

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

**Increasing the *Lactococcus lactis* Biomass through
Aerobic Growth**

**A thesis presented in partial fulfilment of the requirement for the
degree of**

Master of Engineering

in

Biotechnology

at Massey University, Palmerston North,

New Zealand

Aravind Giridhar

2013

ABSTRACT

Starter cultures for dairy fermentations are commonly made by anaerobic fermentation in New Zealand. Anaerobic fermentation involves glycolysis and it is a very inefficient pathway due to the formation of energy rich products such as lactic acid. This pathway only produces 2 moles of ATP per glycolysis and to conserve energy, the amount of biomass produced is less. Aerobic fermentation on the other hand can produce up to 36 moles of ATP per cycle, and the amount of biomass produced will be higher compared to anaerobic fermentation. Lactic acid bacteria do not possess a functional electron transport chain for aerobic respiration to be efficient. It requires the addition of heme, for the electron transport chain to work. The heme addition is a patented process.

The aim of this study was to optimise the aerobic fermentation process for *Lactococcus lactis* for biomass production. An extensive literature search shows that there has been no study in optimising the heme concentration or using other alternatives for heme. Alternatives to heme, that are food grade, are an attractive option, as there is sourcing issues with heme in New Zealand.

A series of shake flask trials were carried out to identify a possible heme replacement. The shake flask trials showed that ammonium ferric citrate is a possible alternative heme replacement. More shake flask trials were then evaluated to optimise the concentration of ammonium ferric citrate. Following that, 1-L fermenter trials were evaluated to optimise heme concentration and to compare the effect of heme and ammonium ferric citrate addition on biomass and activity of the harvested biomass following a freeze and thaw cycle.

It was shown that 44 µg/mL ammonium ferric citrate resulted in the most biomass of the concentrations tested. For heme, the optimum concentration was 1 µg/mL. It was found

that fermentations using heme resulted in more biomass after 5 h compared to using ammonium ferric citrate. But, cells grown by adding ammonium ferric citrate was equally as active.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisor Associate Professor Pak-Lam Yu for all his help and encouragement throughout the duration of this project and for all the advice and answering my questions and being patient with me.

I would also like to thank Andrew Patrick at the Microbial Fermentation Unit (MFU) of Fonterra, Palmerston North for helping me with the cultures, fermentation runs, and activity testing. I would also like to thank all the members of the MFU, especially Vicki Lander for helping me with my experiments

A special thank for all the members of the Microbiology lab, especially Ann-Marie and Julia for helping me with answering my questions and helping me with analysis

This acknowledgement will not be complete without mentioning my wonderful friends who have helped me through thick and thin and encouraging me to complete this thesis. A very special thanks to Lutfi and Fatima. I would also like to thank Neilen and Daniel for all the encouragement and support.

A lot of gratitude towards my parents for helping me throughout the degree. My dad for giving me valuable advice. My mother for everything she has done for me from so far away. My brother for the energy drinks.

Last, but not least, a very special thanks to Krishneel and his family for looking after me during my stay in Palmy. I would have been totally lost without your support and encouragement.

TABLE OF CONTENTS

ABSTRACT.....	iv
ACKNOWLEDGEMENTS.....	vii
TABLE OF CONTENTS.....	viii
LIST OF TABLES.....	x
LIST OF FIGURES.....	xi
1.0 INTRODUCTION.....	1
2.0 LITERATURE REVIEW.....	5
2.1 Starter culture.....	5
2.1.1 Lactic acid bacteria.....	6
2.1.1.1 <i>Lactococcus lactis</i>	7
2.1.2 Nutritional requirements of lactic acid bacteria.....	8
2.2 Production of starter culture.....	8
2.2.1 Fermentation.....	9
2.3 Respiration.....	12
2.3.1 Respiration in LAB.....	14
2.3.2 Heme.....	16
2.3.3 The role of heme in respiration.....	17
2.3.4 Impact of respiration on LAB.....	18
2.4 Improving starter culture production by respiration technology.....	20
2.5 Conclusion.....	22
3.0 MATERIALS AND METHODS.....	23
3.1 <i>Lactococcus</i> strain.....	23
3.2 M17 Media.....	23
3.3 Heme.....	23
3.4 Alternate heme.....	23
3.5 Analytical techniques.....	24
3.5.1 Biomass.....	24
3.5.2 Glucose.....	24
3.6 Fermentation.....	24
3.6.1 Equipment.....	24
3.6.2 Operational Procedure.....	24
3.7.1 Activity testing.....	26

3.7.2	Direct set milk preparation	26
3.7.3	Inoculum preparation for activity testing	27
3.7.4	Operating procedure.....	27
4.0	RESULTS AND DISCUSSION.....	28
4.1	Heme alternatives	28
4.2	Effect of different concentrations of ammonium ferric citrate	30
4.3	Heme Optimisation	34
4.4	Comparison between ammonium ferric citrate and heme.....	36
4.5	Activity Testing	37
5.0	CONCLUSIONS.....	40
6.0	REFERENCES	42
	APPENDIX.....	47
	Appendix 1: Raw data for duplicate runs of heme optimisation	48
	Appendix 2: Activity result graph	50

LIST OF TABLES

Table 4.1: The effect of various heme alternatives on aerobic growth of <i>L.lactis</i> in shake flasks.....	28
Table 4.2: Effect of ammonium ferric citrate concentration on aerobic growth of <i>L.lactis</i> in shake flasks.....	30
Table 4.3: Time taken to reach final pH of 4.605 for <i>L.lactis</i> cultures grown by adding ammonium ferric citrate and heme, and for control.....	36

LIST OF FIGURES

Figure 2.1: <i>Lactococcus lactis</i> (Todar, 2012).....	7
Figure 2.2: Simplified fermentation pathway of lactic acid bacteria (Broojimans, 2008).....	10
Figure 2.3: Conversion of pyruvate to lactate by the action of lactate dehydrogenase (Lechardeur et al. 2011)	11
Figure 2.4: Electron transport chain of lactic acid bacteria.....	15
Figure 2.5: Structure of heme showing the iron centre.....	16
Figure 4.1: Effect of ammonium ferric citrate concentration on aerobic growth of <i>L.lactis</i> in shake flasks.....	30
Figure 4.2: Effect of ammonium ferric citrate concentration on aerobic growth of <i>L.lactis</i> in shake flasks.....	33
Figure 4.3: Effect of different concentrations of heme on biomass of <i>L.lactis</i> grown aerobically.....	33
Figure 4.4: The effect of 44 µg/mL ammonium ferric citrate and 1 µg/mL heme on aerobic growth of <i>L.lactis</i> in a 1-L fermenter.....	35

1.0 INTRODUCTION

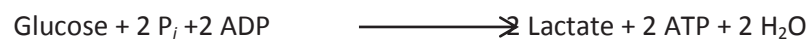
Starter cultures are a term given to microorganisms that are used to initiate fermentation to produce various fermented foods such as cheese, yoghurt, and sourdough bread. The bacteria used in the manufacture of fermented dairy products are generally Lactic Acid Bacteria (LAB), although exceptions exist, such as *Propionibacterium shermanii* and *Bifidobacterium spp.*, which are not lactic acid bacteria.

Most starter cultures currently used presently comes from LAB originally present in the contaminating microflora of milk. Sandine et al. (1972) has noted that the lactic acid bacteria have probably originated from vegetation in the case of Lactococci. Modern starter cultures have developed from the practice of retaining small quantities of the fermented substance and using them as an inoculum or starter on the preceding day's production. Modern starter cultures are usually sold as frozen concentrated cell cultures in the form of either direct set or bulk set. Bulk sets are used for intermediate bulk starter production and direct sets are used for final production (Mullan, 2001).

The current method of starter culture production in New Zealand is through fermentation. This involves growing the lactic acid bacterium in milk at low agitation in an oxygen free environment. Since lactic acid is produced during the fermentation, which can inhibit the growth of bacteria, pH is maintained in the region of 6.0-6.30 by adding ammonium hydroxide. The temperature of fermentation is maintained at 30° C. The cells are harvested after 24h and are concentrated using a steam sterilised centrifuge. The concentrated cell mass is then pumped through a sterile stainless steel filter. Cryoprotectant is then added to the mixture and is filled into 500 mL trays. The trays are then blast frozen at -40° C. The frozen trays are held at -30° C until quality control is completed and then the starter culture is dispatched as required. The quality control involves measuring the activity of the

strains, which is the time taken for the culture to drop the pH of milk to 4.5 and the strain is also checked for contamination and phage.

During LAB fermentation, lactose, the sugar present in milk, is broken down by an enzyme, β -galactosidase to form galactose and glucose. Galactose is converted to glucose 6-phosphate and together with glucose enters the Embden-Mayerhoff-Parnas (EMP) pathway, most commonly known as glycolysis. The end product of glycolysis is lactic acid. One mole of glucose is converted to two moles of lactic acid according to the following equation:



In glycolysis, pyruvate acts as the terminal electron acceptor and allows the fermentation to continue by oxidising NADH to NAD^+ . This results in the formation of products such as lactic acid, which are very wasteful in terms of energy recovery, since no additional ATP is produced. Thus, glycolysis only produces 2 ATP and most of the energy is conserved in the end product, lactic acid. Hence, the amount of biomass produced is less than respiration.

Respiration involves glycolysis, the citric acid (Krebs) cycle and an electron transport chain. In the case of aerobic respiration, oxygen is the terminal electron acceptor. In most aerobic bacteria, glucose is oxidised to form carbon dioxide and oxygen is reduced to form water. Respiration is an energy producing process. Up to 36 moles of ATP can be produced by respiration, compared to 2 moles of ATP for glycolysis (Garrett & Grisham, 2008). Aerobic growth also promotes more biomass growth compared to fermentation. Thus for production of starter cultures, changing the current method from fermentation to respiration has the potential to produce more biomass.

Lactic acid bacteria, not being traditional aerobic bacteria, lack many of the essential components of respiration that other aerobic bacteria possess. Lactic acid bacteria lack a

complete Krebs cycle (Pedersen et al. 2012). Thus, NADH, which is needed for the respiratory chain is produced by carbon catabolism, meaning the bacteria follows the fermentation pathway first. The respiratory chain of the respiration competent lactic acid bacteria is incomplete. Firstly, LAB requires exogenous heme since they lack genes for heme biosynthesis. Some LAB also requires exogenous menaquinone due to the lack of menaquinone biosynthesis genes. Thus, the respiration metabolism is strongly dependent on the addition of those compounds. Secondly, respiring LAB encodes a single cytochrome oxidase, the quinol oxidase CydAB, which is known to work at low oxygen concentrations. This might contribute to elimination of oxygen from the environment.

There are many advantages of growing LAB aerobically compared to fermentation. The first advantage is increased biomass. Gaudu et al. (2002) has found that when growing *Lactococcus lactis* aerobically, the biomass doubled by the end of the growth period, compared to fermentation. Gaudu et al. (2002) also noted less lactic acid production, but accumulation of minor end products such as acetic acid and acetoin were found.

The second advantage is improved oxygen tolerance and long term survival. Hansen et al. (2001) has shown that despite aeration of the culture, oxygen levels are maintained at low levels in the cells, thus reducing the amount of oxygen available for the formation of toxic radicals. This improved tolerance can be explained by the scavenging of oxygen by cytochrome, thus eliminating oxygen and also the presence of a more neutral intracellular pH due to the change in metabolism (Hansen et al, 2001).

The key to long term survival of bacteria is also the presence of cytochromes which protect cells from oxidative stress. The *L.lactis* superoxidase dismutase, the manganese dependent SodA detoxifies the cytoplasm by removing oxygen radicals. Kim et.al (2001) has noted that cell survival improved up to 10 fold after 6 weeks of storage at 4 °C for respiration- grown cultures, compared to fermentation grown cultures. Duwat et al. (2001) suggested that

cytochrome d oxidase activity may serve as an oxygen trap to reduce oxygen toxicity and may also be involved in the activation of other survival genes.

The aim of the project was to improve the biomass production of the starter culture, *Lactococcus lactis*, which was used in the dairy manufacturing industry for producing casein. This was achieved by completing the following objectives:

1. Use of aerobic respiration to grow the starter cultures using small scale fermenters
2. Explore alternatives to heme such as other iron containing compounds like ammonium ferric citrate to supplement aerobic growth
3. Measuring the activity of the aerobically grown culture by looking at the time it takes for the culture to lower the pH of milk to 4.5

2.0 LITERATURE REVIEW

2.1 Starter culture

A starter culture is a preparation containing a large number of viable microorganisms, which accelerates a fermentation process and facilitates improved control of a fermentation process and predictability of its products (Holzapfel, 1997).

The earliest fermentations were caused by spontaneous fermentation, i.e. fermentations initiated without the use for a starter inoculum. Spontaneous fermentations result from the competitive activities of various contaminating microorganisms. The microorganisms that are best adapted to the food substrate and the conditions of storage dominate the fermentation process. Most bacteria produce organic acids, which proves inhibitory to other microorganisms and provides additional advantages during fermentation (Holzapfel, 2002). This process is easily prone to contamination, which can lead to production of unappetising aroma and flavour and may lead to the production of potentially toxic substances. To better control the fermentation process, the microorganism of interest must be added initially to give it a competitive advantage over other microorganisms.

The process of using starter cultures has been developed from the practice of retaining small quantities of the successful manufacture of the fermented product and using it as an inoculum for subsequent fermentations, commonly known as back-slopping (Mullan, 2001). This practice helps to shorten the lag phase of the fermentation and reduce the risk of fermentation failure due to contamination. Through repeated back-slopping, the best adapted strains are selected, of which some may possess features that are desirable for use as starter cultures.

The starter cultures used in the dairy industry are generally lactic acid bacteria (LAB), although exceptions exist such as *Propionibacterium shermanii* and *Bifidobacterium spp*,

which are not LAB. Three main types of starter culture are commonly used in Australia, New Zealand, North America and the UK (Lawrence et.al 1976):

1. Single-strain starters: single strain starter cultures are composed of *Lactococcus cremoris* and less commonly *Lactococcus lactis*. Some factories in NZ use them as pairs, while those in Australia use them as single strains.
2. Multi-strain starters: in multi-strain starters, defined mixtures of three or more single strains of *L.cremoris* and/or *L.lactis* are used. In some cases, *Leuconostoc* and *L.diacetylactis* are also used.
3. Mixed-strain starters: Mixtures of strains of *L.cremoris*, *L.lactis*, *L.diacetylactis* and *leuconostocs* are used as mixed-strain starters. The identity of the component strains are usually kept as a commercial secret and their composition may vary on subculture.

This classification is now of limited value. As more information was obtained on the microbes, the composition of starters became increasingly complex to achieve desired performance and flavour profiles. The classification is now simplified to define starters as either defined when the strain and species of the component strains are known or as undefined cultures (Mullan, 2001, Heap, 1998)

2.1.1 Lactic acid bacteria

Lactic Acid Bacteria consists of a group of gram-positive bacteria that are united by certain morphological, metabolic, and physiological characteristics. LAB are nonsporulating, nonrespiring organisms which can appear as cocci or rods and produce lactic acid as one of their main fermentation products (von Wright, 2012). They lack catalase and are devoid of cytochromes. LAB belong to phylum *Firmicutes*, class *Bacilli* and order *Lactobacillales*. Phylogenetically, LAB can be clustered on the basis of molecular biological criteria such as

rRNA sequencing. Such sequencing has shown that the ancestral LAB have been Bacillus-like soil organisms, which seems to have lost several genes and the associated physiological functions while adapting to the nutritionally rich ecological niches (von Wright, 2012).

2.1.1.1 *Lactococcus lactis*

Lactococcus lactis belongs to the genus *Lactococcus*, which is shared by four other species: *L.garvieae*, *L.piscium*, *L.plantarum*, and *L.raffinolactis*. Among these species, only three species are used for starter culture production: *L.lactis*, *L.lactis ssp.cremoris* and *L.lactis ssp lactis biovar diacetylactis*.

Lactococcus lactis appear ovoid and are typically 0.5-1.5µm in size (Figure 2.1). They are non-spore forming and are non-motile. *L.lactis* is commonly used in the dairy industry to produce dairy products like yoghurt, buttermilk and certain types of cheese. In New Zealand, *L.lactis* is also used to make lactic casein.

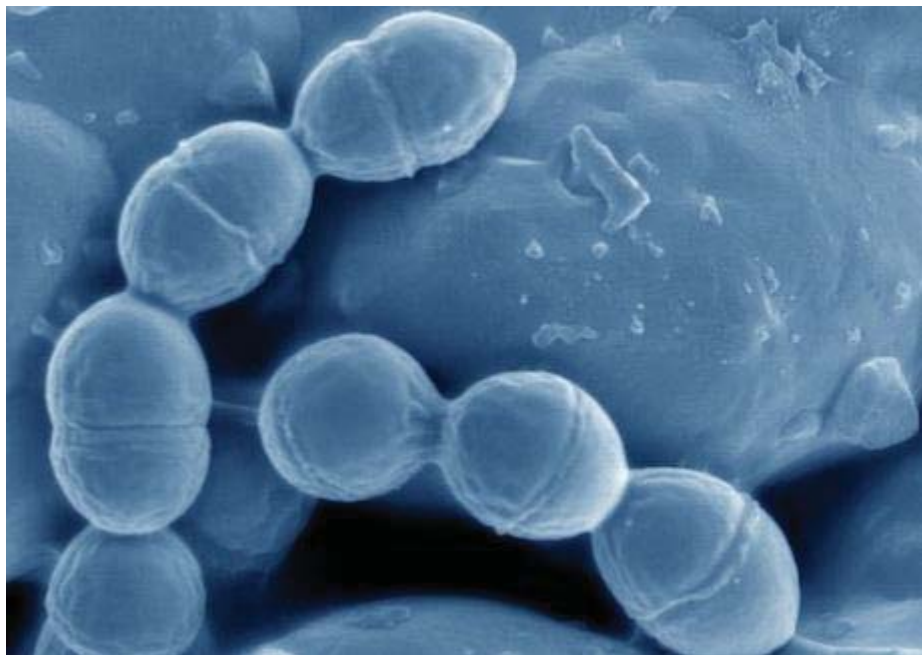


Figure 2.1: *Lactococcus lactis* (Todar, 2012)

Lactococcus lactis are also used in other industries to produce pickled vegetables, beer, some breads and other fermented foods. They are well studied and a complete genomic sequence is available for the strain *L. lactis* ssp. *Lactis* IL 1403 (Bolotin et al. 2001).

2.1.2 Nutritional requirements of lactic acid bacteria

Lactic acid bacteria have complex nutrient requirements, due to their limited ability to synthesize B-vitamins and amino acids (Chopin, 1993). The usual procedure to achieve good growth, is to add the LAB to a complex media such as yeast extract, which provides the essential nutrients for bacterial growth. In industry, LAB is most commonly grown in milk media, which provides the carbon source (lactose), and nitrogen source (proteins from milk) and other essential nutrients. The various amino acids (Dressaire et al. 2011; Law, 1976; Reiter, 1962), vitamins (Ledeshma et al. 1977), nucleic acid bases (Cocaign-Bousquet et al. 1995) and peptide (Tanaka et al. 1995) requirements for lactic acid bacteria have been extensively studied. The conclusion from all the studies is that the nutrient requirement of LAB is strain dependent. Therefore, it is more economically feasible to supply the LAB with a complex nutrient in the form of yeast extract or similar mixture which can provide a variety of nutrients.

2.2 Production of starter culture

There are two common starter systems: bulk set culture and direct vat set culture. In bulk set starter culture systems, the culture is produced as a frozen concentrate, which is partially thawed and added directly to milk. In this method, the culture can be produced at one facility and then shipped to other factories. Direct vat set (DVS) cultures are directly

added to milk, eliminating the need for a mother culture. The cultures can be processed as a frozen concentrate or as a freeze-dried powder (Kindstedt, 2005).

The most common method for production of DVS starter cultures for use in the dairy industry is through fermentation in a milk-based medium. Both pH and temperature are controlled to provide optimum growth conditions. The cells are harvested after optimal growth and are concentrated by passing through a centrifuge. Cryoprotectants are then added to the mixture to prevent cell damage during freezing and the mixture is filled into 500 mL trays. The trays are then blast frozen at -40°C . The frozen trays are held at -30°C until QA is completed and then the starter is dispatched as required

2.2.1 Fermentation

In fermentation, carbohydrates are broken down anaerobically, with an organic molecule acting as the final electron acceptor. Fermentation does not involve an electron transport system (Campbell & Reece, 2008). It provides little energy due to the partial break down of glucose. Glycolysis is the initial stage of fermentation. In Glycolysis, a six-carbon glucose molecule is partially broken down into two, three-carbon molecules of pyruvate, 2 NADH, 2 H^+ and 2 ATP as a result of substrate level phosphorylation (Campbell & Reece, 2008). The overall equation for glycolysis is:



If NADH is not oxidised back to NAD^+ , there will be no further catabolism. Therefore a suitable terminal electron acceptor must be used. The protons and electrons produced in the glycolytic pathway combine with NAD^+ and the organic molecule that serves as the final electron acceptor (pyruvate), reducing NAD^+ to NADH and H^+ . The 2 pyruvic acids are then

converted to different fermentation end products in several non-energy producing steps. A simplified fermentation pathway is shown in Figure 2.2:

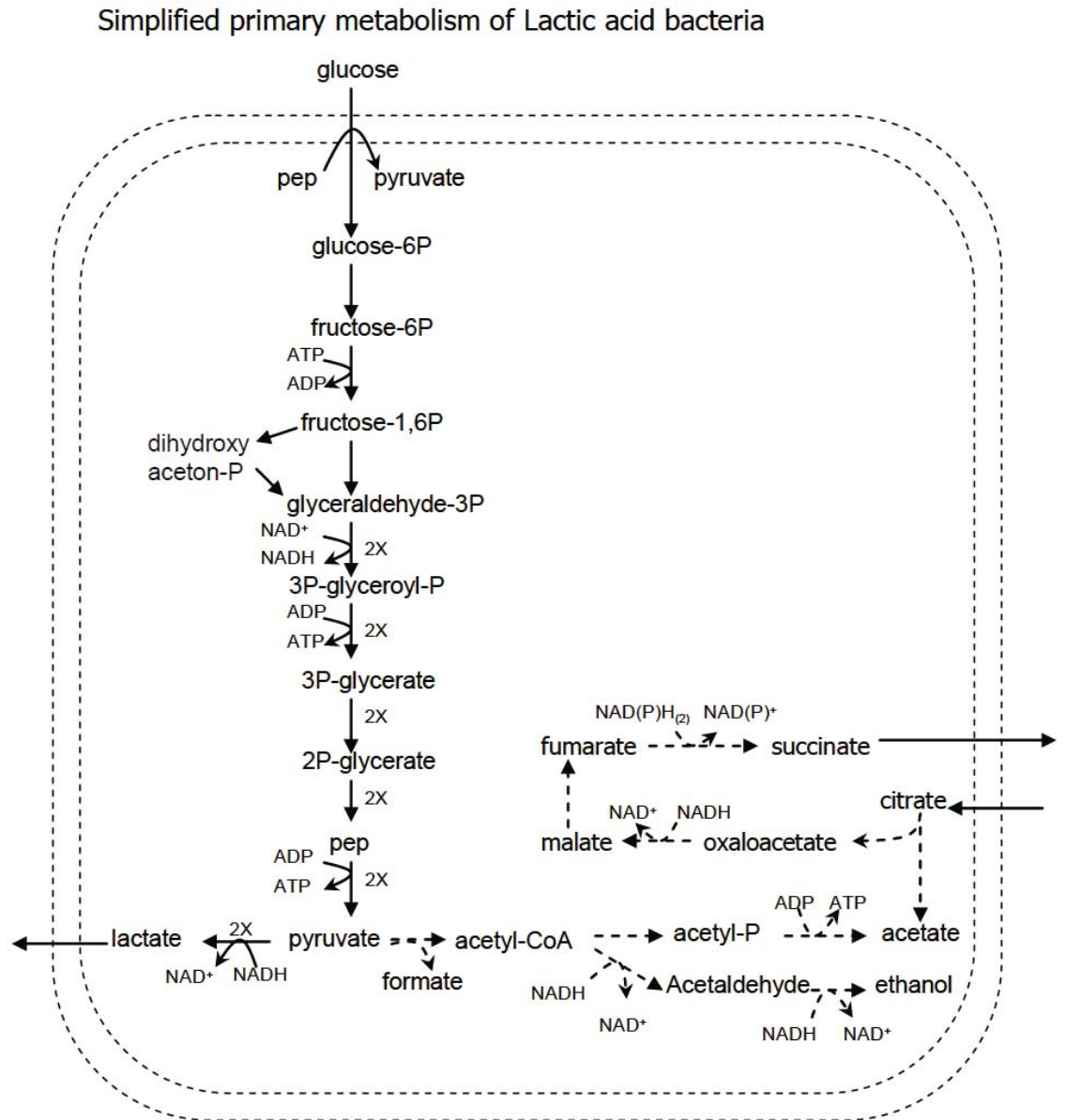


Figure 2.2: Simplified fermentation pathway of lactic acid bacteria (Broojimans, 2008)

If sugars other than glucose are present, they need to be converted to glucose through various reactions into either glucose or intermediates in a glucose catabolic pathway. In milk, where the carbon source is lactose, lactose is cleaved into glucose and galactose by the action of the enzyme β -galactosidase. Glucose can then enter the glycolysis pathway

directly, while galactose is converted to glucose 6-phosphate, which also enters glycolytic cycle.

2.2.1.1 Lactic acid fermentation

One molecule of glucose is oxidised to form two molecules of pyruvic acid in glycolysis, producing 2 moles of ATP as energy. The two moles of pyruvic acid are then reduced by two molecules of NADH to form two molecules of lactic acid, which is catalysed by the enzyme lactate dehydrogenase (Figure 2.3). Lactic acid is the end point of the reaction and no further oxidation happens. The overall reaction of conversion from glucose to lactate can be written as:



Therefore, most of the energy produced by the reaction remains stored in the lactic acid and thus generates only very little energy.

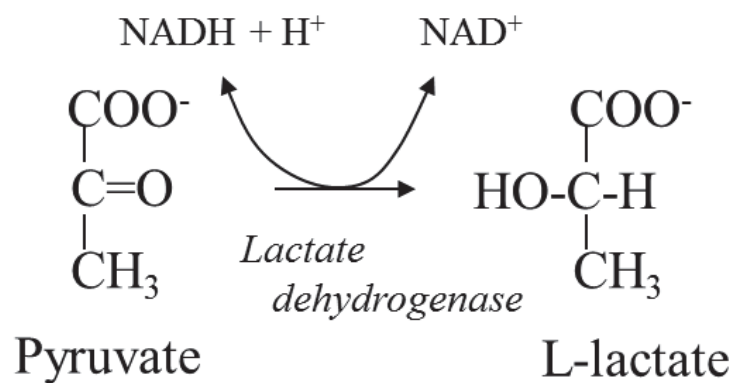


Figure 2.3: Conversion of pyruvate to lactate by the action of lactate dehydrogenase

There are two types of lactic acid fermentation: homolactic, where all pyruvate generated by glycolysis is reduced to lactic acid and heterolactic, where other products such as ethanol and acetate are generated along with lactic acid.

As the fermentation progresses, more lactic acid is produced. Lactic acid lowers the pH of the media to a level that eventually becomes toxic to the bacteria and no further biomass will be produced. Thus, in industry, pH is controlled using an alkali such as ammonium hydroxide to neutralise the acid produced.

Since the fermentation is a low energy yielding pathway, the amount of biomass produced will be low. The carbon source is used for the formation of biomass and product. Since the metabolism produces less energy, more carbon source is directed towards product formation, thus producing more energy than directing towards biomass formation, which is energy intensive.

Since the purpose of this project is to increase the amount of biomass produced, the metabolism of glucose must be directed to a pathway that produces more energy. This will have two benefits. The first one is the production of more biomass, since a greater amount of energy can be directed towards biomass production. The second benefit is that a lesser amount of acid will be produced since pyruvate will no longer be the terminal electron acceptor and thus won't be converted to energy rich acids. This in turn slows the pH drop in the media, allowing for more biomass production.

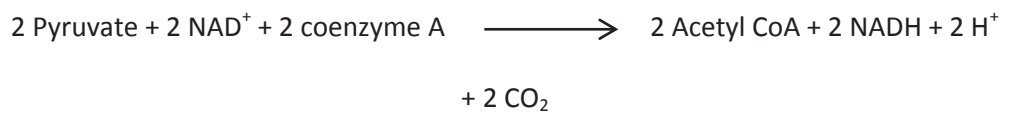
2.3 Respiration

Respiration is an energy-producing process in which organic or reduced inorganic compounds are oxidised by inorganic compounds (Campbell & Reece, 2008). Aerobic respiration involves glycolysis, citric acid (Krebs cycle) and an electron transport chain.

Respiration can be anaerobic, where other chemicals except oxygen is the terminal electron acceptor or aerobic, where oxygen is the terminal electron acceptor (Campbell & Reece, 2008). The function of the glycolysis, citric acid (Krebs) cycle and electron transport chain are explained in detail below (Campbell & Reece, 2008).

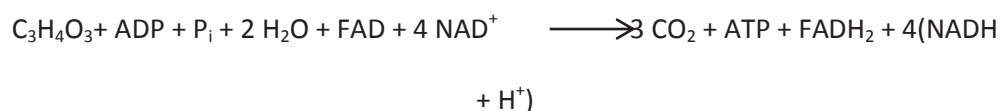
1. Glycolysis

The first part of glycolysis is exactly the same as in fermentation. The 6-C sugar (or its intermediates) is converted to pyruvate. In aerobic respiration, the first step is to convert pyruvate to acetyl coenzyme A, shortened as acetyl CoA, which is catalysed by the pyruvate dehydrogenase complex. Acetyl CoA then enters the citric acid cycle. The reaction for this transition cycle is:



2. Citric acid (Krebs) cycle

The citric acid cycle or Krebs cycle, also known as the tricarboxylic acid pathway, completes the oxidation of pyruvate to CO₂ and reduces the electron carriers to produce NADH and FADH₂. These electron carriers are then used for further ATP synthesis in respiration in the electron transport chain. The Krebs cycle also produces C-4 and C-5 intermediates, which can be used for the biosynthesis of amino acids, purines and pyrimidines. The overall reaction for citric acid cycle is:



3. Electron transport chain

In the electron transport chain, the electrons from NADH are passed to coenzyme Q (also cytochrome reductase). From coenzyme Q, electrons pass through a sequence of cytochromes, which are proteins containing heme groups. ATP is

generated twice for each electron pair along the way. The final step is the combination of hydrogen atoms with dissolved oxygen to yield water as the final product of the oxidation.

The aerobic respiration process involving glycolysis, citric acid cycle and the electron transport chain can produce up to 36 ATP, compared to 2 ATP for glycolysis alone. Thus, it is beneficial to convert the metabolism of LAB to aerobic respiration if more biomass is to be produced. But, since LAB is not a traditional aerobic bacterium, problems exist, which will be explained in detail below.

2.3.1 Respiration in LAB

Lactic acid bacteria are not grown traditionally in aerobic environments. Lactic acid bacteria were first shown to be able to respire by (Sijpesteijn, 1970). This observation was largely ignored until almost 28 years later when different *Lactococcus* strains were found to be able to undergo respiration and thrive when grown in the presence of oxygen and a heme source (Duwat et al. 1999; Duwat et al. 2001; Duwat et al. 1998)

There are a few key reasons for LAB not being a traditional aerobic bacterium:

1. LAB lacks a functioning citric acid cycle. The purpose of citric acid cycle is to produce NADH, which can be fed into the electron transport chain. Therefore LAB requires a sugar carbon source and glycolysis to generate the necessary NADH for further respiration (Gaudu et al. 2002; Pedersen et al. 2012). Thus, LAB undergoes fermentation before moving onto respiration growth.
2. The cytochromes in the electron transport chain require heme to function. Most aerobic bacteria have the ability to synthesise heme. But, the LAB lacks heme synthesis genes. Therefore heme must be added exogenously for the cytochromes to function properly. Some LAB also requires menaquinone due to the lack of

menFDXBEC gene required for menaquinone biosynthesis (Lechardeur et al. 2011; Rezaïki et al. 2008)

3. The respiring LAB only encodes a single type of cytochrome oxidase, the quinol oxidase, CydAB. This enzyme complex is found to work at low oxygen concentrations and may contribute to eliminating oxygen from the bacterial environment (Rezaïki et al. 2004). This may prevent oxygen from being the terminal electron acceptor, thus making the respiration ineffective.
4. Oxygen can itself be a problem for the bacterium. Oxygen can inhibit growth and prolonged aeration of lactococcal cultures can cause cell death and DNA degradation (Duwat et al. 1995). Oxygen toxicity can be attributed to formation of hydrogen peroxide and hydroxyl radicals (Duwat et al. 1995). *L.lactis* possesses a single superoxide dismutase (SOD) (Sanders et.al. 1995) and no catalyase. It was found that the addition of exogenous catalyase improved the survival of *L.lactis* exposed to oxygen (Duwat et al. 1995)

The electron transport chain (Figure 2.3) in LAB is composed of (Lechardeur et al., 2011) : an electron donor (NADH dehydrogenase), an electron shuttle (menaquinone) and a heme-requiring terminal electron acceptor (cytochrome quinol oxidase, CydAB).

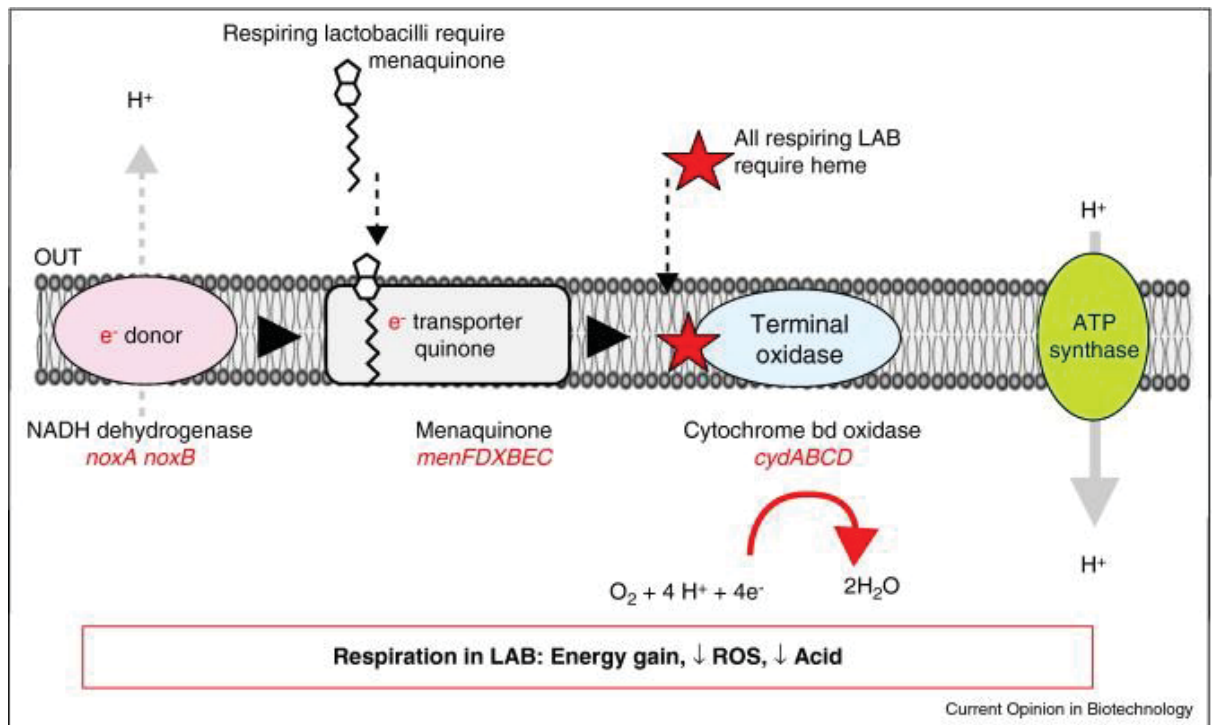


Figure 2.3: Electron transport chain of lactic acid bacteria (Lechardeur et al. 2011)

2.3.2 Heme

Heme is a type porphyrin, which are a group of naturally occurring organic compounds.

Porphyrins are aromatic and are composed of four modified pyrrole subunits

interconnected at their α carbons via methine bridges (=CH-) (Ziang, 2011). Not all

porphyrins contains iron. But, hemoproteins are a large fraction of the porphyrin containing metalloproteins that has heme as their prosthetic group. The center of heme contains an

iron ion (Figure 2.5). Heme is present as the component of haemoglobin, the red pigment in blood (Ziang, 2011).

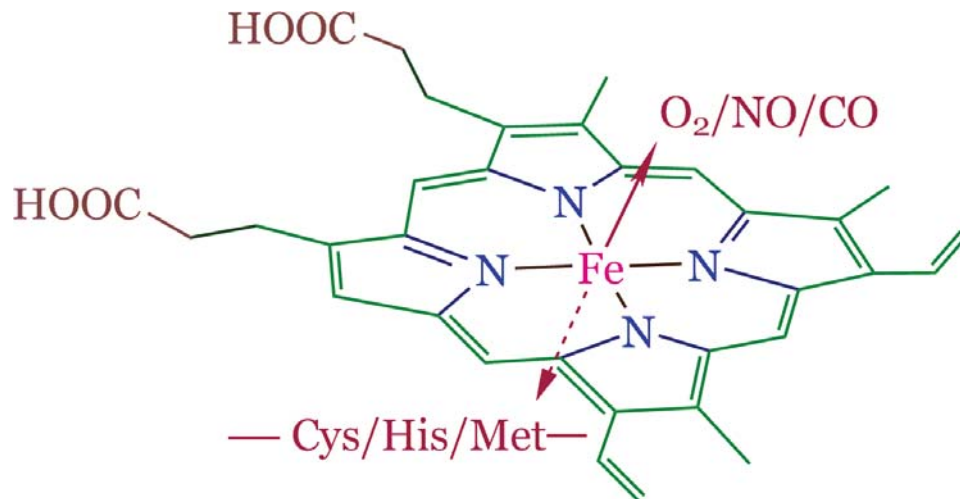


Figure 2.4: Structure of heme showing the iron centre (Ziang, 2011)

2.3.3 The role of heme in respiration

Since heme is not synthesised by any LAB, a heme uptake system is required. In *L.lactis*, this is accomplished by involving the *fhuDBAR* operon (Gaudu et al. 2003). However, despite the existence of *fhu* homologues in different bacteria, it appears that the heme mechanisms are not sufficiently conserved to make functional predictions (Pederson et al. 2012).

Chaperone proteins are involved in the insertion of heme into membrane cytochrome oxidases (Pedersen et al., 2012). Chaperone proteins recognize and selectively bind non-native proteins to form relatively stable complexes (Ellis, 1990). Molecular chaperones comprise of several highly conserved families of unrelated proteins and many chaperones are also heat shock (stress) proteins (Fink, 1999).

One candidate for heme chaperone is AhpC. AhpC is the peroxiredoxin reductase needed for oxygen stress response. AhpC is proposed to be a heme chaperone that protects intercellular heme from degradation (Lechardeur et al. 2010). Another candidate, the CydCD complex, is implicated in cytochrome oxidase (CydAB) activity. Many researchers

(Pittman et al. 2002; Pittman et al. 2005) have come to the conclusion that CydCD transports cysteine and glutathione, thus contributing to a reducing environment, and facilitating CydAB-heme interactions.

Although heme is an essential for the cell as it is used for oxygen sensing and numerous enzymatic reactions, it is toxic to the cell in its free form. Therefore, it is essential that *L.lactis* manages the heme pools by efficient systems for heme homeostasis. This is required to avoid the toxic effects of heme. *L.lactis* lacks the enzymes required to degrade heme for iron recovery. However, it encodes a dedicated heme efflux system (YgfCBA, now called HrtRBA) (Pedersen et al. 2008) that effluxes excess heme to avoid accumulation. However, Pederson et al. (2012) noted that among the numerous LAB that encodes the HrtRBA and/or Pef homologs, some do not have respiration potential, which raises the question whether LAB assimilate and use heme for purposes other than respiration

Therefore, respiration maybe considered as a highly regulated process since it only occurs in the presence of heme in the cells. Heme levels must be strictly controlled to avoid accumulation of heme, thus becoming toxic to the cells, but have sufficient amounts of heme to mediate respiration and growth.

2.3.4 Impact of respiration on LAB

There are two main consequences of respiration on *L.lactis*: doubling of biomass yield and increase in long term stability. These benefits are attributed to four main features of respiration:

1. Respiration is more efficient than fermentation.

2. Less acid stress is generated. In respiration, NADH is oxidised to NAD^+ , which stops the activity of lactose dehydrogenase which uses NADH to produce lactic acid.
3. Less oxidative stress is generated since oxygen is reduced to water, thus reducing intracellular oxygen content.
4. It acts as a sink for free electrons produced by the membrane NADH dehydrogenase. In the absence of respiration, these electrons reduce oxygen to superoxide, Fe^{3+} to toxic Fe^{2+} and Cu^{2+} to Cu^1 .

The advantage of the respiration lifestyle can be augmented by other factors: Koebmann et al. (2008) has noted improved biomass yield when maltose was used as carbon source. Increasing the peptide-nitrogen sources were also used to optimise biomass of respiring Lactococci. (Kringelum et al 2006), doubled the biomass yield by adding inosine to respiring cultures of lactococci.

2.3.3.1 Metabolic changes

1. Carbon metabolism

Proteome and transcriptome analyses have shown that despite the effects of *L.lactis* respiration on cell density and long term survival, respiration attributed gene changes are limited. The major changes occurring via respiration are attributed to a shift in metabolite pools affected by electron transport chain activity. NADH, an electron donor in various enzymatic reactions are depleted by respiration. As a result, the $\text{NADH}:\text{NAD}^+$ ratio, the driving force in dominant metabolic pathway determinant is decreased. For example, the low amount of lactic acid produced. Since lactic acid cannot be produced, a part of pyruvate is directed towards the production of acetoin and acetate.

2. Nitrogen metabolism

Regulation of nitrogen homeostasis in *L.lactis* during respiration is more complex than what is considered presently. Data points to the existence of another regulator in respiration conditions in addition to CodY regulator (Vido et al., 2004). The expression of ammonium/ammoniac uptake (via amtB-glnK), glutamate synthesis (via glnA) , glutamine transport (via glnP) and other functions is controlled by GlnR (Larsen, 2006). Vido et al. (2005) noticed low glutamine levels in spent respiration culture medium. This might explain the observed amtB-glnK induction via the GlnR pathway, however expression of other genes were not affected (Pedersen et al. 2008)

Another interesting observation is the high amount of proline detected in the medium during respiration growth (Vido et al. 2004). This observation might be connected to PepO1 expression since this peptidase cleaves proline-rich peptides. In the cell, proline stabilise proteins and prevent the loss of viability during stress conditions. Proline expulsion during respiration may contribute towards alleviating oxidation stress and might help in bacterial survival (Pedersen et al. 2012)

2.4 Improving starter culture production by respiration technology

The respiration technology was first investigated in the late 1990s and was implemented until the early 2000s at Chr.Hansen A/S (Denmark) (Pedersen et al. 2005). The researchers primarily focused on the organism, *L.lactis*, which was used for the initial proof of the principle (Duwat et al. 2001; Duwat, 1998). Since respiration technology drastically changed bacterial physiology, numerous investigations were carried out to ensure that the respiration starter cultures performed as expected when inoculated in milk and other food substances (Pedersen et al. 2005). Soon after, the complete genome sequence of one LAB, *L.lactis* subsp *lactis* IL1403 was published. This became the first whole genome platform

used to investigate LAB respiration vs anaerobic growth (Pedersen, et al. 2008; Pedersen et al. 2005).

From an industry point of view, the improvement in bacterial yield of starter cultures over traditional fermentation was the important focus. The long term survivability of the thus grown cultures are also an added benefit. The initial invention of respiration growth for starter culture preparation led to approximately doubling in the yield on the industrial scale (Pedersen et al. 2005).

Research is being conducted into further understanding the respiration mechanism so that more biomass yield could be obtained. One interesting observation by Pederson et al. (2012) was that cells were starved for purines early in the respiration growth. This can be alleviated by adding exogenous sources of purine such as inosine and IMP. Adding purines increased the already increased yield of respiratory culture to almost double the amount of product per fermenter volume. More research by Kringelum et al. (2005) has shown that this effect is unique under respiration. More research is required to understand this phenomenon.

More research being conducted is on using a slowly metabolising sugar such as maltose instead of the easily metabolised lactose (Koebmann et al. 2008). This seemed to have increased the biomass yield, but when the cultures were subsequently inoculated, the acidification activity reduced greatly, which is unacceptable in a starter culture used in industry.

Another interesting observation is the ability of respiration growth to improve survival of non-respiring co-cultured strains (Rezaïki et al. 2004). Studies have shown that survival of a *cydA* lactococcal mutant is much improved by co-culture with a respiring wild-type strain in

the presence of heme and oxygen (Pedersen et al. 2005). This co-culture can be used for improving the robustness of an otherwise fragile strain.

2.5 Conclusion

The traditional method of starter culture production involves growing in an anaerobic environment in a complex medium such as milk. This anaerobic growth, also known as fermentation, involves the glycolytic pathway, with pyruvate being the terminal electron acceptor. Pyruvate is then converted to lactic acid, which is rich in energy. Only 2 ATPs are produced in this step. This low energy yield means less biomass is produced. Therefore to improve the biomass growth, an alternative pathway has to be found.

Respiration is an energy producing pathway, which can produce up to 38 ATPs. Various researchers have shown that various LAB, including *L.lactis* has the ability to respire, albeit with the addition of heme and in some cases menaquinone. The consequences of *L.lactis* being grown aerobically is increased biomass yield, improved stress resistance and increased long term survival. All these features are desirable in the production of starter cultures.

3.0 MATERIALS AND METHODS

3.1 *Lactococcus* strain

The strain used in this experiment is *subsp lactis* 383, obtained from the Microbial Fermentation Unit, Fonterra, based in Palmerston North. The strains were obtained in frozen vials and were stored at -80°C until needed. Working cultures were produced from this by streaking a small loop full of the sample from the vial into an M17 plates, with glucose as the sugar source. The plates were then incubated for 24-48 h at 30°C. A new fresh plate was made every week.

3.2 M17 Media

M17 media was reconstituted from Difco M17 powder and Milli-Q water and was autoclaved. Glucose was used as the sugar source and 50 mL of 10% glucose solution was made and autoclaved separately and this was added to the autoclaved M17 media.

3.3 Heme

Heme was purchased as hemin chloride from Calbiochem. A 0.5 mg/mL stock solution was made by dissolving hemin chloride in 0.05M sodium hydroxide solution.

3.4 Alternate heme

The alternate heme used in this experiment is ammonium ferric citrate. A 50 mg/L stock solution was prepared by dissolving ammonium ferric citrate in MilliQ water. It was of analytical grade purchased from JT Baker.

3.5 Analytical techniques

3.5.1 Biomass

The biomass was measured as optical density (OD) at 600 nm (OD_{600}) by using a spectrophotometer (T90 + UV/Vis Spectrometer PS19-2, PG Instruments Ltd). 0.2 mL of the media was added to 6.8 mL distilled water to achieve a 35 fold dilution. If the OD of the diluted sample was greater than 1, a further 35 fold dilution was done.

3.5.2 Glucose

The glucose concentration was measured using a YSI 2700 glucose analyser. Samples were diluted 10 fold and duplicates of each sample were obtained.

3.6 Fermentation

3.6.1 Equipment

The cells were grown in 1.5- L (fermenter volume) Applikon fermenters (Applikon Biotechnology B.V., Schiedam, Holland). The fermenters have a working volume of 1.5 L and is water jacket heated. They have ports for a pH probe, acid/alkali addition, air/oxygen addition, a Dissolved Oxygen probe and stirred using Rushton turbine impellers. Control of the fermenter is by Applikon computer software. Temperature and pH are set and are controlled automatically. The pH is controlled using the addition of 12.5% ammonium hydroxide solution.

3.6.2 Operational Procedure

For all the fermenter runs, M17 media was prepared by adding 37.25 g M17 media to 950 mL of MilliQ water, mixed thoroughly until the mix had dissolved and was poured into the fermenter. The fermenter was then fitted with the top plate and all the ports were covered

with aluminium foil and this was autoclaved at 121 °C for 15 minutes. The water jacket was filled prior to autoclaving to ensure that the media was properly autoclaved. The inoculum was prepared the day prior to the fermentation.

On the day of the fermentation, the fermenter was connected to the controller (figure 3.1) and temperature and pH were set. While the temperature was coming up to the set temperature (30 °C), the sugar solution was prepared. Industrial grade dextrose was used to prepare a 50 mL 10% solution and this was filter through a 0.2 micron filter and added to the fermenter. The appropriate heme/heme alternative were added at this point.



Figure 3.1: Fermentation set up

Two drops of polypropylene glycol was added as an antifoam before sparging air at 82.5 mL/min, which is 35 on the gauge. The inoculum was added when the temperature reached 30 °C. The media was then allowed to mix for 15 min before a sample at time zero was taken. Samples were then taken every hour and the OD was measured promptly to avoid contamination. The samples were then frozen and stored for further analysis.

For aerobic fermentations, the media was mixed at 600 rpm to ensure perfect mixing and air at 82.5 ml/min was also sparged into the media. For anaerobic fermentations, the media was mixed at 150 rpm and no air was sparged into the system.

For the shake flask trials, 150 mL conical flasks were used. All the conical flasks used were of the same shape. A total of 20 mL including separately autoclaved media and sugar were aseptically transferred to the flask. The appropriate additive and inoculum were added and the flasks were shaken overnight at 250 rpm in a rotary shaker at 30°C

3.7.1 Activity testing

3.7.2 Direct set milk preparation

A sufficient quantity of Pasteurised Reconstituted Skim Milk (PRSM) was prepared for activity testing by adding 102g skim milk powder to 928 mL Milli-Q water for each litre required. The milk was autoclaved for 15 min at 121°C. 200 mL of PRSM were dispensed into Schott bottles for each activity testing and for two blanks.

An inoculum of culture was added to each bottle. A pH probe was placed in each bottle and the pH continuously recorded. The activity was defined as the time to reach pH 4.6.

3.7.3 Inoculum preparation for activity testing

The cells were harvested from each fermenter after 5 hrs. Duplicate samples were harvested from each fermenter in blue top falcon tubes. The samples were centrifuged (15000 g for 10 min) and the supernatant was removed. The industrial clarifier has a concentration factor of 15 times, which is not as concentrated as the lab centrifuge. A calculated amount of supernatant was added back to the sample to ensure the final concentrate was a mimic of the large scale concentrate.

Cryoprotectant was then added, at the correct cell to cyroprotectant ratio, to the tubes and mixed thoroughly to make sure all the cells were suspended. The tubes were then placed in a -80°C freezer overnight.

After freezing, the cells were thawed and 1 mL of the sample was aseptically added to 9 mL of sterile peptone water and mixed thoroughly. This stock was further diluted by adding 0.1 mL into 9.9 mL sterile peptone water. One mL of this sample was then added to 200 mL of the PRSM in the Schott bottle prepared earlier.

3.7.4 Operating procedure

The Schott bottles were then placed in a water bath set at 27°C. A number of pH probes were then calibrated and sanitised by placing in chlorine solution for 5 min. The pH probe was then inserted into the bottle through the hole in the lid and the time was noted. Once the activity test had reached the desired pH, the activity was stopped. The data was then transferred to Excel for further analysis.

4.0 RESULTS AND DISCUSSION

4.1 Heme alternatives

The use of Heme or heme derived chemicals for supplementing aerobic fermentation for biomass production is a patented process. The use of heme or blood derived products for the manufacture of dairy products should follow strict regulations, both safety and religious. In order to produce dairy product to be consumed by Muslims, the blood-derived components used in the dairy fermentations should not be derived from any non-halal sources such as porcine. Any blood product derived from bovine sources must be sourced from a halal-approved farm. For the product to be sold to Jewish community, the product has to be kosher. Hence the blood product has to be kosher as well. Blood can carry a variety of pathogens. Hence, it is important that any blood product used is thoroughly tested and purified, which can add to the cost of production. Hence, a major part of this thesis was to look at alternatives to heme.

Antioxidants belong to a group of chemicals that can prevent the harmful oxidative effects of oxygen. Humans take antioxidants in the form of tablets or naturally occurring in certain fruits and vegetables to reduce the harmful effects of oxygen which can prevent premature ageing and certain diseases. Since *L.lactis* cannot deal with the harmful effects of oxygen, growing *L.lactis* aerobically can cause cell death due to the harmful effects of oxygen. Hence, the theory was to limit the harmful effects of reactive oxygen species by using an antioxidant

The other theory was to try supplementing the media with other sources of iron. A literature search shows that ammonium ferric citrate has been tried as an iron source to grow human cells and other pathogenic bacteria (Richardson and Baker, 1991; Krom et al. 2000). Three chemicals were chosen for testing in shake flask trials to find out which

chemical gave the highest biomass compared to heme. The chemicals that were chosen for testing were: glutathione, hydroquinone and ammonium ferric citrate.

There is no published information on the quantity of these chemicals required for effective removal of reactive oxygen species except for glutathione, which was 3.2 mM (Li et al. 2003). The concentrations of the other chemicals to be used were assumed to be closer to the value for glutathione.

The shake flask trials were conducted in duplicates. Each shake flask consisted of 18 mL of M17 media and 2 mL glucose solution. The solutions were autoclaved separately and were transferred aseptically into the conical flasks under the laminar hood.

To each conical flask, glutathione and ammonium ferric citrate were added to give a final concentration of 0.935 mg/mL. Hydroquinone was added to give a final concentration of 0.5 mg/mL and heme was added to a final concentration of 0.0098 mg/mL.

Once the appropriate chemical was added, 100 μ L of the inoculum grown overnight on M17 media was added. The contents of the shake flask were placed in an orbiter shaker at 250 rpm and 30°C overnight. The OD was measured on the following day. The effect of various heme alternatives on the growth of *L.lactis* is shown in Table 4.1

Table 4.1: The effect of various heme alternatives on aerobic growth of *L.lactis* in shake flasks

Sample	Glu	Glu	AFC	AFC	HQ	HQ	Heme	Heme	Aerobic	Aerobic
pH initial	7.06	7.06	7.06	7.06	7.06	7.06	7.06	7.06	7.06	7.06
pH final	4.54	4.53	4.48	4.48	4.66	4.66	4.70	4.67	4.60	4.57
Average OD ₆₀₀	0.281		0.308		0.285		0.358		0.272	

Glu: Glutathione; AFC: Ammonium ferric citrate; HQ: Hydroquinone

It can be seen from Table 4.1 that all the chemicals tested: glutathione, ammonium ferric citrate, heme and hydroquinone showed an increase in biomass (as measured by the OD) as compared to not adding any heme alternatives during aerobic fermentations. Adding glutathione showed a 3.13% increase in OD. Whereas, adding ammonium ferric citrate showed a 13.05% increase in OD. Hydroquinone increased the biomass by 4.78%. Heme had the highest increase of 31.43% in OD compared to aerobic fermentations without the addition of any chemicals. Both glutathione and hydroquinone are antioxidants and might have had some effect in neutralising some of the free oxygen radicals that was formed during the fermentation, which has led to an increase in the biomass.

Ammonium ferric citrate could have acted as a source of iron for the fermentation, albeit not as effective as heme as per the results obtained in this study. Since the purpose of this project was not to find out why there was an increase in the above cases and because of the sheer possibility of chemicals that could be tested as heme alternatives, this part of the experiment was done to prove that alternatives to heme exists, although the reasons for the increase is unknown. Further experiments could be done to study the role of these chemicals in enhancing biomass formation.

The conclusion from Table 4.1 was that ammonium ferric citrate was found to show the greatest increase in OD relative to the aerobic control, of the three chemicals tested. Ammonium ferric citrate was chosen to be used in further experiments.

4.2 Effect of different concentrations of ammonium ferric citrate

Since ammonium ferric citrate was shown to produce the maximum increase in biomass (Table 4.1), further shake flask trials were conducted to determine the optimal concentration of ammonium ferric citrate as an alternate heme source.

A literature search on ammonium ferric citrate indicated that this compound was used to study the possible interaction between ammonium ferric citrate and the aerobic growth of *Listeria monocytogenes* (Dykes and Dworaczek, 2002). Three concentrations of ammonium ferric citrate: 44 mg/mL, 88 mg/mL and 176 mg/mL were tried by Dykes and Dworaczek (2002). The results of the experiments found that increasing ammonium ferric citrate concentration resulted in improved growth of *L.monocytogenes*. In addition to these concentrations, 250 mg/mL and 500 mg/mL were also tested in this project. For the growth of *L.monocytogenes*, the optimum ammonium ferric citrate concentration was found to be 88 mg/ mL (Premaratne et al. 1991).

The shake flask fermentations to optimise the concentration of ammonium ferric citrate consisted of 18 mL of autoclaved M17 media, 2 mL of industrial dextrose. Ammonium ferric citrate was added to the above media to give a final concentration given in the table 4.2. 100 µl of inoculum grown overnight in M17 media was added to the growth medium, mixed thoroughly and incubated overnight in an orbital shaker at 30 °C and 250 rpm. The OD was measured after 18 hours and the results are shown in Table 4.2.

Table 4.2: The effect of ammonium ferric citrate concentration on aerobic growth of *L.lactis* in shake flasks

Ammonium ferric citrate (µg/mL)	0	44	88	176	250	500
OD ₆₀₀	2.91	3.32	3.18	3.22	3.21	3.22

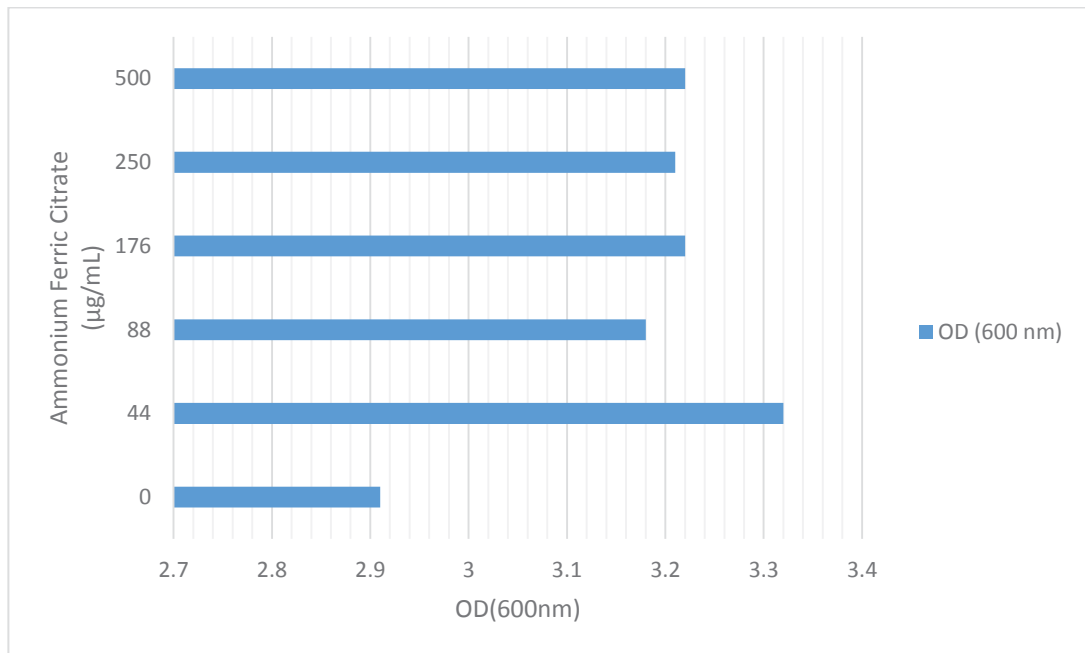


Figure 4.1: Effect of ammonium ferric citrate concentration on aerobic growth of *L.lactis* in shake flasks

It can be seen from Figure 4.1 that there was a sharp increase in OD when 44 µg/mL of ammonium ferric citrate was added compared to *L.lactis* grown in the absence of ammonium ferric citrate. The subsequent increase in ammonium ferric citrate concentration decreased the OD until the OD plateaued.

The reason for increase in biomass by the addition of ammonium ferric citrate is unknown. It is widely known that iron in the form of heme is required for the growth of *L.lactis* under aerobic conditions and the process by which heme is utilised is also well studied (Pedersen et al. 2011). The idea that other forms of iron can be used has never been trialled before. Therefore the process by which ammonium ferric citrate is metabolised and used by bacteria is unknown. It is also not known whether ammonium ferric citrate is metabolised within the cell or outside the cell. Studying that mechanism is beyond the scope of this project. But, certain conclusions can be arrived at from this study:

Firstly, since heme is not added to the media, and remembering that for *L.lactis* electron transport chain to be complete, heme is absolutely required, it can be hypothesised that *L.lactis* could use other forms of iron in the electron transport chain through a hitherto unknown process.

Secondly, iron could be taken up in the cell by systems other than the ones that is already known to exist in *L.lactis*. This idea is not new as Messenger and Ratledge (1981) realised in the case of *Mycobacterium smegmatis* that the bacteria was able to transport iron into cells by a system that was independent of the iron transport systems that was already known to exist in *M.smegmatis*.

Citrate is thought to have a function that is similar to that of siderophores in coliform bacteria, even though citrate is neither excreted nor assimilated by these bacteria (Bishop et al, 1976; Griffiths and Humphreys, 1977). Siderophores are low molecular weight ferric iron specific chelating agents that are used by bacteria and fungi to assimilate iron in an iron limited environment (Neilands, 1995). Citrate is known to be needed for iron uptake in humans (Sarker, 1970) and has also been implicated in transferring iron to and from transferrin (Bates et al, 1967; Charley et al, 1960).

Various bacteria show the ability to uptake iron via citrate mediation in the presence of citrate in the media. For example, *Neisseria meningitidis* can uptake iron from previously unavailable iron chelates by adding citrate in the media (Archibald and DeVoe, 1980). *Escherichia coli* (Frost and Rosenberg, 1973 and 1975) and *Neurospora crassa* (Winklemann and Zahner, 1973) are also known to have similar systems.

One explanation for the decrease in OD as the amount of ammonium ferric citrate is increased may be due to the fact that high levels of iron are harmful for bacteria. Iron can

participate in the formation of reactive species via Fenton reaction and can cause oxidative stress for the bacteria and can damage the cells (Touati, 2000).

4.3 Heme Optimisation

There is no literature evidence on the effect of different concentrations of heme on *L.lactis* biomass when grown aerobically. There has been no published study done on the optimum level of heme required for aerobic fermentation. Hence, it was decided to do 1-L fermenter trials using different concentrations of heme. All the experiments were done in duplicates (appendix 1) and the average results are presented. The concentrations of heme chosen for the study are: 0 $\mu\text{g}/\text{mL}$, 1 $\mu\text{g}/\text{mL}$, 3 $\mu\text{g}/\text{mL}$ and 5 $\mu\text{g}/\text{mL}$. All fermentations were conducted under aerobic conditions. The results are shown in Figure 4.2 below:

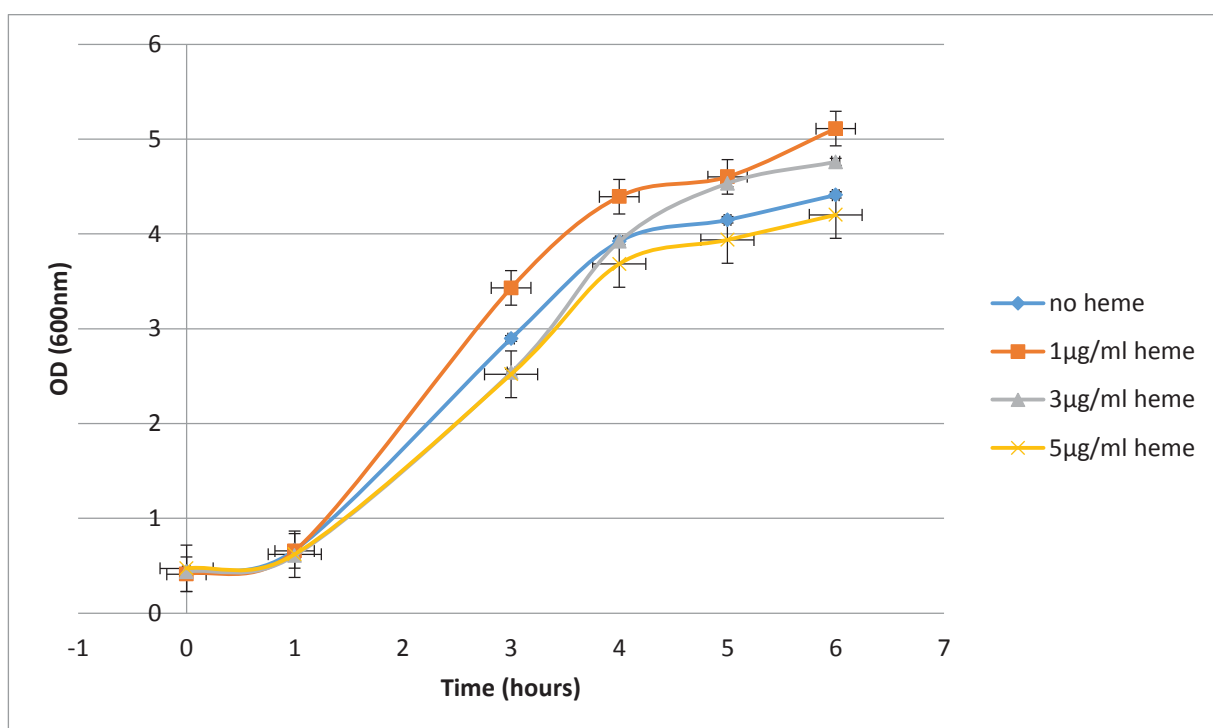


Figure 4.2: Effect of different concentrations of heme on biomass of *L.lactis* grown aerobically

The OD of culture broth from each fermenter was measured over the period of 6 h. This time for measuring OD was chosen because the glucose is all consumed by the 5-6 h into

the fermentation and for further fermentation to proceed, more glucose has to be added.

The glucose course graph for a typical aerobic fermentation using heme is given in figure

4.3. The glucose was consumed in 5-6h regardless of the concentration of heme.

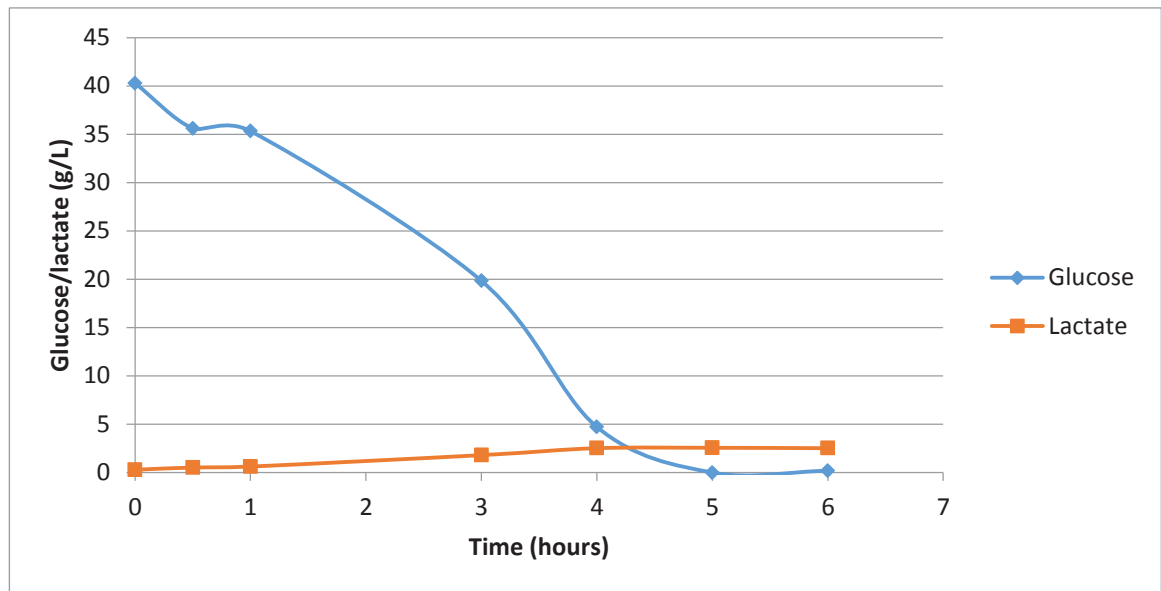


Figure 4.3: Glucose and Lactic acid time course for a typical aerobic fermentation using heme

All the four concentrations of heme tested resulted in very similar lag phases of about 30-45 min. The growth phase was more prominent in 1 $\mu\text{g}/\text{mL}$ heme, followed by the growth without any heme. Cultures grown using 3 $\mu\text{g}/\text{mL}$ and 5 $\mu\text{g}/\text{mL}$ had very similar growth. After 3 h of growth, the culture grown using 3 $\mu\text{g}/\text{mL}$ accelerated growth resulting in second highest final OD, followed by the culture grown without any heme, closely followed by culture grown using 5 $\mu\text{g}/\text{mL}$.

These results showed that the lower the heme concentration, the better the biomass after 6hr was compared to not adding any heme to the growth medium. Adding heme above 3 $\mu\text{g}/\text{mL}$, decreased the biomass below the control. This could be because higher amounts of heme might lead to iron toxicity and may be detrimental to the cells. Hence, it is

recommended to use 1 µg/mL heme concentration for further fermentations if heme was chosen to be added to the growth medium.

4.4 Comparison between ammonium ferric citrate and heme

The next set of experiments were done to compare the effect of 44 µg/mL ammonium ferric citrate and 1 µg/mL heme on the biomass production and final pH when *L.lactis* was grown aerobically in a 1-L fermenter. The results are given in Figure 4.4

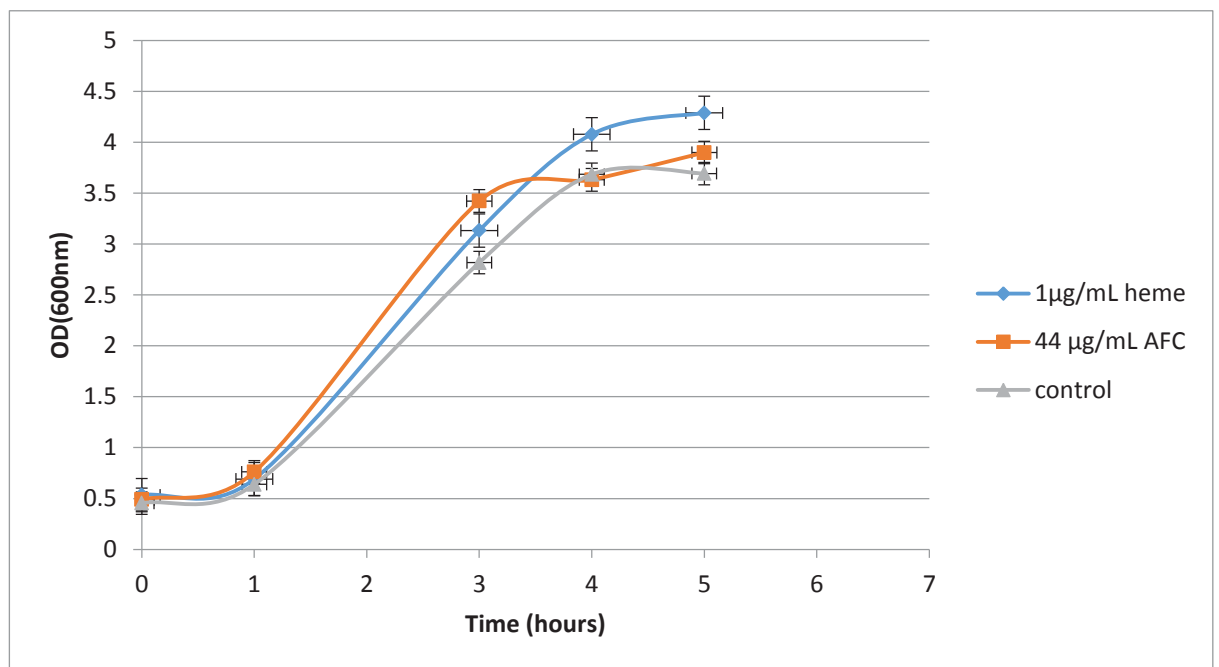


Figure 4.4: The effect of 44 µg/mL ammonium ferric citrate and 1 µg/mL heme on aerobic growth of *L.lactis* in a 1-L fermenter

The control fermentation had no heme or ammonium ferric citrate added. It can be seen from Figure 4.4 that fermentations carried out using heme clearly produced more biomass than those without the addition of ammonium ferric citrate. This may indicate that ammonium ferric citrate is clearly inferior to heme when it comes to enhancing aerobic

growth. This could be because the mode of action of ammonium ferric citrate and heme on the aerobic growth are quite different. But, with regards to using a strain as a starter culture, activity of the strain is also very important. There is no point in producing lot of biomass if it takes a long time to ferment the substrate. Therefore, even though using heme in the fermentations is better, it was decided to do experiments testing the activity to assess how the strains react in a real scenario.

4.5 Activity Testing

The last set of experiments were to test the activity of the cells that were harvested from the fermenter at the end of 5 h growth.

The cells were spun down at 5000rpm for 5 minutes. The final spun weight was calculated based on an industrial clarifier concentrating the cells 15 times. The bench centrifuge concentrates much greater than this, so a calculated amount of supernatant was added back.

To this pellet was added a cyroprotectant system of 20% sucrose. The cells to cyroprotectant was added at a ratio of 60:40. The mix was then frozen. This simulates how starters are made on the industrial scale.

The activity of the cells was measured after a freeze-thaw cycle (overnight freezing). The data from pH probes were continuously recorded and was saved to an Excel file at the end of the fermentation. The data was then processed to make sure that there were no errors within the range of measurement by the probes. This was done by measuring the difference between subsequent readings. The usual error was ± 0.02 . Any bigger difference might show that the probe malfunctioned and the data cannot be used.

A graph was then plotted (Appendix 2) showing the time it took for each probe to reach pH 4.60. Each culture was done in triplicates and the values were averaged. The total time it took for each differently produced cells to reach a pH of 4.60. The summary of the results obtained are given in Table 4.3.

Table 4.3: Time taken to reach final pH of 4.60 for *L.lactis* cultures grown by adding ammonium ferric citrate and heme, and for the control

Condition	Final pH	Time taken to reach final pH (h)
44 µg/ml ammonium ferric citrate	4.60	14.38
1 µg/ml Heme	4.60	14.5
Control	4.60	14.88

It can be seen from Table 4.3 that *L.lactis* cells grown by using ammonium ferric citrate or heme has higher activity compared to the control. This means that even though using ammonium ferric citrate produced less biomass than using heme, the *L.lactis* cells were metabolically equally active compared to the cells grown by using heme. This makes ammonium ferric citrate an alternative to heme in aerobic fermentations of *L. lactis* for starter culture production.

5.0 CONCLUSIONS

Starter cultures are used to control the fermentation to produce desirable products and avoid contamination by other bacteria. Most of the starter cultures that are used for fermentation of dairy products belong to lactic acid bacteria, with some exceptions. Modern starter cultures come from the anecdotal usage of saving a portion of the fermented material and using it for subsequent fermentations.

Lactic acid bacteria are not a traditional aerobic bacteria. As such, they lack the machinery that aerobic bacteria possesses. This causes a problem trying to grow *Lactococcus lactis* aerobically. *L.lactis* does not possess a functional electron transport chain. Therefore, *L.lactis* requires the addition of exogenous heme for efficient aerobic fermentation. Aerobic respiration can lead to the formation of reactive oxygen species that can cause cell death and DNA damage.

A literature search has shown that the mechanism behind aerobic respiration in *L.lactis* is not very well understood. Studies are still being conducted to understand the process and how to manipulate it to obtain maximum biomass and stability. There has been no study published about the effects of alternative compounds to heme on aerobic respiration.

This study found:

1. Ammonium ferric citrate is a viable alternative for heme and has an optimum concentration of 44 $\mu\text{g}/\text{mL}$
2. The optimum heme concentration is 1 $\mu\text{g}/\text{mL}$
3. Although 1 $\mu\text{g} /\text{mL}$ of Heme addition produces more biomass than 44 $\mu\text{g}/\text{mL}$ ammonium ferric citrate, *L.lactis* grown with ammonium ferric citrate are equally active after a freeze-thaw cycle compared to heme

The reason for why ammonium ferric citrate helps aerobic fermentation is unknown. There is no published data on other compounds other than heme used for aerobic fermentation of *L.lactis*. Although, ammonium ferric citrate is extensively used for growing aerobic bacteria such as *Bacillus subtilis*, *Listeria monocytogenes* and for growing human melanoma cells.

All the experiments done in this study in in either shake flask or on 1-L fermenters. Further research needs be done to understand:

- how the aerobic fermentation behaves in larger fermenters
- to adjust the concentration of heme/ ammonium ferric citrate accordingly.

Since most of *L.lactis* is grown in skim milk media in the industry, fermentations need be done in milk media using ammonium ferric citrate and heme to understand their effects in milk. The optimisation of these compounds must be done in milk media.

More study must be done to understand the effect of ammonium ferric citrate and why it is contributing to increased biomass in aerobic growth.

6.0 REFERENCES

- Archibald, F.S., & DeVoe, I.W. (1980). Iron acquisition by *Nisseria meningiditis* in vitro. *Infection and Immunity*, 27, 322-334.
- Bates, G.W., Billups, C., & Saltman, P. (1967). Kinetics and mechanisms of iron (III) exchange between chelates and transferrin .I. Complexes of citrate and nitrilotriacetic acid. *Journal of Biological Chemistry*, 242, 2810-2815
- Bishop, J.G., Schanbacher, F.L., Ferguson, L.C., & Smith, K.L. (1976). In vitro growth inhibiton of mastitis – causing coliform bacteria by bovine apo-lactoferrin and reversal by citrate and high concentrations of apo-lactoferrin. *Infection and Immunity*, 149, 911-918
- Bolotin, A., Wincker, P., Mauger, S., Jailon, O., Malarme, K., Weissenbach, J., Ehrich, S.D., & Sorokin, A (2001). The complete genome sequence of the lactic acid bacterium *Lactococcus lactis ssp. lactis* IL 1403. *Genome Research*, 11(5), 751-753.
- Brooijmans, R.J.W. (2008) Electron Transport Chains of Lactic Acid Bacteria (PhD thesis, Wageningen University, The Netherlands)
- Campbell, N.A & Reece, J.B. (2008). *Biology*. United States: Pearson Benjamin Cummings
- Charley, P., Rosenstein, M., Shore, E., & Saltman, P. (1960). The role of chelation and binding equilibria in iron metabolism. *Archives of Biochemistry and Biophysics*, 88, 222-226.
- Chopin, A. (1993). Organisation and regulation of genes for amino acid biosynthesis in lactic acid bacteria. *FEMS Microbiology Review*, 12, 21-38.
- Cocaign-Bousquet, M., Garrigues, C., Novak, L., Lindley, N.D., & Loubiere, P (1995). Rational development of a simple synthetic medium for the sustained growth of *Lactococcus lactis*. *Journal of Applied Bacteriology*, 79, 108-116.
- Dressaire, C., Redon, E., Gitton, C., Loubière, P., Monnet, V., & Cocaign-Bousquet, M. (2011). Investigation of the adaptation of *Lactococcus lactis* to isoleucine starvation integrating dynamic transcriptome and proteome information. *Microbial Cell Factories*, 10, Supplement 1-18.
- Duwat, P., De Oliveira, R., Ehrlich, D., & Boiteux, S. (1995). Repair of oxidative DNA damage in Grampositive bacteria: The *Lactococcus lactis* Fpg protein. *Microbiology*, 141(2), 411-417.

- Duwat, P., Ehrlich, D., & Gruss, A. (1999). Effects of metabolic flux on stress response pathways in *Lactococcus lactis*. *Molecular Biology* 31(3), 845-848.
- Duwat, P., Sourice, S., & Gruss, A. (1998). Process for preparing starter cultures for lactic acid bacteria. France Patent No. FR9809463.1998
- Duwat, P., Sourice, S., Cesselin, B., Lamberet, G., Vido, K., & Gaudu, P. (2001). Respiration capacity of the fermenting bacterium *Lactococcus lactis* and its positive effects on growth and survival. *Journal of Bacteriology*, 183(15), 4509-4516.
- Duwat, P., Sourice, S., Cesselin, B., Lamberet, G., Vido, K., Gaudu, P., Le Loir, Y., Violet, F., Loubiere, P., & Gruss, A. (2001). Respiration capacity of the fermenting bacterium *Lactococcus lactis* and its positive effects on growth and survival. *Journal of Bacteriology*, 183(15), 4509-4516.
- Ellis, R.J (1990). The molecular chaperone concept. *Seminars in Cell Biology*., 1, 1-9.
- Fink, A.L. (1999). Chaperone-Mediated Protein Folding. *Physiological Reviews*, 79 (2), 425-449.
- Frost, G.E., & Rosenberg, H. (1973). The inducible citrate dependent iron transport system in *Escherichia coli* K12. *Biochemica et Biophysica Acta*, 330, 90-101.
- Frost, G.E., & Rosenberg, H. (1975). Relationship between the *tonB* locus and iron transport in *Escherichia coli*. *Journal of Bacteriology*, 124, 704-712.
- Garrett, R.H. & Grisham, C.M. (2008). *Biochemistry* (4th ed.). Boston, Massachusetts: Brooks/Cole
- Gaudu, P., Lamberet, G., Poncet, S., & Gruss, A. (2003). CcpA regulation of aerobic and respiration growth in *Lactococcus lactis*. *Molecular Microbiology*, 50(1), 183-192.
- Gaudu, P., Vido, K., Cesselin, B., Kulakauskas, S., Tremblay, J., & Rezaïki, L., (2002). Respiration capacity and consequences in *Lactococcus lactis*. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, 82(1-4), 263-269.
- Griffiths, E., & Humphreys, J. (1977). Bacteriostatic effect of human milk and bovine colostrum on *Escherichia coli*: importance of bicarbonate. *Infection and Immunity*, 15, 396-401.
- Hansen, M.C., Palmer, R.J.Jr., Udsen, C., White, D.C., & Molin, S. (2001). Assessment of GFP fluorescence in cells of *Streptococcus gordonii* under conditions of low pH and low oxygen concentration. *Microbiology*, 147, 1383-1391
- Heap, H.A. (1998). Optimising starter culture performance in NZ cheese plants. *The Australian Journal of Dairy Technology*, 53, 77-79.
- Holzappel, W. H. (1997). Use of starter cultures in fermentation on a household scale. *Food Control*, 8, 241-258.

- Holzapfel, W. H. (2002). Appropriate starter culture technologies for small-scale fermentation in developing countries. *International Journal of Food Microbiology*, 75(3), 197-212.
- Kim, W.S., Park, J.H., Ren, J., Su, P., & Dunn, N.W. (2001). Survival response and rearrangement of plasmid DNA of *Lactococcus lactis* during long-term starvation. *Applied and Environmental Microbiology*, 67, 4594-4602.
- Kindstedt, P. (2005). *American Farmstead Cheese: The Complete Guide to Making and Selling Artisan Cheeses*. Vermont: Chelsea Green Publication.
- Koebmann, B., Blank, L.M., Solem, C., Petranovic, D., Nielsen, L.K., & Jensen, P.R. (2008). Increased biomass yield of *Lactococcus lactis* during energetically limited growth and respiratory conditions. *Biotechnology and Applied Biochemistry*, 50(1), 25-33.
- Kringelum, B.W., Sørensen, N.M., Garrigues, C., Pedersen, M.B., Grøn, S., (2005). Use of compounds involved in biosynthesis of nucleic acids to increase yield of bacterial cultures. *International Patent Publication*. WO2006072257.
- Krom, B.P., Warner, J.B., Konings, W.K., & Lolkema, J.S. (2000). Complementary metal ion specificity of the metal-citrate transporters *CitM* and *CitH* of *Bacillus subtilis*. *Journal of Bacteriology*, 182(22), 6374-6381.
- Law, B. A., Sezgin, E., & Sharpe, M.E. (1976). Amino acid nutrition of some commercial cheese starters in relation to their growth on peptone-supplemented whey media. *Journal of Dairy Research*, 43, 291-300.
- Lawrence, R.C., Thomas, T.D., & Terzaghi, B.E. (1976). Reviews of the progress of dairy science: cheese starters. *Journal of Dairy Research*, 43, 141-193
- Lechardeur, D., Cesselin, B., Fernandez, A., Lamberet, G., Garrigues, C., Pedersen, M., Gaudu, P., & Gruss, A. (2011). Using heme as an energy boost for lactic acid bacteria. *Current Opinion in Biotechnology*, 22(2), 143-149.
- Lechardeur, D., Fernandez, A., Robert, B., Gaudu, P., & Trieu-Cuot, P., et al. (2010). The 2-cys peroxiredoxin alkyl hydroperoxide reductase c binds heme and participates in its intracellular availability in *Streptococcus agalactiae*. *Journal of Biological Chemistry*, 285, 16032-16041.
- Ledesma, O.V., Ruiz Holgado, A.de., Oliver, G., Giori, S.de., Raibaud, P., & Galpin, J.V. (1977). A synthetic medium for comparative studies of lactobacilli. *Journal of Applied Bacteriology*, 42, 123-133.
- Li, Y., Hugenholtz, J., Abee, T & Molenaar, D. (2003). Gluthathione protects *Lactococcus lactis* against Oxidative stress. *Applied and Environmental Microbiology*, 69 (10), 5739-5745.
- Li, Z. (2011). *Heme Biology: The Secret Life of Heme in Regulating Diverse Biological Processes*. New Jersey: World Scientific Publishing Company

Messenger, A.J.M., & Ratledge, C. (1981). Iron transport in *Mycobacterium smegmatis*: Uptake of iron from ferric citrate. *Journal of Bacteriology*, 149(1), 131-135.

Mullan, W.M.A. (2001). *Microbiology of starter cultures*. Retrieved from: <http://www.dairyscience.info/cheese-starters/49-cheese-starters.html>.

Neilands, J.B. (1995). Siderophores: Structure and function of Microbial iron transport compounds. *The Journal of Biological Chemistry*, 270, 26723-26726.

Pedersen, M. B., Garrigues, C., Tuphile, K., Brun, C., Vido, K., Bennedsen, M., Møllgaard, H., Gaudu, P., & Gruss, A. (2008). Impact of aeration and heme-activated respiration on *Lactococcus lactis* gene expression: Identification of a heme-responsive operon. *Journal of Bacteriology*, 190(14), 4903-4911.

Pedersen, M. B., Iversen, S.L., Sorensen, K.I., Johansen, E. (2005). The long and winding road from research laboratory to industrial applications of lactic acid bacteria. *FEMS Microbiology Reviews*, 29, 611-624.

Pedersen, M., Gaudu, P., Lechardeur, D., Petit, M., & Gruss, A. (2012). Aerobic Respiration Metabolism in Lactic Acid Bacteria and Uses in Biotechnology. *Annual Review of Food Science and Technology*, 3, 37-58.

Pittman, M. S., Corker, H., Wu, G., Binet, M. B., Moir, A. J., & Poole, R. K. (2002). Cysteine is exported from the *Escherichia coli* cytoplasm by CydDC, an ATP-binding cassette-type transporter required for cytochrome assembly. *Journal of Biological Chemistry*, 277, 49841-49849.

Pittman, M. S., Robinson, H. C., & Poole, R. K. (2005). A bacterial glutathione transporter (*Escherichia coli* CydDC) exports reductant to the periplasm. *Journal of Biological Chemistry*, 280, 32254-32261.

Reiter, B., & Oram, J.D. (1962). Nutritional studies on starter cultures: Vitamin and amino acid requirements of single strain starters. *Journal of Dairy Research*, 29, 63-77.

Rezaïki, L., Cesselin, B., Yamamoto, Y., Vido, K., Van West, E., Gaudu, P., & Gruss, A. (2004). Respiration metabolism reduces oxidative and acid stress to improve long-term survival of *Lactococcus lactis*. *Molecular Microbiology*, 53(5), 1331-1342.

Rezaïki, L., Lamberet, G., Derré, A., Gruss, A., & Gaudu, P. (2008). *Lactococcus lactis* produces short-chain quinones that cross-feed Group B Streptococcus to activate respiration growth. *Molecular Microbiology*, 67(5), 947-957.

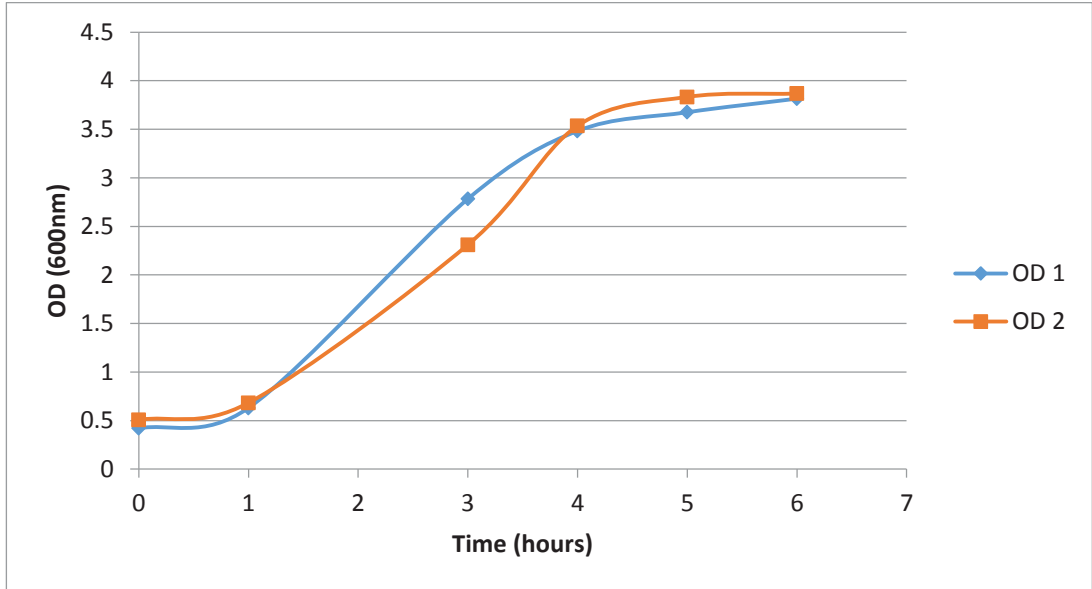
Richardson, D., & Baker, E. (1992). Two mechanisms of Iron uptake from transferrin by Melanoma cells: The effect of desferrioxamine and ferric ammonium citrate. *The Journal of Biological Chemistry*, 267(20), 13972-13979.

Sandine, W.E., Radich, P.C., & Eliker, P.R. (1972). Ecology of the lactic streptococci: A review. *Journal of Milk and Food Technology*, 35, 176-185.

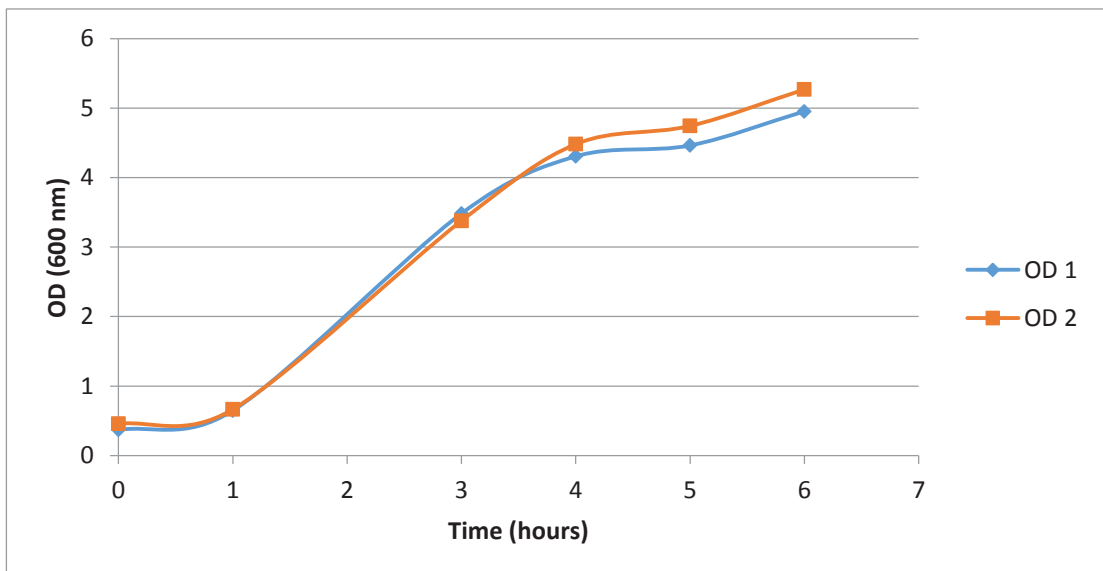
- Sarker, B. (1970). State of Iron (III) in normal human serum: low molecular weight and protein ligands besides transferrin. *Canadian Journal of Biochemistry*, 48, 1339-1350.
- Tanaka, K., Ohta, T., & Ishizaki, A. (1995). Effect of nitrogen sources in culture medium on L-lactate fermentation employing *Lactococcus lactis* IO-I. *Journal of the Faculty of Agriculture*, 39, 131-138
- Todar, K., 2012. *Todar's Online Textbook of Bacteriology: Lactococcus lactis*. Retrieved from www.textbookofbacteriology.net.
- Touati, D. (2000). Iron and oxidative stress in bacteria. *Archives of Biochemistry and Biophysics*, 373, 1-6.
- Vido, K., Les Bars, D., Mistou, M., Anglade, P., Gruss, A., & Gaudu, P. (2004). Proteome analyses of heme-dependent respiration in *Lactococcus lactis*: involvement of the proteolytic system. *Journal of Bacteriology*, 186, 1648-1657.
- von Wright, A., Axelsson, L. (Ed.). (2012). *Lactic Acid Bacteria: An Introduction* (4 ed.). Florida: CRC Press.
- Winklemann, G., & Zahner, H. (1973). Uptake of iron by *Neurospora crassa*. The specificity of iron transport, *Archives of Mikrobiologie*, 88, 49-60.

APPENDIX

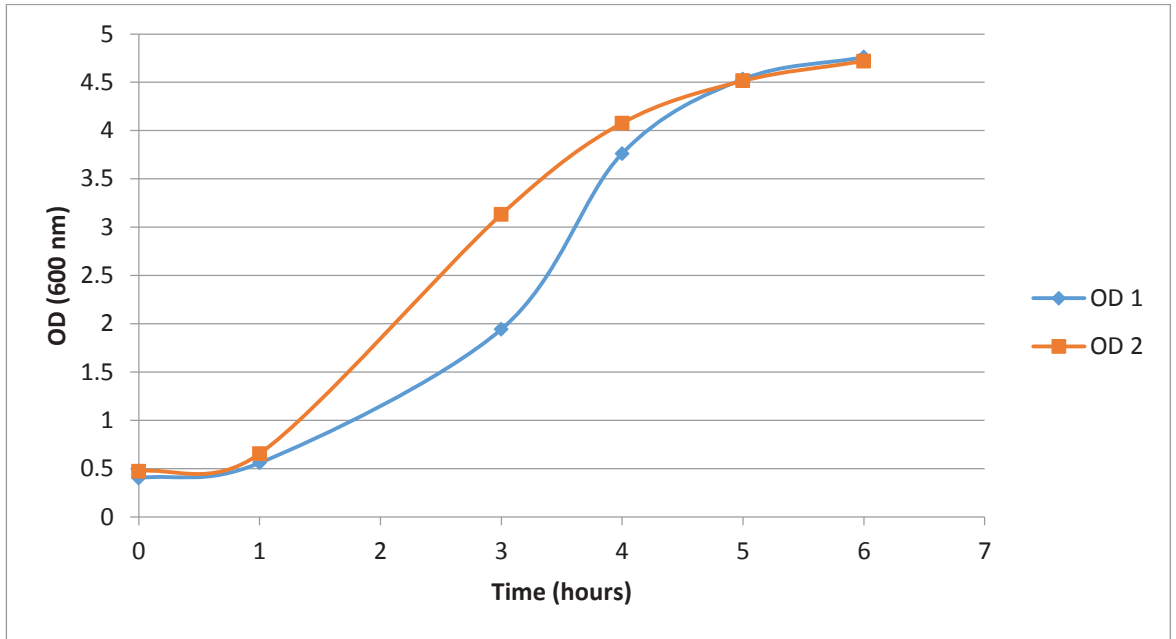
Appendix 1: Raw data for duplicate runs of heme optimisation



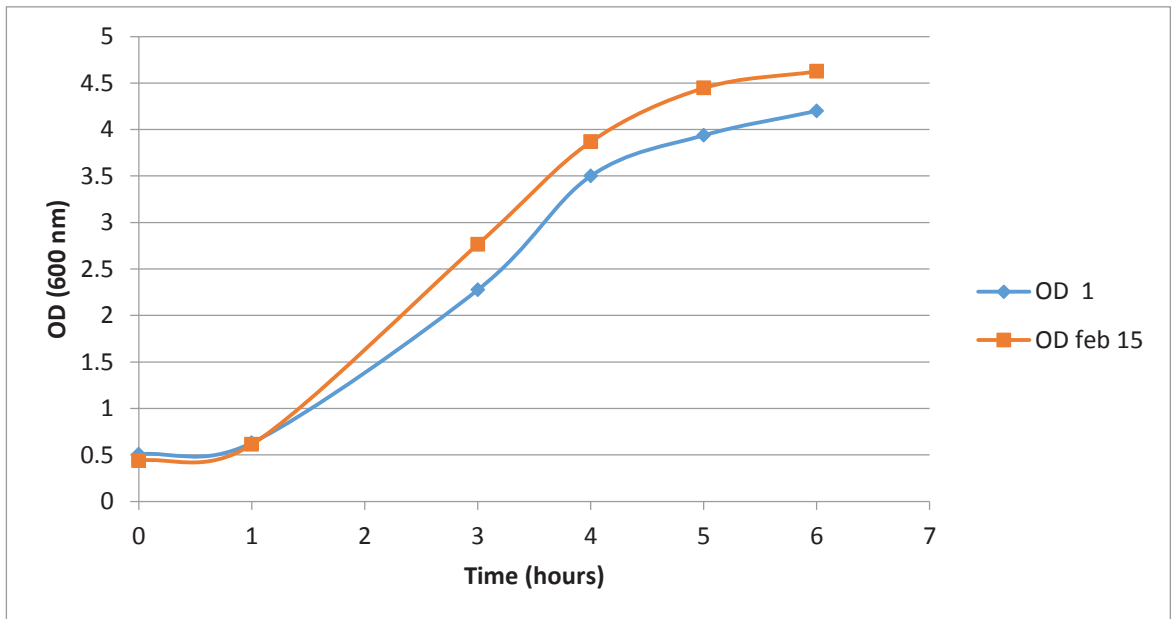
Duplicate OD graphs for aerobic fermentation with no heme added.



Duplicate OD graphs for aerobic fermentation with 1µg/mL heme added

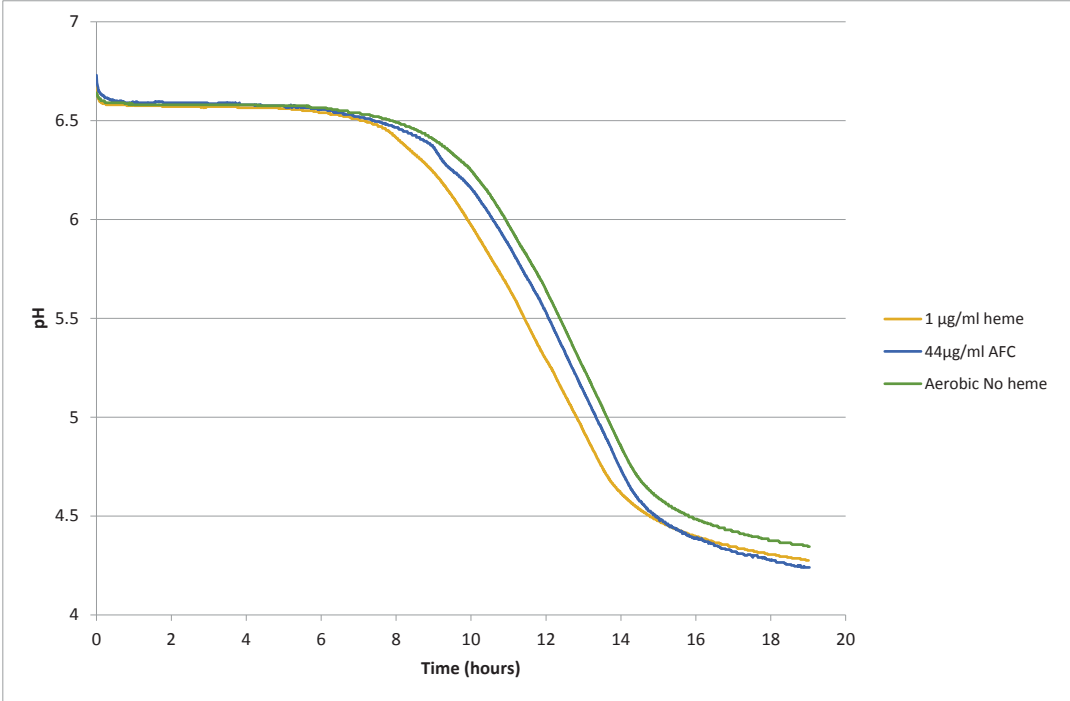


Duplicate OD graphs for aerobic fermentation with 3 µg/mL heme added



Duplicate OD graphs for aerobic fermentation with 5 µg/mL heme added

Appendix 2: Activity result graph



Activity graph for the three culture samples tested. Aerobic No heme is the control sample.