:

4.1.1 Chemical Digestion of Lignocellulose

The method described by Roman *et al* regarding chemical degradation of lignocellulose sources such as wheat bran and sugar cane bagasse, which allows for the release of the encapsulated sugars within the lignin barriers can be utilized to transform agricultural waste into a viable feedstock source for filamentous fungi to utilize in order to generate chemicals/metabolites of interest. Also due to the glucose concentration being in the range of 25-30 g/l it is sufficient enough to sustain fungal growth and provide these fungal species with enough nutrients to generate citric acid, itaconic acid and oxalic acid.

4.1.2 Citric Acid and Itaconic Acid Turnover

The current rate of citric acid production reported by Magnuson *et al* stated a 80% turn-over for *A. niger* when utilizing pure glucose and sucrose syrup as a substrate source, our study depicted a 200% citric acid turn-over from *A. terreus* in the airlift bioreactor environment and most importantly to note that agricultural waste of very low quality is used instead, thereby receiving a 200% turnover is a very good result, this is however only compared to the glucose present in the fermentation chamber, however in it assumed that due to our second generation feedstock being sugar cane

bagasse, which contains high levels of sucrose, which therefore explains the resulting high turn over rate. The turnover rate of itaconic acid production by *A. terreus* patented in 1962 by Nubel and Rabajak with a reported yield of 70% and utilized sucrose in molasses as its primary substrate source, our study showed a maximum yield of also around 200% for *A. terreus*, which can also be attributed to the sucrose being present in the feedstock source. Considering that we were able to obtain common organic acids and a more rare and expensive example such as itaconic acid and in such high quantities from essentially waste, therefore determining that there is potential for industry to adopt similar methods. As currently the reason why industry hasn't adopted these methods already is down to the simple fact that money plays an issue, being that the lignocellulose did come from agricultural waste which is very abundant and almost cost free, however the chemicals used to degrade it would far out weigh the cost of simply adding pure glucose and/or sucrose syrup directly. This is only for the time being as the worlds population is increasing exponentially and the requirement for energy and substrates sources are depleting as mentioned earlier, therefore this research is aimed at improving the burden that will be placed on the agricultural industry in the future. With predictions of global populations to increase to the ten billion range within the next 100 years, this would mean that the costs of agricultural yield in all forms will increase due to the high demand, that includes substrates to produce organic acids, as glucose and sucrose could be put to better use if it was providing humans with nutrition instead of supplying fungi with nutrient sources.

4.1.3 Potential of Fungal Bioreactors for the Future

As mentioned before our study would not only lower the burden of food production in the future, but it can also be utilized to eliminate huge amounts of agricultural waste that will be generated due to the increase in agricultural developments in the future and in turn generate more useful products to aid the ever-increasing human population, which has recently reached to over 7 billion. Therefore many aspects of this study must be optimized in further research as only then can this study fulfill its full potential to better mankind in the years to come.

4.1.4 Methods to Enhance Metabolite Production

When looking at our study, one can determine a number of aspects that may be altered in order to achieve a better result, the first being, the experimental parameters, as our

experiment, one could only control a number of parameters, that being the pH, the Dissolved Oxygen Levels, the temperature and the flow rate of the nutrient source. The current method for citric acid production utilizing *A. niger* took over 100 years to perfect and generate the high yields of organic acids (18). Therefore it would take further experiment to test all the present variables individually and then simultaneously to determine what effects they have on the organic acid yield. Ideally itaconic acid should be focused on as it is a more rare and expensive platform chemical, and is generated in much lower quantities than citric acid, therefore in our study we have chosen to further investigate the metabolite producing capabilities of *A. terreus* due to it's ability to secrete large amounts of itaconic acid. As citric acid production worldwide was recorded at 879 000 metric tons in 1998, where as the production of itaconic acid is only recorded at around 15 000 metric tons, as it's price is very high due to the costs involved to manufacture it (18). An alternative method to optimize acid production utilizing agricultural waste is by genetically modifying the fungal species to generate larger quantities of organic acids via homologous recombination, for example using plasmid vectors to knock out genes that delay or decrease organic acid production and/or by enhancing promoters that increase the metabolism of citric acid and itaconic acid. Therefore after identifying the growth parameters that the fungi require in order to secrete the metabolites of interest in high quantities and making them more efficient when scaled up to an industrial level, thus we have only covered part of the process, as fungi found naturally in the wild are only designed by evolution to be able to produce a certain amount of organic acids as it only requires it in small amounts to ensure it's prolonged survival, however humans have tried to transform this microorganism into a metabolite factory and maximize it's organic acid, hormone or protein producing capabilities. This can only be done with genetic engineering, as by altering the genes of the fungi, one can truly transform it into a metabolite producing factory, this would be accomplished by shifting it's priorities from simply producing these organic acids, for example to survive, to only producing organic acids and putting it's survival second.

*New

Chapter 4

4.1 Discussion:

4.1.1 Chemical Digestion of Lignocellulose

The method described by Roman *et al* (24) regarding chemical degradation of lignocellulose sources such as wheat bran and sugar cane bagasse, which allows for the release of the encapsulated sugars within the lignin barriers can be utilized to transform agricultural waste into a viable feedstock source for filamentous fungi to utilize in order to generate chemicals/metabolites of interest. Also due to the glucose concentration being in the range of 25-30 g/l it is sufficient to sustain fungal growth and provide these fungal species with enough nutrients to generate organic acids of interest; citric acid, itaconic acid and oxalic acid.

4.1.2 Operating the 5 l Airlift Bioreactor

Stirred tank fermentors are currently the most important type of bioreactor used in industrial production processes (37) (38). The stirred tank fermentor has it's own disadvantages and has been proved to be unsuitable for certain production systems. The reasons are due to technical, economical and biological factors (38). The airlift reactor has been widely studied. An airlift bioreactor does not require mechanical agitation and does not have moving parts. Therefore the energy demand of running such a reactor is considerably reduced when compared to running a stirred tank fermentor (38). Airlift bioreactor's only requires about a third of the energy needed for stirred tank fermentors and also airlift bioreactors can be easily assembled and operated (38).

The airlift bioreactor for this study was successfully set up to run independently. The fungal growth parameters were recorded and maintained using the DataTaker DT50, this was done to ensure that the fermentation chamber was at a stable temperature and had sufficient amount of dissolved oxygen to ensure maximum fungal proliferation. Previous fermentation studies involving filamentous fungi utilized solid-state fermentation procedures. Submerged fungal fermentation experiments within a bioreactor environment tend to encounter problems with regards to pellet formation and microbial washout; both these issues were addressed by first performing a shake flask fermentation experiment where the fungal pellets can be pre-formed prior to

inoculation. The reason for the pellets being pre-formed is so that the spores being used in the experiment aren't washed out of the fermentation chamber of the airlift bioreactor. However during the initial run of the experiment, washout did occur and the fungus began to proliferate within the collection chamber. Thereby causing the product, IA to be broken down and metabolized due to lack of fresh nutrients available to the washed out spores/pellets.

The problems encountered with the initial run included the complete blockage of the exit piping leading from the fermentation chamber to the collection chamber. This caused the top of the fermentation chamber to blow up due to the build up of pressure and fresh nutrients being pumped in constantly. To solve this particular problem, a plastic cage was constructed out of 1-2 mm plastic wire gauze, with the measurements 5 cm diameter, 20 cm high and this cage was submerged into the center of the fermentation and the pellets were seeded directly into this cage. After which the cage was sealed. This aided the experiment in three ways, firstly it prevented the previous scenario from reoccurring, and secondly it also prevented the fungal pellets from depositing onto the oxygen feeder tubes and causing blockage. Finally the third reason why the plastic cage was so successful is that if confined the fungi to the optimum location within the fermentation chamber, i.e. it was located right next to where the warm water from the water bath was being circulated, where the temperature was most stable. The location also was away from the oxygen feeders where shear forces were at a minimum to maintain the pellet's structural rigidity, as the pellet size and structure is vital to obtain maximum metabolite output.

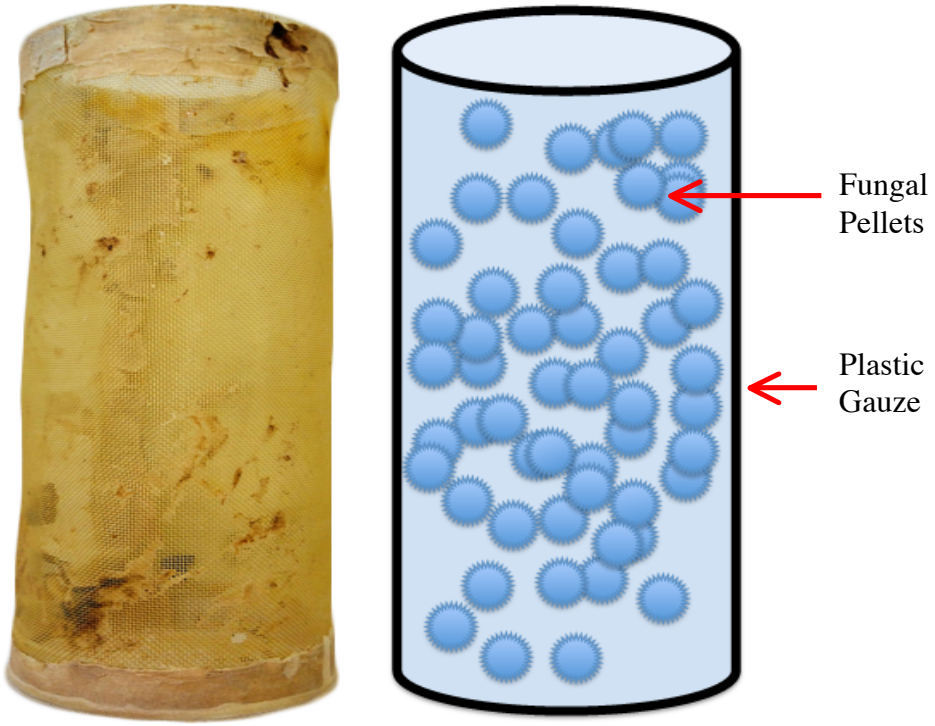

Figure 9. The Fungal Pellet Cage made from Plastic Gauze

*Note: New figures were added.

Figure 9 shows both the original fungal pellet cage constructed from plastic gauze and the diagram of the fungal pellet cage within the running bioreactor. The concept of the draft tube was adopted from the study conducted by Okabe, M. *et al*. in 1993 where they developed a draft tube that was inserted into the fermentation tank to perform a similar function as the fungal pellet cage in out experiment.

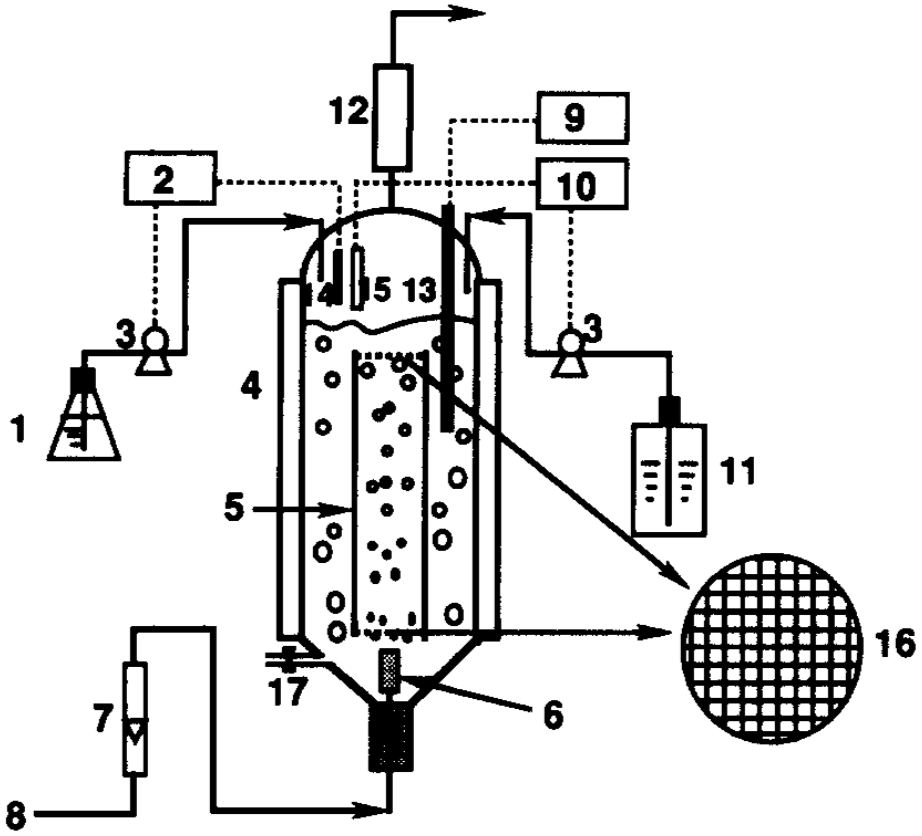

Figure 10. Schematic diagram of the Airlift bioreactor with the draft tube (38)

*Note: New figures were added.

Figure 10 shows the airlift bioreactor Okabe, M. *et al*. (38) utilized with their draft tube. No. 1 on the diagram is the Antifoam reservoir, No. 2 is the antifoam controller, 3 is the pump, 4 is the water jacket circulating heated water to maintain the fermentation chamber's temperature, 5 is the draft tube of interest that is the concept of the fungal pellet cage (38). No. 6 is the stainless steel sparger, no. 7 is the flow meter and no. 8 is the air compressor, used to maintain oxygen levels within the fermentation chamber to be 50% of the saturated value. No. 9 is the Dissolved Oxygen meter, no. 10 is the level controller, no. 11 is the sterilized water tank and no. 12 is the condenser. No. 13 works in conjunction with Dissolved Oxygen meter and it is the Dissolved Oxygen probe, no. 14 is the antifoam sensor, no. 15 is the level sensor and no. 16 is the stainless steel sieve that makes up the top and bottom of the draft tube. No. 17 is the sampling port of the airlift bioreactor (38).

The results yielded by Okabe, M. *et al*. (38) showed that the maximum itaconic acid concentration was 63 g/l after 4 d, and the IA production rate was 0.66 g/l/h, which is two fold higher than for draft tubes with one sieve or without the sieve which is

usually 0.26- 0.30 g/l/h (38). Therefore from the results of Okabe, M. *et al*. (38) we can't really compare that to our study as in the case of the fungal pellet cage, if it was removed the entire experiment would not work as pellet over flow and blockage would occur, however when the stainless steel sieve was not used or was used at the bottom of the draft tube in Okabe, M. *et al*. experiment (38), cell growth, itaconic acid concentration and glucose consumption were similar. However, when the draft tube was covered with the sieves at the bottom and top, the product yield based on the consumption of glucose with and without the stainless steel sieve was 0.53 and 0.45 g/g, respectively (38). The reason why the airlift bioreactor with stainless steel sieves yielded a high itaconic acid production rate remains unclear (38). This could be another reason why our organic acid production level was so high when utilizing such low-grade substrate, as the inclusion of such a apparatus seems to enhance the IA production rate as it concentrates the fungal pellets in the optimum environment within the fermentation chamber.

Also from the study conducted by Okabe, M. *et al*. they determined that to achieve maximum itaconic acid production, an intermediate state between pellet and the pulp state of *A*. terreus must be achieved. Due to if the mycelia form pellets, the nutrients being provided are limited inside the pellets, while if the mycelia form a pulp state, oxygen transfer decreases due to the increase in viscosity in culture broth (38). Therefore, it is desirable that the mycelia form an intermediate state between pellets and the pulp state for the efficient production of itaconic acid (38).

The intermediate state of mycelia was achieved using a draft tube covered with stainless steel sieves on the top and bottom (38), a similar effect was created when utilizing the fungal pellet cage in our study. Although the fungi were seeded into the fermentation chamber in pellet form they can quickly adapt to the new environment. A portion of the seeded fungi began to form such intermediate structures by growing on the walls of the cage and spreading out, instead of all forming pellets. As this intermediate structure was optimally suited to the airlift bioreactor environment, it could also explain the high product yields of 59.35 g/l (200 % turnover) reported in this study.

4.1.3 Citric Acid and Itaconic Acid Turnover

The current rate of citric acid production reported by Magnuson *et al* (8) stated a 80% turn-over for *A. niger* when utilizing pure glucose and sucrose syrup as a substrate source, our study depicted a 200% citric acid turn-over, which is considered very high concentrations of acid being produced with regards to the amount of glucose available within the hydrolysate. When considering the amount of glucose used and the amount of IA produced from *A. terreus* in the airlift bioreactor environment and most importantly to note that agricultural waste of very low quality is used instead, thereby receiving a 200% turnover is a very good result, this is however only compared to the glucose present in the fermentation chamber, however it is assumed that due to our second generation feedstock being sugar cane bagasse, which contains high levels of sucrose that were not detected by the HPLC, which therefore explains the resulting high turn over rate.

The turnover rate of itaconic acid production by *A. terreus* patented in 1962 by Nubel and Rabajak with a reported yield of 70% utilized sucrose in molasses as its primary substrate source (8). Our maximum yield of 200% for *A. terreus* can also be attributed to the undetected sucrose being present in the feedstock source. Considering that we were able to obtain common organic acids and a rare organic acid such as IA, in such high quantities, therefore makes this study significant to the organic acid industry. These products were metabolized from essentially waste, therefore determining that there is potential for industry to adopt similar methods to produce these organic acids of interest. As currently the reason why industry has not adopted these methods is due to the issues such as operating costs and the economical aspect, even though the lignocellulose obtained did come from agricultural waste, which is very abundant and almost cost free, however the chemicals used to degrade it would far out weigh the cost of adding pure glucose and/or sucrose syrup directly. With predictions of global populations to increase to the ten billion range within the next 100 years, this would mean that the costs of agricultural yield in all forms will increase due to the high demand, that includes the substrates from which organic acids are produced, as glucose and sucrose could be put to better use if it was providing humans with nutrition instead of supplying fungi with nutrient sources. Thereby creating the need to find an alternative substrate from which organic acids of interest like IA can be synthesized from.

4.1.4 Methods to Enhance Metabolite Production

When looking at our study, one can determine a number of aspects that may be altered in order to achieve a better result, the first being, the experimental parameters, as our experiment, one could only control a number of parameters, that being the pH, the Dissolved Oxygen Levels, the temperature and the flow rate of the nutrient source. The current method for citric acid production utilizing *A. niger* took over 100 years to perfect and generate the high yields of organic acids (8). Therefore it would take further experiment to test all the present variables individually and then simultaneously to determine what effects they have on the organic acid yield. Greater focus should be put on IA as it is a more rare and expensive platform chemical and it is produced in much lower quantities than citric acid. As citric acid production worldwide was recorded at 879 000 metric tons in 1998, where as the production of itaconic acid is only recorded at around 15 000 metric tons, IA's price is very high due to the costs involved to manufacture it (8).

An alternative method to optimize acid production from agricultural waste is by genetically modifying the fungal species to generate larger quantities of organic acids via homologous recombination, by using plasmid vectors to knock out genes that delay or decrease organic acid production and enhancing promoters that increase the metabolism of citric acid and itaconic acid.

Therefore after identifying the growth parameters that the fungi require in order to secrete the metabolites of interest in high quantities and making them more efficient when scaled up to an industrial level. As fungi found naturally in the wild are only designed by evolution to be able to produce a certain amount of organic acids, as it only requires it in small amounts to ensure it's prolonged survival. Humans have tried to transform this microorganism into a metabolite factory and maximize its organic acid, hormone or protein producing capabilities. This can only be done with genetic engineering, as by altering the genes of the fungi, one can truly transform it into a metabolite producing factory, this would be accomplished by shifting its priorities from simply producing these organic acids to survive, instead to only producing organic acids and putting it's survival second.

Another method previously mentioned in the literature review on how metabolite production can be increased is strain modification, as many studies have been

conducted focusing on IA production, but very few have focused on strain modification. Kobayashi *et al*. reported that IA production was sup- pressed during cultivation since the produced IA significantly inhibited the growth of A. terreus (36). Therefore in order to overcome product inhibition during the growth of the fungus, it is preferable to select an IA resistant mutant strain, which will lead to improvement of IA production. A study conducted by Yahiro, K. *et al*. focused on the IA production by the mutant *A. terreus* strain TN-484 in a 2.5 l Airlift Bioreactor, where they achieved a maximum IA concentration of 72.5 g/l after a period of six days (37). Yahiro, K. *et al*. results showed a higher IA concentration than this study, as their substrate used for fungal metabolism was pure glucose, which is a very important factor when considering up scaling this technology to an industrial level. As the substrate accounts for more than 25% of the total production cost, therefore by using agricultural waste we can significantly reduce the total production costs (37).

6. Chapter 5 was reviewed and moved to the literature review section and more conclusions were added to this chapter.

   *Original

Chapter 5

Outlook

5.1 Genetic Transformation:

The general method of genetic transformation of filamentous fungi, begins firstly by removing the permeability barrier presented by the cell wall of the fungus, either by treatment with lithium acetate or by enzyme degradation using crude extracts found in snail gut (23) or by using a *Trichoderma* extract commercially known as Novozym 234 (23) to produce protoplasts. Some filamentous fungi require a mixture of enzyme extracts to produce a sufficient amount of protoplasts. This is followed by the removal of the cell wall by utilizing an osmotic stabilizer such as 0.6 potassium chloride or 1.2M sorbitol, the choice of which stabilizer is used depends on the species of fungus, the two Aspergillus species, *A. niger* and *A. terreus* make use of 1.2M and 0.55M sorbitol respectively as their osmotic stabilizers (23). DNA is then added to the protoplast suspension in the presence of 10-50mM calcium chloride and then followed by the addition of a solution of polyethylene glycerol, which initiates the uptake of the

DNA by the protoplasts. The treated protoplasts are then allowed to proliferate of selective medium that selects for only the transformed cells (23). The selectable markers that can be used are genes that complement a nutrition requirement mutation, which allows the growth of the fungus in absence of the required nutritional factor (15).

5.2 Limitations regarding Fungal Genetic transformation:

There is a limitation to this methodology as it requires the recipient fungal species to be a mutant strain and this is often undesirable in industrial strains (34). The solution to this drawback is the use of positive selection systems, which is based on supplementation of antimetabolites, for example; oligomycin resistance, prokaryotic antibiotic resistance genes such as kanamycin or G418 resistance (23). Another alternative system utilized the *amdS* gene isolated from *A. nidulans, which* codes for acetamidase, an enzyme that allows the growth on acetamide as a sole nitrogen or carbon source (5). Several other fungal species such as *A. niger* lack the *amdS* gene, which allows for easier selection of transformants, therefore making it a useful marker for transformation (23). The frequency of transformants is 10-100 stable transformants per μg. A similar technique to transform industrial fungi is to disrupt the gene functioning by homologous recombination using a defective gene or a gene that has been disrupted by a selectable marker, this method produces null mutations that eliminate the chance of undesirable traits developing in industrial strains of filamentous fungi (15) (26).

5.3 Future Prospects and other Transformation Methods:

Future prospects involving the heterologous expression of genes in filamentous fungi, where current gene expression systems rely on either powerful inducible or constitutive promoters, and homologous promoters are preferred to the production host as mentioned above to enhance organic acid production (24). There is however a disadvantage involving the constitutive expression of housekeeping gene promoters is that the promoters are functional during growth and therefore unsuitable for over expression of foreign proteins that might be toxic to the host cells. Another major disadvantage of inducible gene promoters, such as the powerful cbh1 gene promoter, is their repression by glucose and other carbon sources therefore they can severely affect the yield of protein secreted. These promoters can be regulated by the induction of carbon and nitrogen compounds and the pH of the growth medium (24).

Experiments have shown a novel metabolically independent expression system that can be regulated by oestrogenic compounds and it has been tested in *Aspergillus*. *A. nidulans* and *A. niger* transformants are highly sensitive with regards to acquiring oestrogen responsive elements or low levels of oestrogenic substances such as diethylstilbestrol, this therefore allows this research to be utilized in the detection of xenoestrogens in food and in the environment (28). Other experiments involving the modification of the traditional method for cultivating filamentous fungi for protein production have been researched. Whereas instead of a submerged fermentation occurring in liquid media, solid-state fermentation, which is supported by high yields of the secreted metabolites, obtained in these systems can be utilized instead (35). Due to the physical mode of growth and gene regulation of fungi in solid-state fermentation, differ from fungi grown in liquid cultures (28).

Another vital factor in the development of transgenic fungal expression systems is the establishment of effective transformation protocols across fungal genera to add efficiency and flexibility to high throughput screening for evolved proteins and different metabolites of interest (33). Advancement in observation procedures including; microscopy technologies, such as fluorescence resonance energy transfer and fluorescent life time imaging, allows scientists to visualize metabolic pathways and protein–protein interactions in living systems therefore allowing greater understanding with regards to these areas, as fluorescent imaging, coupled with molecular biology, bioinformatics, biochemistry, genomics and proteomics, will redesign the concept of microbial metabolite production (33).

Another transformation method, which is being used more commonly in experiments involving the genetic transformation of filamentous fungi, is biolistic bombardment and inclusion of the seven barrels Hepta adaptor system has noticeably increased the number of transformations achieved (31). *Agrobacterium*-mediated transformation method has also gained a lot of interest, and claims of up to 100–1000 times greater efficiency of the T-DNA transfer and chromosomal integration, compared with conventional methods mentioned before, as one is aware that the T-DNA of the bacterium *Agrobacterium tumefaciens* can be transferred to plants, yeasts, fungi and human cells (13). We are entering an era of accelerating development of novel fungal fermentations and transformations due to the exponential increase and development of

information and tools to exploit nature. Therefore there are many methods our study can be expanded with regards to higher yield of organic acids from agricultural waste.

Chapter 5: Conclusions
*The conclusions have been reworked.

*Original

Conclusions:

The organic acids mentioned in this study have the potential to be utilized more in the near future. The production of these acids by this alternative fermentation method gives industry a new option to generate commodity chemicals from renewable resources. Many current research programs studying the production of these "green" chemicals are still in the experimental stage, however with the advances made towards self-sustainable industrial processes with better knowledge of biological and biochemical regulations, better biocatalysts can be generated to overcome the current limitations. Therefore with further research, agricultural and food residues along with municipal waste can be utilized as useful feedstocks and efficiently converted into valuable products. In conclusion the production of organic acids presents a great opportunity to recycle and reutilize natural resources, thus lifting the strain on non-renewable resources.

*New

Chapter 5

Conclusions:

The fungus successfully produced the metabolites of interest IA and CA in an airlift bioreactor environment that utilized the hydrolysate produced from chemical digestion. The concentrations produced are of sufficient quantity, being 59.4 g/l and 59.2 g/l for IA and CA respectively as the objective was to produce both metabolites in excess of 60 g/l. Therefore showing that agricultural waste, sugar cane bagasse and wheat bran can be hydrolyzed and subsequently used to produce high concentrations of metabolites of interest. The production methods of the two organic acids in this study have the potential to be utilized in industry. As the production by this alternative fermentation method gives industry a new option to generate commodity chemicals from renewable and inexpensive resources.

Current industrial research programs studying the production of these "green" chemicals are still in the experimental stage, limitations, such as operating cost have restricted the progress of such research to be up scaled to an industrial level, however with the advances made towards self-sustainable industrial processes coupled with

better knowledge of biological and biochemical regulations, better biocatalysts can be discovered and produced to overcome the current limitation of operating costs by increasing and sustaining product yield. Therefore with further research, agricultural and food residues along with municipal waste can be used as feedstocks for industrial acid production. In conclusion the production of organic acids presents a great opportunity to recycle and reutilize natural resources, thus lifting the strain on non-renewable and/or expensive resources.

References: Were reworked, as the original was in Alphabetical order and it was changed to the order of appearance in the text.

*Note: Additional References were added.

*Original: Alphabetical

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Appendix: An Appendix was added as per the request from both examiners.

*New

Appendix

Table 2: HPLC Analysis Results for the 48 Samples (g/l) extracted from the Airlift Bioreactor during the 16 Days of Fermentation

HPLC Sample No.	Xylose	Glucose	Itaconic Acid	Citric Acid
1	0.00	29.58	0.00	0.00
2	0.00	28.75	0.00	0.00
3	0.00	29.27	0.00	0.00
4	0.00	29.04	0.00	0.00
5	0.00	28.59	0.00	1.46
6	0.00	28.87	0.00	0.00
7	0.00	29.10	0.00	0.00
8	0.00	29.12	0.00	2.14
9	0.00	28.84	0.00	10.10
10	0.00	27.52	0.00	13.84
11	0.00	28.12	0.00	12.67
12	0.00	26.67	8.94	11.86
13	0.00	27.32	9.14	13.21
14	2.91	22.92	25.74	14.33
15	0.00	26.11	21.28	20.15
16	2.27	16.98	25.80	21.13
17	2.52	18.23	27.00	21.81
18	2.19	15.82	23.96	26.88
19	2.22	15.98	25.01	23.95
20	2.49	15.01	42.51	25.01
21	2.26	14.61	31.45	42.02
22	2.18	14.10	30.24	32.41
23	2.49	15.78	33.60	31.31
24	2.11	12.67	35.81	34.81
25	1.99	11.69	29.13	29.00
26	2.51	13.23	52.35	25.17
27	1.90	10.33	34.23	35.01
28	1.87	9.95	34.53	34.10
29	1.78	8.57	40.08	41.27
30	1.77	8.42	41.02	40.11
31	2.09	8.12	54.97	45.48
32	1.67	6.69	46.03	46.11
33	1.69	4.10	53.45	56.01
34	1.72	5.10	51.04	51.11
35	1.65	2.97	55.46	55.09
36	1.63	2.68	55.53	57.04
37	1.54	0.00	56.67	57.00
38	1.59	2.09	55.48	56.89
39	1.83	2.31	61.68	63.61
40	1.51	0.00	55.90	57.70
41	1.89	0.00	66.08	56.85
42	1.52	0.00	56.05	52.11
43	0.73	0.00	32.91	29.10
44	0.75	0.00	34.07	24.68
45	0.59	0.00	0.00	22.10
46	0.61	0.00	0.00	18.31
47	0.60	0.00	0.00	0.00
48	0.00	0.00	0.00	0.00