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Azher Razvi

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.A.Sc. (Chemical Engineering)

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TITRE DE LA THÈSE / TITLE OF THESIS

Dr. C. Lan

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

Dr. J. Zhang

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

Dr. J. Thibault

Dr. X. Cao

Dr. A. Tremblay (Chair)

Gary W. Slater

Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

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Azher Razvi

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Name of Faculty
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Abstract

The influence of initial glucose concentration: 60, 80, and 90 g/L on the hemin-stimulated respirative growth of *Lactococcus lactis* was studied in batch culture. Quantification of substrate consumption, biomass and product formation kinetics revealed that energy-spilling reactions may to contribute to a significant amount of non-growth associated or non-maintenance associated substrate utilization. The maximum specific growth rate was unaffected by initial substrate concentration, while the maintenance coefficient, m_S, and the maximum yield coefficient, $Y^M_{X/S}$, were strongly affected. The value of m_S increased from 1.56 to 1.73 g glucose/g DCW-h when the substrate concentration was increased. Product formation kinetics were also affected with the growth associated product formation (α) increasing from 1.9 to 2.8 g lactate/g DCW, while the non-growth associated product formation (β) value increasing from 0.74 to 0.81 g lactate/g DCW-h. The total biomass accumulated was also improved by a systematic modification of the growth medium utilized. A 50 % increase in the concentration of yeast extract, peptone, and tryptone against the base medium used increased the final biomass concentration from 4.2 to 6.3 g/L.

An analytical solution to the differential equations developed by Pirt for substrate consumption, Leudeking-Piret for production formation, and the differential logistic growth equation was successfully developed in this work. The solution represents a nonlinear system of equations that allows the simultaneous modeling of cell growth, substrate consumption, and product formation for substrate-limited growth.

A medium for improved growth of *L. lactis* and lactic acid bacteria was also developed for small-scale microbiological applications. By modification of a popular commercial medium used for its cultivation, not only was improved growth of *L. lactis* observed, but the final extracellular environment for long term cell survival was improved. Increasing the concentration of the salt disodium glycerophosphate from 19 to 59 g/L and addition of 7.5 g/L glucose increased the amount of biomass accumulated by a factor of 4 while maintaining a final pH of 5.1, which is ideal for growth.

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1 Introduction

The production of recombinant proteins has to follow an economic and qualitative justification dictated by the characteristics and the expected applicability of the compound produced. In the case of technical enzymes or food additives, genetic engineering must provide an approach which has to outperform the mass production of such compounds from traditional sources. Consequently, procedures have to be developed employing highly efficient platforms leaning on the use of economical media components in the fermentation processes. The production of pharmaceuticals that are developed for administration to humans must of course be novel, but at the same time their impact on public safety cannot be ignored. The demand for appropriate expression systems has increased as an increasing number of gene targets for a variety of industrial applications have been identified; this is especially true in regards to the pharmaceutical industry. To date, the production of approved pharmaceuticals is mostly restricted to *Escherichia coli*, several food grade species of yeast, and mammalian cells. Given the pathogenic nature of many *E. coli* strains and the genetic complexity of eukaryotes such as yeast and higher eukaryotic systems such as mammalian cells, the need for an expression system that allows for ease of genetic manipulation while presenting no deleterious health risk to the human population, is evident. A variety of expression platforms exist ranging from Gram-negative and Gram-positive prokaryotes, several yeasts and filamentous fungi to mammalian and plant cells. This diverse group of expression system is characterized by increased complexity from prokaryotic microbes to mammalian cell culture, each with their distinct advantages and disadvantages. Many of the systems that will be mentioned represent a distinguished group with an impressive

track record as hosts for production of a variety of valuable proteins that have achieved widespread commercial applications, while others are newly developed systems that have yet to establish themselves but demonstrate a great potential for industrial applications. In particular, lactic acid bacteria, and more specifically *Lactococcus lactis*, embody this new generation of possible hosts for widespread application in the ever expanding and diverse area of recombinant protein expression, not only as research tools for microbiologist and molecular biologist, but also as biocatalysts in full-scale commercial production operations.

The focus of this thesis lies with *Lactococcus lactis* and its potential use a potential host for large-scale protein production and/or direct dietary consumption for improved health. The starting point for this Masters research was work performed by Dr. Christopher Q. Lan and coworkers while at the University of California Davis. They studied the implications of aeration and exogenous hemin concentration on growth and metabolite formation of *L. lactis* LM0230. They verified the pseudo-diauxic nature of *L. lactis* growth in the presence of hemin under aerobic conditions which uses acetoin as a carbon sink and quantified the growth kinetics of *L. lactis* under the aforementioned environmental conditions. This thesis is divided as follows: Chapter 2 contains a literature review beginning with a review the most popular expression systems utilized commercially, as well as, those that are in development, comparing the various advantages and disadvantages of the most popular. The focus shifts to lactic acid bacteria and their applications to industry, specifically, the pharmaceutical industry, which is follow by a discussion on the current research initiatives undertaken with respect to LAB and more specifically *L. lactis*. The use of *L. lactis* as an expression system for a variety

of pharmaceutical products is then discussed followed by an explanation of *L. lactis* metabolism. The implications of hemin and aeration on redox balance, bioenergy production, and product formation are described. An in-depth description of hemin stimulated growth under aerobic conditions is given, as this is the primary focus of the experimental work presented here.

Chapter 3 contains a detailed description of a newly developed analytical model solution and its application to the growth kinetics of *L. lactis*. A discussion of the kinetics of cell growth, substrate utilization, and product formation is presented, which is concluded with the development of a new model to predict cell, substrate, and product formation.

Chapter 4 describes the materials and methods used for the experiments that were performed.

Chapter 5 contains the results and discussion. The first sub-chapter discusses the effect of substrate concentration on respirative *L. lactis* cell growth, substrate, utilization, and product formation under energy-sufficient conditions. The second sub-chapter describes the development of a new medium for growth of LAB and specifically *L. lactis* based on the optimization of the medium composition.

Chapter 6 presents conclusions and recommendations; specifically, in regards to the further development of a *L. lactis* expression system with respect to applications to the pharmaceutical industry. It also discusses the necessity to further develop the model of L. lactis cell growth based on the unique nature of *L. lactis* growth.

Based on the previous discussion, the objectives of this thesis become apparent. First, an investigation into the effects of increased concentrations of substrate on the cell

growth and metabolite formation kinetics of *L. lactis* IL 1403 under hemin-stimulated respirative conditions is performed in an attempt to improve biomass yield based upon experiments with differing medium compositions using a novel solution to the equations describing cell growth, substrate consumption, and product formation. The results of this study illustrate the tolerance *L. lactis* has for elevated substrate concentrations, and identifies the limiting nutrient within the fermentation medium. Furthermore, the influence of initial substrate concentration on substrate consumption and product formation kinetics are investigated. These results will provide valuable guidelines for the development of a medium for improved growth characteristics for use of the species in food-grade biotechnology applications.

Second, based on the delicate balance of sugar concentration and buffering capacity, a medium for LAB cultivation that results in a significant increase of cell growth, while at the same time containing sufficient buffering capacity as to maintain a close-to-neutral final pH for the benefits of cell survival was developed. Furthermore, the beneficial affects of hemin supplementation in the medium, which further enhances the cell growth and final pH for *L. lactis*, is demonstrated.

2 Literature Review

2.1 Commonly Utilized Recombinant Expressions Systems

All species have special favorable characteristics, but also limitations and drawbacks, as is often the case with all known systems when applied to the production of recombinant proteins. As no single system exists for all possible proteins, predictions for a successful development can only be made to a certain extent and indeed is often hypothetical, which can lead to inappropriate host/vector system for a particular protein product leading to loss of time, money, and raw materials. As such, it is advisable to assess several selected organisms or cells in parallel for their capability to produce a particular protein in desired amounts and quality.

A direct relationship between the desired functionality and/or complexity of a particular protein is tightly associated with the complexity of the expression system to be utilized. For example, single-subunit proteins can easily be produced in bacterial hosts, whereas proteins that require an authentic complex mammalian glycosylation or the presence of several disulfide bonds necessitate a higher eukaryote as host. However, research into the development of new platforms and improvement of preexisting system might render alternative microbes of a lower evolutionary scale suitable for production of more complex biopharmaceuticals. For instance, *E. coli*-based production systems have successfully been applied to a tissue plasminogen activator (t-PA) production process (Saito et al. 1994). This protein is used in the removal of blood clots, which is the cause of heart attack and stokes. Strains of yeast species *Pichia pastoris* and *Hansenula polymorpha* have been engineered to synthesize core-glycosylated proteins or those with

a "humanized" N-glycosylation pattern . Protein gylcosylation is a common phenomenon carried out by higher eukaryotes. It involves the addition of saccharides (carbohydrates) to the protein, and occurs in a post-translational processing that is not carried out by prokaryotic bacteria or eukaryotic yeast. Thus, it is clear that microbial systems provide ease in terms of genetic manipulations; however, this simplicity may be insufficient when attempting express proteins found in higher eukaryotes.

The Gram-negative bacterium *E. coli* was the first organism to be employed for recombinant protein production because of its long tradition as a scientific organism, the ease of genetic manipulations, and the availability of well-established fermentation procedures (Walsh 1998). However, the lack of both secretory systems and gylcosylation necessitate limitations on its general use. Furthermore, recombinant products are often retained as inclusion bodies (Hu et al. 2004). For the purposes of purification and downstream processing inclusion bodies represent a good starting point; however, this aggregation of insoluble, often misfolded, protein may render the product in partially or completely inactive. As such, costly, sophisticated renaturation steps are often required to activate the product. Nonetheless, numerous complex, *E-coli*-derived biopharmaceutical products have successfully entered the market.

Fungi combine the advantages of a microbial system such as a simple fermentability with the capability of secreting proteins that are modified according to a general eukaryotic scheme. Filamentous fungi such as *Aspergillus* or *Trichoderma* sp. efficiently secrete genuine proteins, but the secretion of recombinant proteins turned out be a difficult task in particular cases. Foreign proteins have to be produced as fusion proteins from which the desired product must be released by subsequent proteolytic processing

(Walsh 1998). Furthermore, spore formation, which is common phenomenon when dealing with fungi, is undesirable in the production of pharmaceuticals. Nevertheless, *Aspergillus* species have successfully been used for the production of phytase (Gargova and Sariyska 2003), an enzyme that is commonly added to animal feed to allow for utilization of inorganic phosphate as an energy source, or for lactoferrin (Ward et al. 1992), which is an antimicrobial agent.

Yeasts represent another broad class of microorganisms that are commonly used for recombinant protein production. Traditional baker's yeast, *Sacchromyces cerevisiae*, has been used for the production of FDA-approved HBsAg (Zhang et al. 2003), which is used as a test for the Hepatitis-B-virus and insulin, which was one of the first biopharmaceutical products and is of tremendous value to diabetics. However, numerous drawbacks are encountered in the application of this system. First, *S. cerevesiae* tends to excessively glycosylate recombinant proteins and often times produce a product that is allergenic. Another yeast, *Hansenula polymorpha*, has a developing list of use as production host for industrial and pharmaceutical proteins, as it has been shown to exhibit a more "humanized" glycosylation pattern as compared to *S. cerevesiae* (Gellissen 2005).

Mammalian cells are capable of authentically modifying heterologous compounds according to a mammalian pattern. Chinese hamster ovary (CHO) cells and baby hamster kidney cells (BHK) represent the most commonly used expression systems in mammalian cell culture (Gellissen 2005). However, as is often the case when dealing with higher evolutionary cell types, the fermentation procedure is expensive and production rates and yields are much lower than those reported for various microbial systems. In addition, mammalian cells are potential targets of infectious viral agents resulting in costly control

of all fermentation and purification steps. To date, the production of industrial compounds is thus restricted to high-price drugs. Nevertheless, very successful pharmaceutical products such as antibodies and their derivatives, or pharmaceuticals such as factor VIII, which is a glycoprotein, clotting, procofactor synthesized and released into the bloodstream; a lack of it results in Hemophilia A, an inherited bleeding disorder. Factor VIII requires authentic glycosylation, and at this point only mammalian cell culture is able to achieve such a result (Gellissen 2005).

Plant suspensions cell cultures carry most of the advantages of terrestrial plants and can be used at present for the production of low or medium amounts of proteins. Benefits include the ability to produce proteins under good manufacturing practice conditions, the ability to isolate proteins continuously from the culture medium, and the use of sterile conditions. However, further improvements in yield and optimization in downstream processing are required before this platform becomes commercially viable.

Currently, a variety of expression systems exist based on the use of not only bacteria, but fungi, yeast, plant, and animal cells as host for said expression system. Table 1.1 summarizes just a few of the host most commonly used in recombinant protein production to date.

Table 2.1 – Types of host cells used for genetic engineering and recombinant protein synthesis (Walsh 1998)

Major Group	Cell Type	Classification	Examples
Bacteria	Prokaryoic	Gram negative Gram positive	*Escherichia coli* *Bacillus substilis* *Streptomyces* spp.
Fungi	Eukaryotic	Microbial Filamentous	*Saccharomyces cerevesaie* *Aspergillus Niger*
Plants	Eukaryotic	Protoplasts	Variety of host systems

Animals	Eukaryotic	Intact cells Whole organsims Insect Cells Mammalian cells Oocytes Whole Organism	Variety of host systems

The discussion herein has been an attempt to identify and illustrate the current state of the art and the future direction it is taking. As was previously mentioned, the Lactic Acid bacterium (LAB), *Lactococcus lactis*, is one such species that has shown potential for use as a host in recombinant protein production, and is the subject of this thesis. LAB comprise a clade of Gram-positive, low-GC, acid tolerant, non-sporulating, non-respiring cocci associated based upon common metabolic and physiological characteristics (Wood and Warner 2003). Usually found in decomposing plants and lactic products, these bacteria produce lactic acid as the major metabolic end product of carbohydrate fermentation (Wood and Warner 2003). This trait has historically linked LAB with food fermentations as acidification inhibits the growth of spoilage agents allowing for their potential use in food grade applications. Proteinaceous compounds known as bacteriocins are produced by several LAB strains and provide an additional hurdle for protection against spoilage and pathogenic microorganisms. Furthermore, lactic acid and other metabolic products may contribute to the organoleptic and textural profile of a food item. The industrial importance of the LAB is further evidenced by their generally regarded as safe (GRAS) status, due to their ubiquitous appearance in food and their contribution to the healthy microflora of human mucosal surfaces. The primary genera that constitute this broad class of bacteria are *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, and *Streptococcus*, as well as, the more peripheral *Aerococcus*,

Carnobacterium, *Enterococcus*, *Oenococcus*, *Teragenococcus*, *Vagococcus*, and *Weisella*; these belong to the order Lactobacillales (Wood and Warner 2003).

LAB are rod-shaped bacilli or coccus characterized by an increased tolerance to a lower pH range. This aspect partially enables LAB to outcompete other bacteria in a natural fermentation, as they can withstand the increased acidity from organic acid production. Laboratory media used for LAB typically includes a carbohydrate source as most species are incapable of respiration (Wood and Warner 2003).

There are two main hexose fermentation pathways that are used for their classification. Under conditions of excess glucose and limited oxygen, homolactic LAB catabolize one mole of glucose in the Embden-Meyerhof-Parnas (EMP) pathway to yield two moles of pyruvate. Intracellular redox balance is maintained through the oxidation of NADH, concomitant with pyruvate reduction to lactic acid. This process yields two moles ATP per glucose consumed. Representative homolactic LAB genera include *Lactococcus*, *Enterococcus*, *Streptococcus*, *Pediococcus* and group I lactobacilli (Wood and Warner 2003).

Heterofermentative LAB utilize the pentose phosphate pathway, alternatively referred to as the pentose phosphoketolase pathway. One mole Glucose-6-phosphate is initially dehydrogenated to 6-phosphogluconate and subsequently decarboxylated to yield one mole of CO_2. The resulting pentose-5-phosphate is cleaved into one mole glyceraldehyde phosphate (GAP) and one mole acetyl phosphate. GAP is further metabolized to lactate as in homofermentation, with the acetyl phosphate reduced to ethanol via acetyl-CoA and acetaldehyde intermediates. Theoretically, end products (including ATP) are produced in equimolar quantities from the catabolism of one mole

glucose. Obligate heterofermentative LAB include *Leuconostoc*, *Oenococcus*, and *Weissella*.

In 1985, members of the diverse genus *Streptococcus* were reclassified into *Lactococcus*, *Enterococcus*, *Vagococcus*, and *Streptococcus* based on biochemical characteristics as well as molecular features. Historically, streptococci were segregated primarily based on serology, which has proven to correlate well with the current taxonomic definitions. Lactococci (formerly Lancefield group N streptococci) are used extensively as starter innocula in dairy fermentations, with humans estimated to consume 10^{18} lactococci annually. Partly due to their industrial relevance, both *Lactococcus lactis* subspecies (*lactis* and *cremoris*) are widely used as generic LAB models for research. *L. lactis* ssp. *cremoris*, used in the production of hard cheeses, is represented by the laboratory strains LM0230 and MG1363. Similarly, *L. lactis* ssp. *lactis* is employed in soft cheese fermentations, with the workhorse strain IL1403 ubiquitous in LAB research laboratories. In 2001, the genome of IL1403 was sequenced coinciding with a significant shift of resources to understanding LAB genomics and related applications (Bolotin et al. 2001). Currently, there are two *L. lactis* ssp. *cremoris* genomes (MG1363 and SK11) that have been sequenced that have been publicly released.

2.2 Traditional and Fermentation Based Applications of Lactic Acid Bacteria and *Lactococcus lactis*

Among LAB, *Lactococcus lactis* is by far the most extensively studied with respect to its physiology, metabolic pathways, and regulatory mechanisms (Bolotin et al. 2001). Traditional uses of LAB and *L. lactis* stem from their fermentative properties. For centuries *L. lactis* has been used in mixed starter cultures for the production of fermented vegetables, meats, and dairy products (Labrie et al. 2005). The economic value of *L. lactis* is substantial. For example, its use in the production of cheese which is produced in quantities over 10^7 tons annually worldwide. A number of its fermentation by-products, diacetyl and to a lesser extent acetoin, are valued for their aromatic properties, and have been applied to both the flavouring and perfume industries (Boumerdassi et al. 1997). It also has applications as a preservative as it produces bacteriocins, small proteineous compounds that act to kill other closely related and non-related bacteria (Amiali et al. 1998; Bromberg et al. 2005; Cabo et al. 2001; Fernandez et al. 2004; Wolf et al. 1997; Yang and Ray 1994). Exopolysaccharides (EPSs) from food-grade lactic acid bacteria have potential for development and exploitation as food additives and functional food ingredients with both health and economic benefits (Korakli and Vogel 2006; Ruas-Madiedo et al. 2002; Velasco et al. 2006; Welman and Maddox 2003). EPS have the potential to play an important role as natural viscosifiers and texture enhancers in yogurts and other fermented milks, low-fat cheeses, and dairy desserts, and are therefore of interest to the food industry (De Vuyst 2000). In addition, EPS such as oat β-d-glucan reduces serum cholesterol levels (Sutherland 1998). Furthermore, *L. lactis* has received

considerable attention due to a number of associated health benefits, as it has probiotic properties (Fang et al. 2000; Salminen et al. 1998). The vast majority of these applications have taken advantage of the capacity of *L. lactis* as a traditional fermentative microorganism. With recent developments in *L. lactis* molecular biology, the use of *L. lactis*, as well as LAB, as food-grade microorganisms and as a host for recombinant protein production is becoming increasingly more probable.

2.3 Current Research and Potential Applications for LAB and *L. lactis*

2.3.1 Food-Grade Microorganisms

Bacteria used in the manufacture of foods often remain viable in the end product. This means they will be eaten, often while still viable, by the consumer. Furthermore, bacteria such as LAB found in our food are known to pass through the digestive system and to be excreted in a viable form. Thus, they survive and may actually colonize or grow in the human digestive tract. Food-grade genetic modifications are the limited types of modifications that would be considered acceptable for food microorganisms with safety as the chief concern.

A strict definition of food-grade has been used in initial applications. According to this definition, a food-grade genetically modified organism (GMO) must contain only DNA from the same genus and possibly small stretches of synthetic DNA. In order for a GMO to be used in food, it must fulfill a number of safety criteria and must be able to attain "generally recognized as safe" (GRAS) status in the United States. At least one *Lactococcus* strain fulfilling this definition has been affirmed as GRAS and brought to the market in the United States (Kondo and Johansen 2002). An important element of this definition is that a food-grade GMO can only contain DNA from the same species. In a broader definition of food-grade, genes from other GRAS food microorganisms would be considered acceptable (Johansen 1999). In either case, the use of antibiotic resistance genes as selectable markers is not allowed. Many of the strains constructed at universities and in research institutions are for "proof of concept" and as such there is

often no need for them to be food-grade. Thus, antibiotic resistance markers have been used due to the ease of working with them. If these strains are to be used in the industry, it is necessary to eliminate all foreign DNA or to reconstruct the strains in a more appropriate manner.

Industrially, LAB, especially *L. lactis*, have been used for years in the production of foods and animal feeds. They produce lactic acid and contribute to the flavour, texture, and shelf life of the food. A few examples of this are the use of LAB in starter cultures, food preservation, and alcohol production; as such, a variety of industrial strains have garnered a food-grade status (Johansen 1999). However, to this point only one known example of a *Lactococcus* food-grade GMO has been utilized industrially (Kondo and Johansen 2002).

With the recent developments in *L. lactis* molecular biology which includes the elucidation of its genome and development of efficient genetic engineering tools (Bolotin et al. 2001; Dunny and McKay 1999; Rong et al. 2004), the door has opened for the utilization of *L. lactis* for a variety of food-grade applications. In addition, a number of products produced by *L. lactis* have been granted the GRAS status by the FDA (Burdock 2000; Burdock and Carabin 2004; Salminen et al. 1998; Wolf et al. 1997). This unique status of *L. lactis*, reinforced by the development of food-grade expression systems (Leenhouts et al. 1998; MacCormick et al. 1995) makes genetically engineered *L. lactis* strains possible hosts for the production of a variety of pharmaceuticals products.

2.3.2 Application of LAB and *L. lactis* in Pharmaceuticals Production

LAB and *L. lactis* may potentially have widespread applications in the biopharmaceutical industry as efficient expression systems for the production of recombinant protein based pharmaceuticals. Examples of these applications include the production of live vaccines, the expression of virolysin (endolysins), the production of growth hormone for animal feeds, the production of genetically modified super-antibiotics, and the production of other recombinant proteins.

2.3.2.1 Live Vaccine Production

Of the numerous uses of *L. lactis*, the production of vaccines has tremendous potential, as LAB have a number of favorable properties. LAB are natural inhabitants of the gastrointestinal tract of many mammals including humans. Thus, by expressing an antigen against a pathogenic organism on the surface of the LAB cell or as a recombinant product that is produced intracellular and subsequently excreted, immunization against a variety of diseases is possible. The concept of a specialized immune system associated to the mucosa is relatively new. The mucosal epithelium represents a barrier between the internal and the external environment and is the primary line of defense against most of the pathogens that use it as a route of infection (Salminen et al. 1998). Oral, respiratory, and genital are the pathways for infection exposing antigens to the mucosa. In response, secretion of antibodies by the epithelium neutralizes the toxicity of pathogens (Salminen et al. 1998). A number of approaches to combat pathogens at mucosal surfaces are desirable and often represent the only way to avoid infection. In other words, it is more

efficient and easy to block the infection at the mucosa, rather than to develop treatments against the organism after infection has taken place.

Recent studies deal with the idea of using food-grade lactic acid bacteria, and specifically *L. lactis*, for delivering live vaccines that act against a variety of pathogens (Mercenier et al. 2000; Miyoshi et al. 2006; Nouaille et al. 2003; Piard 1998; Seegers 2002). One of the most extensively studied examples of *L. lactis* use in this capacity is for a live vaccine for immunization against tetanus. More specifically, Tetanus Toxin Fragment C (TTFC) has been expressed in large amounts as a heterologous antigen to confer immunity against infection, and could be a novel alternative to the painful route currently utilized (Grangette et al. 2002; Grangette et al. 2004; Norton et al. 1996; Norton et al. 1995; Norton et al. 1997; Robinson et al. 1997; Steidler et al. 1998; Wells et al. 1993). The development of a live vaccine delivery system for human papillomavirus (HPV), which is the cause of cervical cancer among women, has also been initiated. Production of HPV type 16 E7 protein in *L. lactis*, mucousally delivered through the nasal passage of mice has been shown to reduce the occurrence of cervical cancer (Bermudez-Humaran and Langella 2006; Bermudez-Humaran et al. 2004; Bermudez-Humaran et al. 2005; Bermudez-Humaran et al. 2002; Cortes-Perez et al. 2005b; Cortes-Perez et al. 2003). Live vaccines acting against pneumococcal infection have also been developed through the expression of pneumococcal surface protein A (PspA) in *L. lactis* (Audouy et al. 2006; Audouy et al. 2007; Gilbert et al. 2000; Hanniffy et al. 2007). A novel protein-based nasal vaccine against *S. pneumoniae*, in which three pneumococcal proteins were displayed on the surface of a non-recombinant, inactive *L. lactis*-derived delivery system (Audouy et al. 2007). (This is different from live vaccines using

recombinants. A transit phrase or sentence would be nice.) Another example of vaccine delivery utilizing *L. lactis* is in the prevention of infection by *Helicobacter pylori*, which causes gastritis, gastric and duodenal ulcer, mucosal-associated lymphoid tissue (MALT) lymphoma, and finally the development of gastric adenocarcinoma in humans, i.e. cancer (Crespo and Suh 2001). Considerable efforts have been made to develop an effective oral vaccine against *H. pylori* infection based on the expression of a diversity of antigens in *L. lactis* (Kim et al. 2006a; Kim et al. 2006b; Lee et al. 2001; Roussel et al. 2007). Immunization against *Plasmodium falciparum* infection, i.e. malaria, is of considerable importance in African nations. A variety of *P. falciparum* antigens have effectively been expressed in *L. lactis*, and presenting a novel route for immunization against malaria (Carvalho et al. 2005; Ramasamy et al. 2006; Theisen et al. 2004; Zhang et al. 2005). DNA vaccines are also being developed for immunization against HIV (Gram et al. 2007; Guimaraes et al. 2005; Guimaraes et al. 2006; Xin et al. 2003). Development of live vaccines acting against a variety of other pathogenic microorganism has also been attempted. For instance, treatment against Group B Streptococcal infections, which are the foremost cause of fatalities in newborns by infectious disease has also be addressed using *L. lactis* as a vaccine delivery system (Buccato et al. 2006). Live vaccines delivery systems based on *L. lactis* against other pathogenic infections including *Salmonella* (Pacheco et al. 2005), cholera (Slos et al. 1998), and Rotavirus (Cortes-Perez et al. 2005a) are also being developed.

2.3.2.2 Virolysin – A Novel Alternative to Antibiotics

For more than half a century, the human society has been relying primarily on antibiotics to treat infectious diseases caused by pathogenic bacteria. However, the emergence of bacterial resistance to antibiotics following widespread clinical, veterinary, and animal agricultural usage (Heuer et al. 2006; Teuber 2001; Witte 2000) has made antibiotics less and less effective. We are now facing the fast approaching of the age of "superbugs", i.e., the pathogenic bacteria acquired resistance to most or all available antibiotic. It was warned by the WHO (World Health Organization) that those multiple antibiotic resistant bacterial pathogens will very likely bring the world back to the pre-antibiotics era (Parisien 2007).

Given the implications of antibiotic resistance, enormous demand has triggered worldwide efforts in developing novel alternative antibacterials. One of the most promising class of alternative antibacterials is the bacterial cell wall hydrolases (BCWHs), more specifically, endolysin (virolysin). Bacterial cell wall hydrolases (BCWHs) are enzymes that degrade the peptidoglycan, the major component of bacterial cell wall and cause bacteriolysis. They can be applied for the treatment of infectious diseases in different forms, including purified native enzymes, denaturized enzymes, partial digests, and recombinant proteins indigenously over-expressed in transgenic animals or plants for the enhanced host defense (Yazawa et al. 2006).

Virolysins are BCWHs encoded by lytic dsDNA phages and produced in phage-infected bacterial cells toward the end of the lytic cycle. They are also capable of degrading peptidoglycan when applied externally (as purified proteins) to the bacterial cell wall, resulting in a rapid lysis of the bacterial cell. They possess several important

features including a narrow antibacterial spectrum and activity against bacteria regardless of their antibiotic sensitivity. It is also important to notice that there are evidences showing that it is unlikely for sensitive bacteria to develop resistance to virolysins. The results of preclinical studies indicate that the most apparent potential problems associated with phage therapy (e.g., their immunogenicity, the potential toxicity, or the development of resistance) may not be a serious problem to the use of virolysins (Borysowski et al. 2006).

A few attempts have been made to express virolysins acting against infectious organisms in *Lactococcus lactis*. Gene cloning, expression, and secretion of *Listeria monocytogenes* bacteriophage-lyric enzymes in *Lactococcus lactis* has been accomplished. Bacteriophage lysins (Ply), or endolysins, are phage-encoded cell wall lyric enzymes which are synthesized late during virus multiplication and mediate the release of progeny virions. Bacteriophages of the pathogen *Listeria monocytogenes* encode endolysin enzymes which specifically hydrolyze the cross-linking peptide bridges in *Listeria* peptidoglycan (Gaeng et al. 2000).

One of the major research initiatives in our group is focused upon virolysin production; specifically, the isolation of DNA encoding for virolysin protein from the bacteriophage of pathogenic bacteria and the subsequent expression of these genes in bacteria, favorably in *L. lactis*, for the cost effective production of virolysins. At present, a protocol for phage propagation and quantification has also been developed and the optimization of phage propagation using a new medium developed for improved *L. lactis* growth is under way. The development of this medium will be discussed in more detail as part of this thesis on *L. lactis* growth. Eventually the development of a food-grade

Lactococcal expression system for virolysin from bacteriophages acting against a variety of pathogenic organisms will be attempted. This represents a novel alternative to antibiotics for treatment of diseases caused by the evolving class of resistant bacteria.

2.3.2.3 Expression of other recombinant proteins as pharmaceuticals

Live vaccine delivery and virolysin expression are only two examples of the applications of food-grade LAB, and more specifically *L. lactis*, to the pharmaceutical industry. There are a number of other examples illustrating the use of food-grade *L. lactis* for the production of a variety of recombinant protein products that have wide range applicability to the pharmaceutical industry. For example, *L. lactis* has become a popular expression system for a variety of recombinant proteins such as green-fluorescent protein, which is commonly expressed for proof of concept purposes to illustrate the capacity for a particular system to express recombinant proteins (Fernandez De Palencia et al. 2000). *L. lactis* is also a potential hosts for the production of plant-bioactive compounds because of this food grade status, efficient expression, and metabolic engineering tools. This has been facilitated by the development of efficient expression systems such as the Nisin-Controlled Expression (NICE) system (Hernandez et al. 2007; Zhou et al. 2006). Another example of recombinant protein production using *L. lactis* as a host is for therapeutic treatment using streptokinase, which is a potent human plasminogen activator secreted by various β-hemolytic *Streptococcus* species. It is widely used as a thrombolytic agent in treatment of acute myocardial infarction (Sriraman and Jayaraman 2006).

However, to date large-scale commercial utilization of *L. lactis* as a host for recombinant protein production for biopharmaceutical applications has yet to be realized. As previously mentioned *L. lactis* and LAB be in general are facultative anaerobes. In other words, they grow in the absence of oxygen to a limited extent. Under increasing aerated conditions *L. lactis* growth is increasingly inhibited. In comparison to other bacterial species such as *Escherichia coli* and yeast species such as *Saccharomyces cerevisiae* that are grown aerobically, they have comparably slower growth rates and yields. As a consequence, much of the current research with *L. lactis* is aimed at improving its growth, which is one of the major objectives of this thesis. The reasons for the poor growth characteristics of *L. lactis* are better understood by a detailed discussion of its physiology and metabolism.

2.4 *Lactococcus lactis* Metabolism

L. lactis has traditionally been classified as a facultative anaerobe (Neves et al. 2005) for which oxygen has strong regulatory effects. It conducts homolactic fermentation under anaerobic conditions converting up to 90 % or more of the available carbon source to lactic acid, and switches to aerobic heterolactic fermentation under aerobic conditions in response to oxygen stress (Cocaign-Bousquet et al. 2003; Lopez De Felipe et al. 1997; Van Niel et al. 2002), producing lower quantities of both biomass and lactic acid and increasing amounts of acetic acid, diacetyl, and acetoin. Cell growth of *L. lactis* under homolactic fermentation or aerobic heterolactic cultivation conditions is typically low due to significant product inhibition and/or oxygen stress (Ishizaki and Ueda 1995; Van Niel et al. 2002). The poor growth characteristics of *L. lactis* could be the single most important factor that makes it a less competitive prokaryotic rprotein expression system than industrially popular strains such as *E. coli*. Respiration is not the traditionally accepted metabolic route for *L. lactis* cell growth, and the discovery of this route (Sijpesteijn 1970) was met by a great deal of skepticism but verified (Duwat et al. 2001). This finding went unnoticed for approximately 30 years until it was further studied in late 1990s and the initial works soon after led to the development of a patented process for producing lactic acid bacteria (Duwat et al. 2001). Further experiments were carried out to determine whether respiration would occur in other species of lactic acid bacteria of interest to the dairy industry (Haier et al. 1999), followed by studies with a broader view on the beneficial effects of hemin stimulated respiration on cell growth and survival (Lan et al. 2006; Rezaiki et al. 2004).

The beneficial effect of hemin-stimulated respiration to cell growth of *L. lactis* has been well established in previous studies, and the significance of this phenomenon to industries utilizing *L. lactis* strains is great. However, most of the previous work in this area has been focused either on producing *L. lactis* as cheese starter cultures (Duwat et al. 2001; Haier et al. 1999) or growth in flasks in which pH control and oxygen supply are poor. Previous studies investigating the effects of aerated and non-aerated cultivation of *L. lactis* in the presence and absence of hemin on growth kinetics and metabolite formation have been performed (Lan et al. 2006). From this work, it was found that there exist three distinct metabolic behaviors for *L. lactis* growth: homolactic fermentation under anaerobic conditions, heterolactic fermentation under aerobic non-hemin stimulated conditions, and respirative growth due to aerobic, hemin-stimulated conditions. These three distinct metabolisms are characterized by differing amounts of accumulated biomass and differing fermentation product spectrums. Furthermore, the idea of biphasic growth with repression of respiration in the primary growth phase due to the presence of glucose was found to perhaps be in error (Duwat et al. 2001). It was shown that in fact respiration and fermentation co-exist in the primary growth phase, and once glucose levels diminish, the accumulated lactic acid is used as a carbon source for a secondary growth phase. These works have added substantially to the understanding of *L. lactis* growth and metabolism, and application of *L. lactis* for food-grade biotechnology, specifically rprotein production appears to be more feasible. Nonetheless, a complete picture of hemin-stimulated respiration has yet to be developed.

2.4.1 Metabolic Pathways

Depending upon the operating conditions within the cultivation system substantially different metabolic behaviour of *L. lactis* is observed (Duwat et al. 2001; Lan et al. 2006). It is well established that under anaerobic conditions *L. lactis* is homofermentative, resulting in conversion of over 90 % of the available carbon sources to lactic acid with minimal biomass accumulation (Cocaign-Bousquet et al. 2003; Duwat et al. 2001; Lan et al. 2006; Sijpesteijn 1970). The redox balance of $NADH/NAD^+$ is maintained by the reduction of pyruvate into lactic acid via lactate dehydrogenase (LDH) with no accompanied ATP production as shown in Figure 2.1 (Salminen et al. 1998). Under aerobic conditions in the absence of hemin, heterolactic fermentation occurs with reduced amounts of lactic acid and biomass accumulation, and increased amounts of acetic acid, diacetyl, and acetoin being formed (Cocaign-Bousquet et al. 2003; Lan et al. 2006; Ruas-Madiedo et al. 2002). Again, the balance of $NADH/NAD^+$ is maintained by the reduction of pyruvate, but in this scenario into a variety of products including lactic acid, acetic acid, and acetoin. Acetic acid and acetoin are formed by reactions with no accompanying ATP production as shown in Figure 2.1. This is because, in addition to LDH, □-acetolactate synthase (ALS), pyruvate dehydrogenase complex (PDH), and pyruvate formate-lyase (PFL) are also able to dissipate pyruvate under different physiological conditions (Lopez De Felipe et al. 1997). For example, ALS is active at high pyruvate concentrations and low pH. Dissipation of pyruvate through this pathway can produce acetoin, diacetyl, and 2,3-butanediol. On the other hand, PDH, which is active in aerobic conditions and at low pH, is inhibited at high NADH concentrations.

The end products from this pathway are acetate and/or ethanol. Under anaerobic conditions and at high pH, PFL is active, which leads to a mixture of formate, acetate, and/or ethanol (Hols et al. 1999). Finally, under aerated conditions and in the presence of hemin, the phenomenon of hemin-stimulated respiration is observed. Larger quantities of biomass are accumulated and acetoin is formed via a non NADH oxidizing pathway with a decrease in the amount of lactate being formed. The addition of hemin permits the utilization of the NADH formed in the respiratory chain as depicted in Figure 2.2 (Blank et al. 2001). This process results in the oxidation of NADH to NAD^+ via the reduction of oxygen to water resulting in the simultaneous production of 3 ATP molecules for every NADH.

In comparison to anaerobic homolactic fermentation and aerobic heterolactic fermentation, hemin-stimulated respiration benefits cell growth in at least the following three ways: (1) bio-energy efficiency of respiration is significantly higher than that of homolactic/heterolactic fermentation since each mole of NADH oxidized through respiration chain will produce three additional moles of ATPs; (2) less by products produced and consequently, more metabolic carbon fluxes directed to the formation of biomass and target products (e.g., rprotein); (3) less product inhibition than homolactic fermentation and less oxygen stress than heterolactic fermentation; and (4) a smaller drop in the pH of the system requires a smaller addition of neutralizing base (Lan et al. 2006). There are a number of implications to the switch to respiration including a higher specific growth rates, as well as, greater biomass and product yields should be possible with hemin-stimulated respiration growth of *L. lactis*.

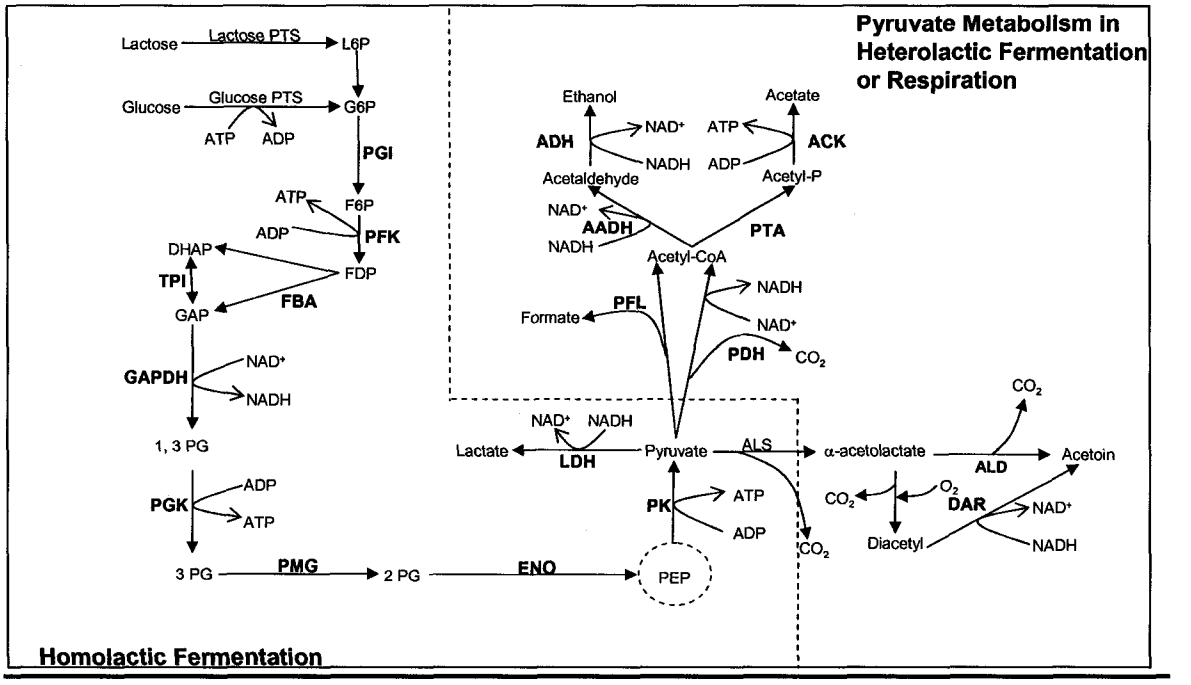

Figure 2.1 - Lactose and glucose catabolism in *L. lactis* under homolactic fermentation, heterolactic fermentation, and hemin-stimulated respiratory conditions (Lan et al. 2006).

Figure 2.2 shows the components of the respiratory chain in *L. lactis* that have been identified so far and the way they are linked to each other. Up to now several dehydrogenases that are involved in generating NADH/NADPH have been identified in *L. lactis*, and some of their functions have been confirmed (Duwat et al. 2001). For instance, the genes for quinone biosynthesis have been deduced from genome sequences and a functional quinone biosynthesis pathway was found (Bolotin et al. 2001; Morishita et al. 1999; Rezaiki et al. 2004). In contrast to respiratory bacteria, which have several types of cytochrome oxidase, *L. lactis* has only one cytochrome oxidase. It expresses a cydAB operon, which encodes cytochrome *bd* oxidase that is essential for respiration in the presence of heme (Duwat et al. 2001).

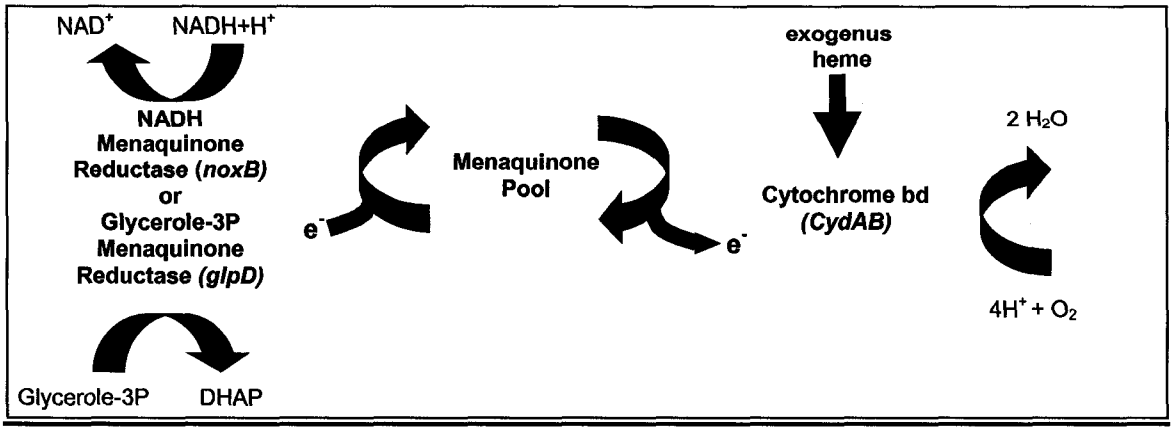

Figure 2.2 – Identified components of the *L. lactis* respiratory chain

The major differences between *L. lactis* and respiratory bacteria like *E. coli* and *B. subtilis* concern the NADH pool and heme sources. First, the tricarboxylic acid (TCA) cycle, which recycles the NADH pool for the respiratory chain, is incomplete in *L. lactis* (Lapujade et al. 1998). Figure 2.3 shows the TCA in respiratory bacteria. So far, citrate synthase and aconitase have been found in different strains of *L. lactis*. The fact that neither IDH nor □- KGDH could be found in any strain to date suggests that *L. lactis* has developed other pathways to provide NADH (Rezaiki et al. 2004).

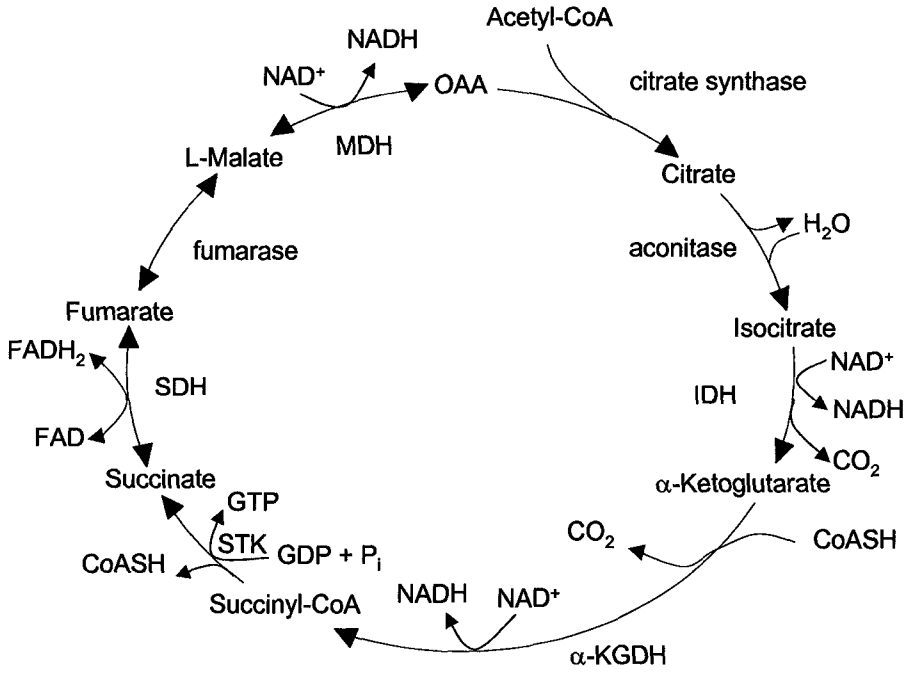

Figure 2.3 - TCA cycle in aerobic bacteria, shown are major substrates, as well as enzymes and co-factors, arrows indicate the direction.

Second, *L. lactis* is not equipped with an intact heme biosynthesis pathway, which allows *L. lactis* respiration growth only in the presence of an external heme source. As some genes are present and functional, for example, ferrochelatase (encoded by hemZ), which catalyses iron incorporation into the heme precursor protoporphyrin IX, the addition of heme or protoporphyrin IX allows *L. lactis* to overcome the absence of heme biosynthesis and enables respiration growth (Duwat et al. 2001).

With the knowledge that hemin-stimulated conditions greatly reduce the amount of lactic acid produced, thus, minimizing product inhibition, the potential to increase the amount of carbon sources in *L. lactis* cultivations to accumulate larger amounts of biomass becomes a possibility. However, the influence of elevated substrate concentrations on respirative growth of *L. lactis* has yet to be investigated.

2.5 Development of a new medium for *L. lactis* and LAB growth

The poor growth characteristics of *L. lactis* have multiple implications for its commercial utilization. One area that clearly needs to be addressed is the poor growth of *L. lactis* in small scale applications. As such there appears to be a clear demand in developing improved media that can provide better cell growth not only for *L. lactis*, but for LAB in general. One clear example of this, as was previously mentioned, is for bacteriophage propagation. LAB are fastidious microorganisms requiring a complex medium for optimal growth. Various media have been developed for the growth of LAB, those include CM medium (De Vuyst and Vandamme, 1992) modified CM (Li et al. 2002; Rodrigues et al. 2006; Sen 1997), SM8 (De Vuyst, 1995), M17 (Terzaghi and Sandine 1975), M17S (Li et al., 2000), and MRS (De Man et al., 1960) media. Among these media, M17 medium (Terzaghi and Sandine 1975) is probably the most widely accepted for the cultivation of LAB for a variety of different purposes (Birollo et al. 2000; Budin-Verneuil et al. 2005; Derzelle et al. 2005; Garcia Fontan et al. 2006; Hernandez et al. 2007; Kucukoner et al. 2006; Mor-Mur et al. 1992; Parente and Hill 1992; Salama et al. 1995).

The poor growth of LAB, especially in small vessels such as Petri dishes, test tubes, culture bottles, and flasks are mainly due to the difficulties in pH control. The accumulation of large amount of organic acids in LAB growth causes the pH of cultures to drop quickly, when sufficient amount of carbon sources are available, to below 4.5, at which point cell growth and metabolic activities are halted. Furthermore, studies illustrate that at such a pH level, LAB cells lose in part or entirely their viability (Hernandez et al.

2007; Terzaghi and Sandine 1975). Thus, not only should a good medium permit for good cell growth but maintain the pH of the culture environment within an optimal range for not only cell survival, but also product formation. The capacity to maintain a relatively neutral final pH is probably the most important reason why M17 medium is still the most popular medium for laboratory growth of LAB after more than three decades of its creation, even though media that allow high final biomass concentration have been formulated (Li et al., 2002).

M17 was developed on the basis of M16 by the addition of 1.9% □-disodium glycerophosphate (GP), an organic pH buffering reagent, into M-16 medium, resulting in improved growth of different LAB species and a prolonged neutral pH period. The creation of M17 was based on qualitative observations and the concentration of carbon source in M17 is kept at a very low level (i.e. 5 g/l lactose) to obtain the relatively high final pH. However, the low carbon source level no doubt set a rather low upper limit for cell growth of LAB, which is for instance, typically around 1.0 OD unit (equivalent to approximately 0.37 g/l DCW per volume) for *L. lactis*, compare very unfavorably to some non-food-grade fast growing prokaryotic expression hosts such as *Escherichia coli*. Clearly, there is a demand of improved media which can sustain better cell growth and, at the same time, has the capacity to maintain a beneficial neutral final pH.

3 Model Development

3.1 Batch Fermentation Kinetics

Modeling of growth kinetics is an important topic not only at a scientific level, but also for the purpose of developing full-scale industrial processes. It permits not only the study of fermentation conditions upon a process by varying medium composition and operating conditions, but also the quantification of essential parameters including maximum specific growth, maximum yield coefficients for both biomass and product, maintenance coefficients, and characterization of product formation for the purpose of scale-up processes.

Typically, a batch cell growth kinetics profile is comprised of six distinct phases including the lag, acceleration, exponential, deceleration, stationary, and death phase. Figure 3.1 contains a typical batch growth curve on a semi-logarithmic scale with log of the cell number on the y-axis and time of fermentation on the x-axis.

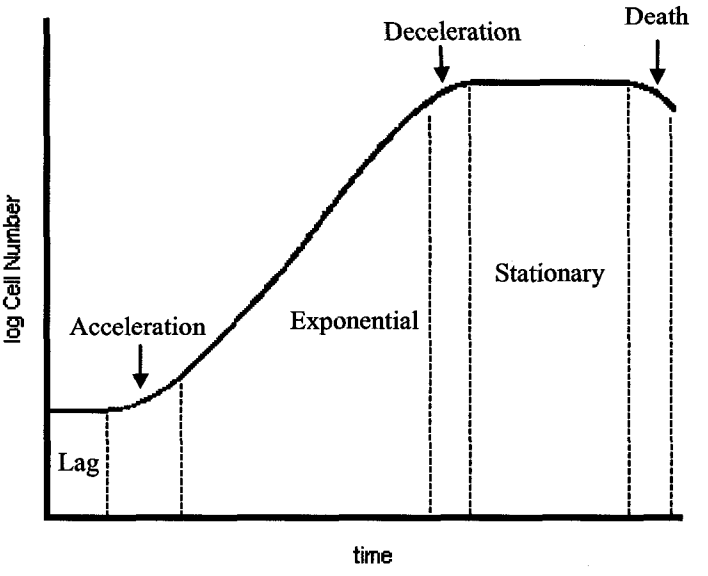

Figure 3.1 – Typical Batch Cell Growth Curve.

The lag phase represents the period that occurs upon inoculation of the bioreactor. The net specific growth rate, μ_{net}, is 0. Nonetheless, this phase of cell growth represents a period in which the cells undergo a period of rapid change. Inoculation represents a change in the environmental conditions the cells are exposed to, and as a consequence, they must adapt to their new environment. This adaptation involves a number of metabolic events including the synthesis of enzymes necessary to metabolize new nutrients. Another cause of the lag phase may be due to the required activation of intracellular enzymes by small molecules (vitamins, cofactors) or ions which may have appreciable permeability through the cell membrane. Transfer of a small culture volume to a larger volume of medium with low concentrations of such molecules would thus cause outward diffusion of these required species, and result in the occurrence of the lag phase until the internal machinery of the cell synthesizes these requisite compounds. After the lag phase the cells enter the acceleration phase where the specific growth increases to a maximum value, μ_m. The value of μ_m of a particular strain is dependent

upon the operating conditions and the composition of the medium. After the acceleration phase, the log or exponential phase occurs where the cells grow exponentially at μ_m. During this time, the compositions of the cells are constant and all cellular processes are assumed to occur at the same rate. In this way, the growth is said to be balanced. The exponential phase continues as long as the substrate concentration is substantially greater than the saturation constant, K_S, which will be discussed in more detail when discussing the Monod Equation. As the exponential phase ends, the specific growth rate begins to decrease, which signals the onset of the deceleration phase. A variety of factors may cause deceleration of growth, most often it is due to the exhaustion of a limiting nutrient or perhaps the accumulation of an inhibitory fermentation by-product. The deceleration phase continues until the net specific growth rate reduces to zero. In this period cells may still grow, but their rate of death, k_d, equals the specific growth rate, μ_g.

The concept of balanced growth along with an average cell approximation represents the most idealized view of cell growth. A conceptual view of different approximations and representations which are useful for the cellular phase of the system is summarized in Figure 3.2.

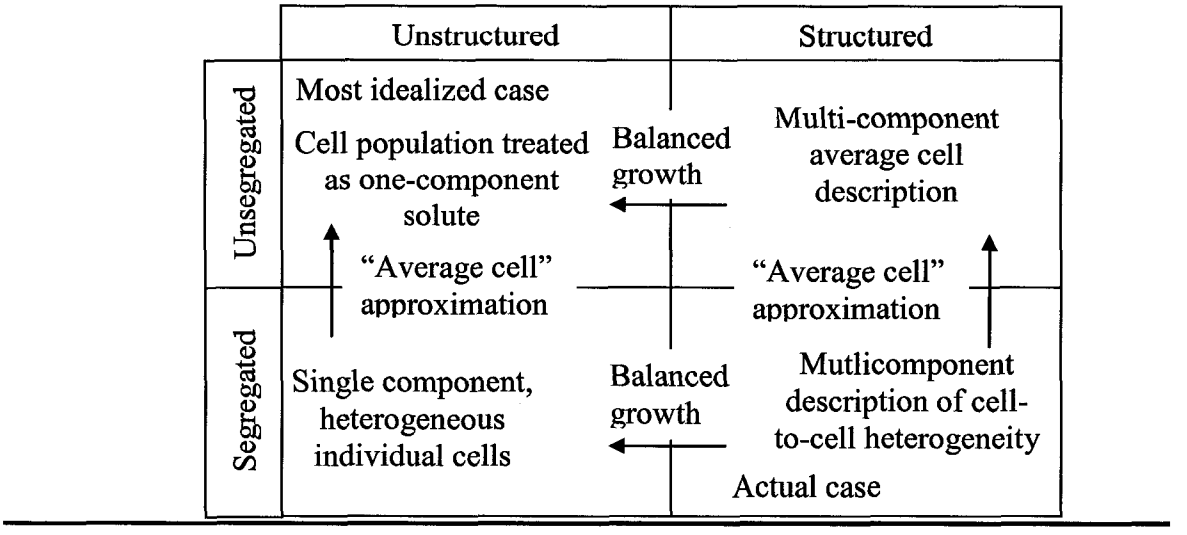

Figure 3.2 – Different perspective for cell population kinetic representations.

This perspective classifies approaches to microbial system according to the number of components used in the cellular representation and whether or not the cells are viewed as a heterogeneous collection of discrete entities, as they really are, or instead as some kind of average cell which becomes almost the same conceptually as a component in solution. Cellular representations which are multi-component are called structured, and single component representations are designated unstructured. Consideration of discrete, heterogeneous cell constitutes a segregated viewpoint, while an unsegregated perspective considers average cellular properties. As indicated in Figure 3.2 the actual situation is a structured, segregated one. If cell-to-cell heterogeneity does not substantially influence kinetic processes of interest, one may introduce the "average cell approximation" and simplify the segregated viewpoint to unsegregated. The aforementioned balanced growth views all cellular synthesis activities as being coordinated in such a way that the average cellular composition is not affected by cell proliferation. It is the common practice, especially when modeling cell growth kinetics, to take the most idealized approach, i.e. an unsegregated, unstructured model, when

analyzing and describing growth of cellular populations as will be done herein when developing the model to describe substrate consumption, as well as product and biomass formation.

The most commonly utilized unstructured model used to describe microbial growth kinetics is the Monod equation, Equation 1. A function relationship between the specific growth rate m and an essential compound's concentration was proposed by Monod in 1942. Of the same form as the Langmuir adsorption isotherm and the standard rate of equation for enzyme-catalyzed reactions with a single substrate, i.e. the Michaelis and Menten equation:

$$\mu_g = \frac{\mu_m S}{K_S + S} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \; 1$$

This equation predicts that the specific growth rate, μ_g, will increase with substrate concentration, S, up to a maximum value, μ_m, furthermore when $S >> K_S$, as S approaches K_S, the specific growth approaches $\frac{1}{2}\mu_m$. As previously indicated, the Monod equation is based upon an idealized system that is unsegregated and unstructured; thus, a number of assumptions must be made when it is utilized, and interpretation of the physical parameters may often not be realistic. Originally, it was proposed for convenience as hyperbolic equations find broad use to develop simple models in phenomena such as adsorption. Indeed, Michaelis-Menten kinetics applied to enzymatic reactions gives mechanistic meaning to the constants. The constant v_m, equivalent to μ_m in the Monod equation for cell growth is the rate of an elementary reaction of product

formation by dissociation of the enzyme-substrate complex, and K_m, equivalent to K_S in Monod equation, is either the equilibrium constant for the enzyme-substrate system (rapid equilibrium assumption), or the combination of the constants corresponding to the elementary reactions of reversible formation of the complex and its dissociation (quasi-steady-state assumption). None of those meanings can be applied readily to a substrate-cell system. The Monod Equation is limited to substrate-limited growth when growth is slow and population density is low. In these situations, environmental conditions can be related directly to the substrate concentrations. If the consumption of a carbon-energy source is rapid then the release of toxic waste products is more likely due to energy-spilling reactions. At high population levels, the build up of toxic metabolite by-products becomes important. The following rate expressions have thus been proposed for rapidly growing dense cultures:

$$\mu_g = \frac{\mu_m S}{K_{S0} S_0 + S} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 2$$

$$\mu_g = \frac{\mu_m S}{K_{S1} + K_{S0} S_0 + S} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots 3$$

where S_0 is the initial substrate concentration, and is a dimensionless constant K_{S0}.

A number of alternative equations to the Monod equation have also been proposed simply based on the better fit they yield depending on the shape of the growth

curve. Equations 4 through 7 represent the Blackman, Tessier, Moser, and Contois equations, which all relate the μ_g to S

$$\mu_g = \mu_m, \qquad iff\ S \geq 2K_S$$
$$\mu_g = \frac{\mu_m}{2K_S} S, \qquad iff\ S < 2K_S \quad \text{..... 4}$$

$$\mu_g = \mu_m (1 - e^{-KS}) \text{..... 5}$$

$$\mu_g = \frac{\mu_m S^n}{K_S + S^n} \text{..... 6}$$

$$\mu_g = \frac{\mu_m S}{K_{SX} X + S} \text{..... 7}$$

In the Contois equation, X (g/L) is the biomass concentration, and K_{SX} (g S/g X-L) is a constant.

As often is the case with microbial fermentation, inhibition may become an issue, either due to high initial substrate concentrations or because of the accumulation of fermentation products. The influence of inhibition can be significant as it relates to cell growth. In situations where a single-substrate enzyme catalyzed reactions is the rate-limiting step in microbial growth, then kinetic constant in the rate expression can take on biologically significant meaning. Most often, however, the underlying mechanism is far more complicated then just described, and the kinetic constants do not have any

biological meaning, and are simply obtained by curve fitting. In regards to substrate inhibition the two most popular equations are based on noncompetitive and competitive inhibition mechanisms, equations 8 and 9, respectively

$$\mu_g = \frac{\mu_m}{\left(1+\frac{K_S}{S}\right)\left(1+\frac{S}{K_I}\right)} \quad \ldots\ldots\ldots\ldots\ldots\ldots 8$$

$$\mu_g = \frac{\mu_m S}{K_S\left(1+\frac{S}{K_I}\right)+S} \quad \ldots\ldots\ldots\ldots\ldots\ldots 9$$

where K_I (g/L) is the substrate inhibition constant

High concentrations of product can be inhibitory for microbial growth. Product inhibition may be competitive (Equation 10) or noncompetitive (Equation 11), and in some cases when the underlying mechanism is unidentifiable, the inhibited growth rate is approximated as an exponential or linear decay expression.

$$\mu_g = \frac{\mu_m S}{K_S\left(1+\frac{P}{K_P}\right)+S} \quad \ldots\ldots\ldots\ldots\ldots\ldots 10$$

$$\mu_g = \frac{\mu_m}{\left(1+\frac{K_S}{S}\right)\left(1+\frac{P}{K_P}\right)} \quad \ldots\ldots\ldots\ldots\ldots\ldots 11$$

where K_P (g/L) is the product inhibition constant

A specific example is ethanol inhibition from glucose by yeasts which exhibits noncompetitive inhibition, where ethanol inhibits above 5 %. Other commonly used inhibition model for ethanol include equations 12 and 13

$$\mu_g = \frac{\mu_m}{\left(1+\frac{K_S}{S}\right)}\left(1-\frac{P}{P_m}\right)^n \quad \ldots\ldots\ldots\ldots\ldots\ldots 12$$

Another form of inhibition is that by toxic compounds, whose mechanisms can either be competitive (Equation 13), noncompetitive (Equation 14), or uncompetitive (Equation 15)

$$\mu_g = \frac{\mu_m S}{S\left(1+\frac{i}{K_i}+S\right)} \quad \ldots\ldots\ldots\ldots\ldots\ldots 13$$

$$\mu_g = \frac{\mu_m}{\left(1+\frac{K_S}{S}\right)\left(1+\frac{i}{K_i}\right)} \quad \ldots\ldots\ldots\ldots\ldots\ldots 14$$

$$\mu_g = \frac{\mu_m}{\left(\frac{K_S}{\left(1+\frac{i}{K_i}\right)}+S\right)\left(1+\frac{i}{K_i}\right)} \quad \ldots\ldots\ldots\ldots\ldots 15$$

where i (g/L) is the concentration of the toxic compound, and K_i (g/L) is the toxic compound inhibition constant. Equations (1) through (15) all come from the same reference (Shuler and Kargi 2002).

The influence of elevated substrate concentration is an important topic, especially when substrate concentration achieves a level at which it is no longer limiting. Under such situation, i.e. energy-sufficient systems, the implications upon cellular metabolism are significant, and the manner in which substrate is utilized is affected. In a substrate-sufficient batch culture, cells are able to over consume substrate, and a much higher substrate consumption rate is observed than in substrate-limited culture (Liu 1996; Liu 1998; Tsai and Lee 1990). A balanced substrate reaction shows that the observed specific substrate consumption rate, q_{obs}, is the sum of three quantities for a substrate-sufficient batch culture, Equation 16

$$q_{obs} = q_g + m_s + \Delta q_w \quad \ldots\ldots\ldots\ldots\ldots 16$$

where q_g is the specific rate of substrate consumption for growth, m_s is the specific maintenance rate, and Δq_w is the specific rate of substrate consumption due to energy spilling. Within the limits of this study, m_s is considered constant. In his study on the

effect of S_0/X_0 ratio on substrate-sufficient batch culture, Liu (1996) developed a S_0/X_0-dependent Δq_w model as follows:

$$\Delta q_w = (\Delta q_w)_m \frac{S_0/X_0 - (S_0/X_0)_{min}}{S_0/X_0 - (S_0/X_0)_{min} + K_{S/X}} \quad \ldots\ldots\ldots\ldots\ldots 17$$

where $(S_0/X_0)_{min}$ is the critical S_0/X_0-ratio for substrate-limited growth, $K_{S/X}$ is the S_0/X_0-ratio-related saturation constant, and $(\Delta q_w)_{max}$ is the maximum energy-spilling-associated substrate consumption rate. It is reasonable to assume that, under substrate-sufficient conditions, the S_0/X_0 ratio would be much greater than $(S_0/X_0)_{min}$. Therefore, Equation 17 can be simplified to the following expression:

$$\Delta q_w = (\Delta q_w)_m \frac{S_0/X_0}{S_0/X_0 + K_{S/X}} \quad \ldots\ldots\ldots\ldots\ldots 18$$

Equation 18 shows that the excessive substrate consumption rate due to energy spilling increases with increasing the S_0/X_0 ratio until it reaches a maximum, $(\Delta q_w)_m$. For the growth-associated substrate consumption the Monod equation can be utilized, expressed in terms of the specific substrate consumption rate

$$q_g = q_{g,m} \frac{S}{S + K_S} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 19$$

where $q_{g,m}$ is the maximum specific substrate consumption rate for growth. Substituting Equations 18 and 19 into 16 gives

$$q_{obs} = q_{g,m}\frac{S}{S+K_S} + (\Delta q_w)_m \frac{S/X_0}{S_0/X_0 + K_{S/X}} + m_S \ldots\ldots\ldots 20$$

Given that the discussion herein is based on the excessiveness of the energy (carbon) source, it is safe to assume that $S >> K_S$. Therefore, Equation 20 will reduce to the following expression under substrate-sufficient conditions:

$$q_{obs} = q_{g,m} + (\Delta q_w)_m \frac{S_0/X_0}{S_0/X_0 + K_{S/X}} + m_S \ldots\ldots\ldots\ldots 21$$

Obviously, model development is simplified while sacrificing little in terms of accuracy (Liu 1996; Liu 1998; Tsai and Lee 1990). Equation 21 can be rearranged as

$$q_{obs} = (\Delta q_{obs})_m + (\Delta q_w)_m \frac{S_0/X_0}{S_0/X_0 + K_{S/X}} \ldots\ldots\ldots\ldots 22$$

where $\Delta q_{obs,m} = q_{g,m} + m_S \ldots\ldots\ldots\ldots 23$

where $(\Delta q_{obs})_{,m}$ is the maximum observed specific substrate consumption rate under substrate-limited conditions. Equation 22 clearly shows that the observed specific substrate consumption rate is a function of the S_0/X_0 ratio in substrate-sufficient batch culture. This provides a plausible explanation for the fact that the determination of kinetic parameters using batch cultures closely depends on the ratio of S_0/X_0 (Chudoba et al. 1992; Simkins and Alexander 1984).

The differential equations governing cell, substrate, and product concentration used here represent simple, unstructured, and non-segregated kinetics based on bulk behaviour of the microbial system (Shuler and Kargi 2002). They have previously been used in numerous studies to model the kinetics of growth for a variety of fermentation and cell culture systems. Modifications of these equations have also been proposed when the basic forms failed to model the observed data, and many plausible explanations for these deviations have also been hypothesized (Lan et al. 2006; Messens et al. 2003; Pirt 1982). Substrate utilization has long been described by the equation introduced by Pirt and has been used in numerous studies. Similar to the equation for substrate consumption, the Luedeking-Piret equation predicting product formation has also been used for decades. The three differential equations describing cell growth, substrate utilization, and product formation form the basis for the development of the analytical model used in this study. The solution presents a novel means by which biomass, substrate, and product concentration can be predicted. The data modeled were obtained from the batch cultivation of *Lactococcus lactis* IL1403 grown under hemin-stimulated respirative conditions.

3.1.1 Cell Growth

A number of relationships have been shown to describe how the specific growth varies with substrate concentration as has just been illustrated. All these equations previously described are similar in that they relate to the μ_g to the substrate concentration S, and all are variations on the Monod Equation. The logistic equation represents an empirical, quantitative evaluation of cell growth that characterizes growth in terms of carrying capacity relating μ_g to the biomass concentration X rather substrate concentration. This concept is borrowed from an ecological concept that denotes the maximum achievable cell mass (population) a given environment can sustain. Typically, they are based on a formulation in which the specific growth rate is related to the amount of unused carrying capacity (Shuler and Kargi 2002).

$$\mu_g = k\left(1-\frac{X}{X_m}\right) \quad \text{..... 24}$$

where k is a constant (h^{-1}), X_m the final (plateau) cell concentration corresponding to the maximum carrying capacity, and $\left(1-\frac{X}{X_m}\right)$ the unused carrying capacity. The value of X_m is a function of the initial substrate concentration, S_0, in the system. The rate of cell growth is defined as

$$\frac{dX}{dt} = \mu_g X \quad \text{..... 25}$$

Combining these two equations yields

$$\frac{dX}{dt} = kX\left(1 - \frac{X}{X_m}\right) \quad \text{..... 26}$$

When integrated, the logistic equation results

$$X = \frac{X_0 e^{kt}}{1 - \frac{X_0}{X_m}(1 - e^{kt})} \quad \text{..... 27}$$

where X_0 is the concentration at $t = 0$ h.

3.1.2 Substrate Consumption

A generalized mass balance on substrate reduces to the Pirt equation (Pirt 1965)

$$\frac{dS}{dt} = -\frac{1}{Y^M_{X/S}}\frac{dX}{dt} - m_S X \quad \text{..... 28}$$

where S (g/L) is the substrate concentration, $Y^M_{X/S}$ (g DCW/g S) the maximum yield quantifying the amount of substrate utilized from cell growth, m_S (g S/g DCW-h) the maintenance coefficient quantifying the amount substrate used to perform non-growth

associated cellular functions. Dividing Equation 28 by Equation 26 yields an equation describing the rate of change in substrate with cell concentration

$$\frac{dS}{dX} = -\frac{1}{Y^{M}_{X/S}} - \frac{m_S X_m}{k(X_m - X)} \quad \ldots\ldots\ldots\ldots\ldots\ldots 29$$

Integrating this equation between X_0 and X yields

$$S = S_0 - \frac{1}{Y^{M}_{X/S}}(X - X_0) + \frac{m_S X_m}{k} \ln \frac{X_m - X}{X_m - X_0} \quad \ldots\ldots\ldots\ldots\ldots\ldots 30$$

This equation represents a nonlinear relationship between X and S. Combining the logistic equation, Equation 27, with Equation 30 gives a relationship between time (t) and substrate concentration (S). The parameters $Y^{M}_{X/S}$ and m_S can subsequently be estimated using any of a variety of nonlinear regression schemes.

3.1.3 Product Formation

A similar mass balance to substrate can be performed on product formation resulting in Equation 31 (Luedeking and Piret 1959)

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \quad \text{..... 31}$$

Dividing equation 31 by 26 yields

$$\frac{dP}{dX} = \alpha + \frac{\beta X_m}{k(X_m - X)} \quad \text{..... 32}$$

which when integrated yields

$$P = P_0 + \alpha(X - X_0) - \frac{\beta X_m}{k} \ln \frac{X_m - X}{X_m - X_0} \quad \text{..... 33}$$

Again, this equation represents a nonlinear relationship between t and P when combined with the logistic equation, Equation 27. Performing a regression analysis similar to that performed for t and S will yield coefficients α (g lactic acid/g DCW) and β (g lactic acid/g DCW-h). The physical meaning of the constants describes the nature of the product formation. For example, α represents maximum product yield associated

with growth-associated process, while, β represents the product formation due to the non-growth-associated (maintenance) processes.

4 Materials and Methods

4.1 Inoculum

Stock cultures of *Lactococcus lactis* ssp. IL1403, kindly provided by Dr. Pierre Renault of INRA in France, were stored at -80 °C in 50% glycerol in a 1:1 (v/v) ratio. Flask cultivations of 100 mL were preformed to activate the cells for 10-12 hours. The inoculum size for the flasks was 1 mL. Flasks were incubated at 30 °C and 200 rpm. The flask medium was a modified M-17 broth formulation adjusted to pH 6.

4.2 Fermentations

4.2.1 Substrate Concentration Study

A 25 mL volume of the flask cultivation was used to inoculate a NBS Bioflo 110 3-litre bioreactor. Batch cultivations with an initial volume of 1-litre were performed at a glucose concentration of 60 g/L in triplicates. The cultivation medium was a formulation containing 10 g/L yeast extract, 5 g/L peptone and tryptone, 1 g/L magnesium sulfate, and 5 mg/L hemin. Temperature, pH, and DO were controlled via a PI controller. An electrically powered heating jacket and cooling water flow were used to maintain the temperature throughout the fermentations at 30 °C. The pH was maintained at 6 with the addition of 5 M NaOH. The DO was maintained at 30 % by sparging air at a rate of 1 L/min, and gradually increasing agitation from 200 to 600 rpm.

4.2.1.1 Analytical Procedures

Samples of 1-3 mL were taken every 30 minutes over an 11 hour period throughout fermentations. The optical density (OD) was measured using a ThermoElectron Gensys10 spectrophotometer at 600 nm. Samples were diluted to obtain an OD between 0.2 and 0.4. Samples were then clarified using 0.22 μm syringe filters. Glucose and lactic acid assays were performed on clarified samples using a YSI 2700 Select analytical instrument.

4.2.1.2 Modeling

The data obtained from the fermentations was modeled by minimizing the total sum of squares of the residuals for biomass, substrate, and product concentration

$$\sum SSR = \sum (X_e - X_p)^2 + \sum (S_e - S_p)^2 + \sum (P_e - P_p)^2 \quad \ldots\ldots\ldots\ldots 34$$

where subscript e denotes the experimental data and subscript p denotes the predicted data. Parameters k, $Y^M_{X/S}$, m_S, α, β, and n, in case of the modified form, of the logistic equation were simultaneously varied using the solver function of Microsoft Excel to minimize ΣSSR for all instances of modeling.

4.2.2 Medium Development

To develop an optimized medium for *L. lactis* growth, the effect of hemin, glucose, and gylcerophosphate concentration on growth rate, final biomass concentration, and final medium pH was evaluated. Shake-flask experiments were performed using the commercially available medium M17, as a control medium, and comparing it to M17 supplemented with varying concentrations of hemin, glucose, and GP. The inoculum for the shake-flask experiment was flask cultivation of 100 mL incubated for 10-12 hours. The inoculation flask was inoculated with the aforementioned stock culture. The flask was incubated at 30°C and 200 rpm. The medium was a modified M-17 broth formulation adjusted to pH 6. For all shake-flasks run the incubation was run at 30 °C at 200 rpm with all cultivations adjusted to pH 6 prior to inoculation and 0.5 mg/L hemin. To evaluate the effect of glycerophosphate (GP) buffer concentration on the performance of different media formulations shake flasks were run with M17 supplemented with 0, 30, 40, 50, and 60 g/L GP and 20 g/L glucose. To evaluate the effect of substrate concentration on the performance of the different media formulations shake-flask were run with M17 supplemented with glucose concentrations of 0, 5, 7.5, 10, and 12.5 g/L glucose and 50 g/L GP.

5 Results and Discussion

5.1 Substrate Concentration Study

5.1.1 Effectiveness of the Analytical Solution

As shown in Figure 5.1, cell growth under such conditions is modeled satisfactorily using the model of logistic cell growth, Equation 27, with a k of 1.0 h^{-1}. This value is similar to the value of the maximum specific growth rate of 1.1 h^{-1} based on the exponential growth phase, i.e. the linear part of the ln$X$ vs. t. The exponential phase started soon after inoculation without noticeable lag phase and lasted for about 3.5 h, followed by the deceleration phase. Cessation of growth appears to be due to something other than substrate exhaustion, as there is almost 5-10 g/L residual glucose when cell growth has leveled off. We speculate that the exhaustion of essential growth factors such as amino acids and vitamins was the cause of growth cessation, which will be discussed later on in more detail. The kinetics of the accumulation of lactic acid exhibited typical kinetics of mixed-growth associated product with an α-value of 1.67 g lactic acid/g DCW and a β-value of 0.90 g lactic acid/g DCW-h. Modeling of lactate formation was satisfactorily performed using Equation 11 with an R^2 of 0.999. Substrate consumption kinetics was also modeled well using Equation 8 with an R^2 of 0.999. A maximum biomass yield coefficient, $Y^M x/s$, of 0.20 g DCW/g glucose and a maintenance coefficient, m_S, of 1.5 g glucose/g DCW-h were observed.

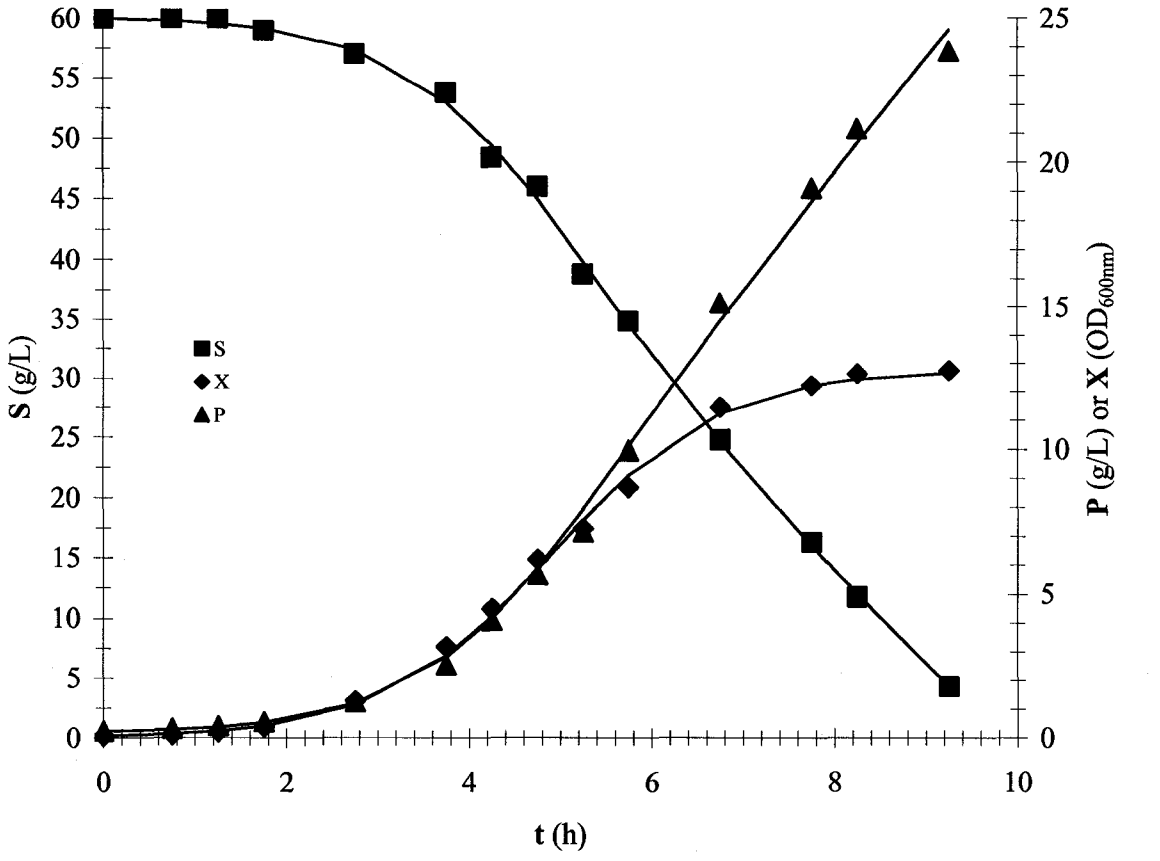

Figure 5.1 - Typical batch cultivation of *L. lactis* under respirative conditions at 30 °C, pH 6.0, initial glucose 60 g/L, 30 % DO, and hemin 0.5 mg/L; (■) S, (▲) P, and (♦) X. Solid lines are best-fit curves of experimental data using corresponding models (i.e., cell growth, Eq. 4; product formation, Eq. 11; and substrate consumption, Eq. 8).

Figure 5.1 shows the typical time courses of cell growth, glucose consumption, and lactic acid accumulation for *L. lactis* cultivation under hemin-stimulated respirative conditions. The batch was run at 30°C, pH 6.0, and a 30 % DO. The control of pH was carried out by addition of 5 M NaOH to the fermentation broth. The initial glucose concentration was 60 g/L. The maximum lactic acid concentration observed at the end of the cultivation with a level of 24 g/L, corresponding to an apparent lactate yield of 50 %. However, the lactic acid concentration had not begun to level off, so presumably a higher concentration of lactic acid would have been achieved resulting in a higher apparent yield

of lactate on biomass. A higher lactate yield would be expected despite the cessation of growth, as lactate can be formed from either growth or non-growth associated processes. Since glucose exhaustion has not occurred, the formation of lactic acid should presumably continue, resulting in a higher yield of lactic acid on glucose. Both the lactic acid concentration and apparent lactic acid yield for *L. lactis* IL1403 differ substantially from that observed by Lan et al. with *L. lactis* LM0230, which were approximately 5.2 g/L and 29 %, respectively.

Four batch runs were performed at an initial glucose concentration of 60 g/L to evaluate the reproducibility of the parameter estimations. The results of parameter estimations for all four batches are summarized in Table 1.1. The average *k*-value for the batches was 1.00 ± 0.07 h^{-1}. The estimation of parameters quantifying substrate consumption also exhibited little variability with an average maximum biomass yield of 0.176 ± 0.005 g DCW/g glucose and an average maintenance coefficient of 1.56 ± 0.14 g glucose/g DCW-h. The contribution of growth-associated processes to lactic acid formation resulted in an average of α-value of 1.94 ± 0.19 g lactic acid/g DCW, while the lactic acid formed due to non-growth-associated processes was represented by a β-value of 0.747 ± 0.219 g lactic acid/g DCW-h.

Table 5.1 - Parameter estimation for replicate batches run with an initial glucose concentration of 60 g/L under respirative conditions at 30 °C, pH 6.0, 30 % DO, and hemin 0.5 mg/L.

Run	k (h^{-1})	$Y^{M}_{X/S}$ (g DCW/g glucose)	m_S (g glucose/g DCW-h)	α (g lactic acid/g DCW)	β (g lactic acid/g DCW-h)
Average	1.00	0.176	1.56	1.94	0.747
s	0.07	0.005	0.14	0.19	0.219
100*s/average	7.0	2.8	9.0	9.8	29.3

5.1.2 Cell growth is not limited or inhibited by the initial glucose concentrations of 60 to 90 g/L

Figure 5.2 summarizes the effect that glucose concentration has on cell growth. It contains plots of cell growth and substrate consumption of batch runs with initial glucose concentrations of 60, 80, and 90 g/L. For all range of glucose concentrations studied the measured final OD was between 12 and 13 absorbance units corresponding to a dry cell concentration of 4.1 to 4.4 g/L. The value of k determined from the fit of the experimental data to the logistic model (Equation 27) was constant over the range of concentration studied varying between 0.95 and 1.05, which is similar to the maximum specific growth rate determined from the linear part of the $\ln X$ vs. t plots. This is significantly higher than values for μ_m reported in previous studies with *L. lactis* under respirative conditions (Duwat et al. 2001; Lan et al. 2006). It is clear from Figure 5.2 that cell growth stops well before the exhaustion of glucose regardless of the initial concentrations of glucose. Moreover, the net consumption of glucose in all three batches is approximately 50-55 g/L, and the apparent yield of biomass is also relatively constant (Table 5.2).

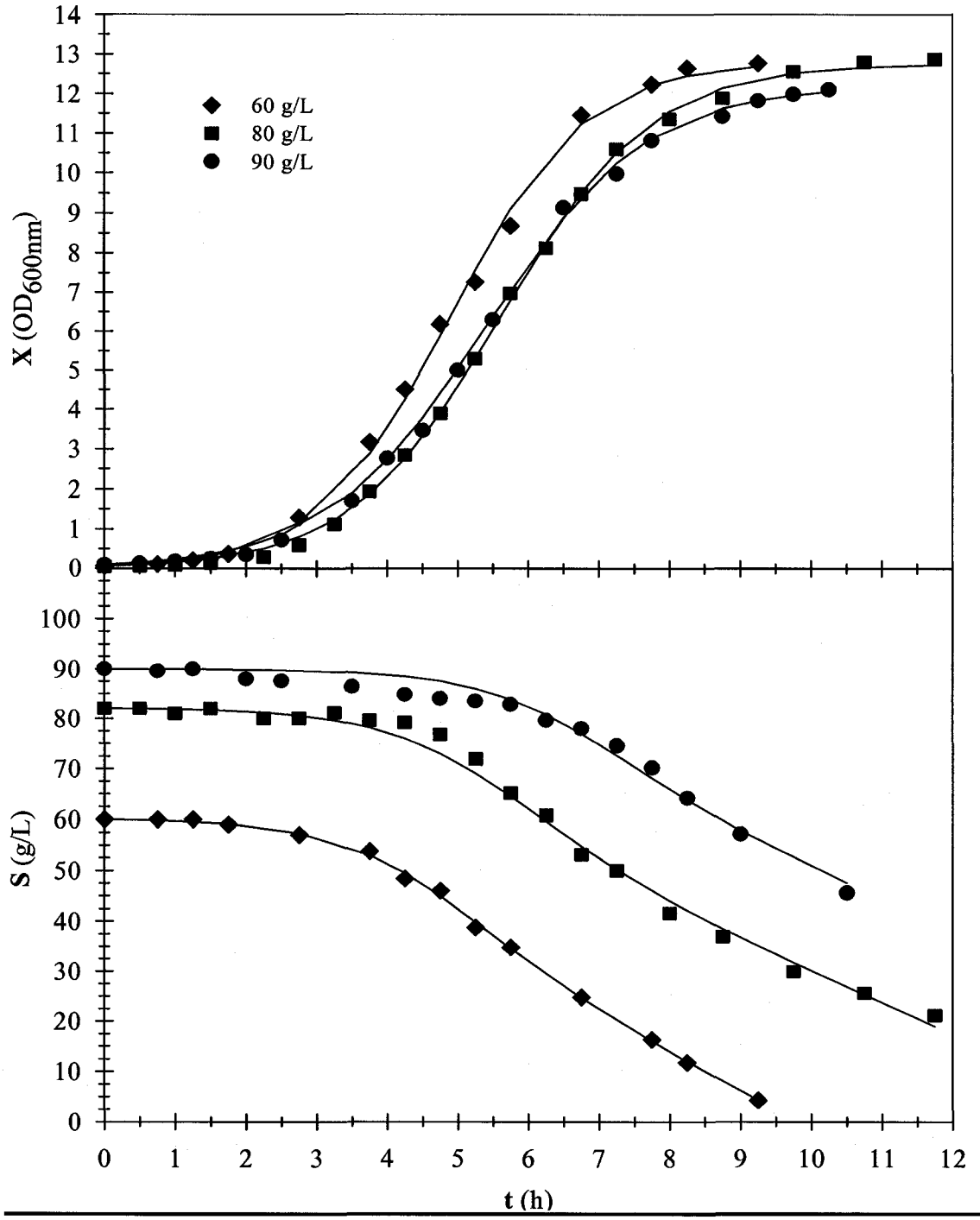

Figure 5.2 – Experimental (data points) and model predictions (lines) of cell growth (top) and substrate concentration (bottom) as initial glucose concentration is increased. For 60 g/L data (♦), 80 g/L (■), and 90 g/L (●).

Given these facts, it would appear that some other component in the system is limiting to cell growth. However, the cessation of cell growth could potentially be due to the accumulation of lactic acid to concentrations that are inhibitory to cell growth. It is well documented that *L. lactis* IL1403 requires the inclusion of a number of growth factors in the cultivation medium, as this subspecies is incapable of synthesizing them on its own. These include nine amino acids: glutamic acid, methionine, valine, leucine, threonine, arginine, isoleucine, histidine, and serine, as well as, five vitamins: biotin, calcium pantothenate, nicotinic acid, pyridoxine and riboflavin (Cocaign-Bousquet et al. 1995; De Vuyst 1995; Loubiere et al. 1996).

In an attempt to determine the cause of the termination of cell growth, the concentrations of yeast extract (YE), peptone (Pep), and tryptone (Trp) were all increased by 50 %, using an initial glucose concentration of 60 g/L. Figure 5.3 contains a plot of the experimental data and model predictions of cell growth, substrate consumption, and lactic acid formation for this modified medium. The concentrations of all other components were kept constant. Figure 5.3 clearly illustrates that the increase in these three components results in a 1.6 times increase in final OD corresponding to a dry cell concentration of 6.63 g/L. Furthermore, the termination of cell growth now corresponds to the exhaustion of glucose. Since the amount of lactate formed did not change when compared to the batch run at 60 g/L with the nominal concentrations of YE, Pep, Trp, it appears that the remaining glucose was directed into the respirative pathway, resulting in an increase in the amount of biomass accumulated.

Based on the results of the experiments with increasing initial glucose concentration, and the experiment carried out with the modified medium with increased

concentrations of YE, Pep, and Trp two conclusions can be made. First, cell growth is indeed limited by some other nutrient source apart from glucose, presumably from the list of essential growth factors that were previously described. In other words, the initial medium composition utilized represents a glucose (energy) sufficient system; the implications of which will soon be discussed. Second, the increase in initial glucose concentration had no effect on the final biomass concentration, the maximum specific growth rate, or the nature of the cell growth curves, indicating that substrate inhibition in this range of glucose concentration is not present.

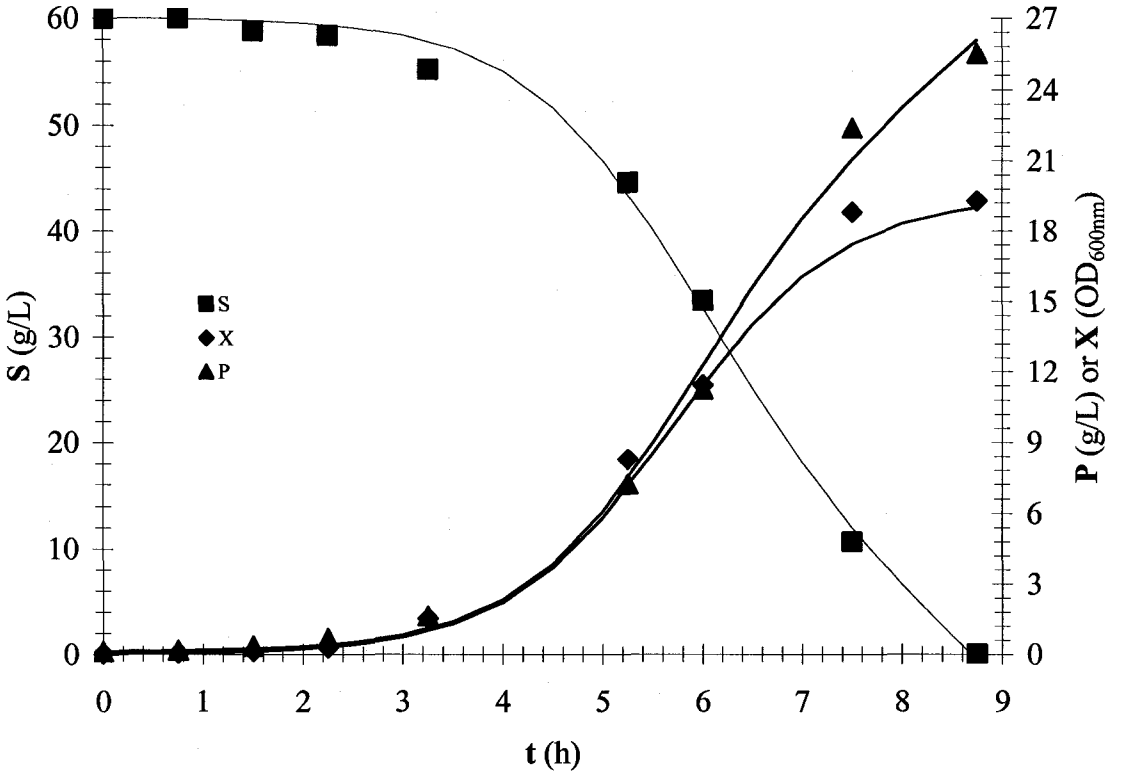

Figure 5.3 – Fermentation profiles for cell growth (♦), glucose concentration (■), and lactic acid concentration (▲) with 1.5 times the concentration of YE, Pep, and Trp, as compared to the standard medium formulation with an initial glucose concentration of 60 g/L.

5.1.3 Implications of energy-sufficiency on substrate consumption kinetics

Table 5.2 summarizes the influence that initial glucose concentration has on kinetics of batch substrate consumption. The maximum biomass yield coefficient remains relatively constant with initial glucose concentrations from 60 to 90 g/L. with an approximate value of 0.18 g DCW/g glucose, there appears to be no apparent trend in the variability of the data as initial glucose concentration is increased. Furthermore, the maximum biomass yield in Table 5.2 is typically over 2-fold greater than the apparent yield biomass yield on substrate, implying that a significant proportion of glucose is being utilized for maintenance processes.

Table 5.2 – The effect of initial glucose concentration on the parameters for substrate consumption kinetics. The maximum yield ($Y^{M}_{X/S}$) and maintenance coefficient (m_S) were determined from Equation 8.

S_0 (g/L)	$Y^{M}_{X/S}$ (g DCW/g glucose)	m_S (g glucose/g DCW-h)	$Y^{APP}_{X/S}$ (g DCW/g glucose)
60	0.176	1.56	0.0836
80	0.179	1.64	0.0801
90	0.168	1.73	0.0781

The maintenance coefficient, conversely, exhibits a markedly different behaviour as initial glucose concentration is increased. It increases from 1.56 to 1.73 g glucose/g DCW-h. This value of the maintenance coefficient may seem unusually high (order of magnitude larger) in comparison with those that have previously been reported (references). However, based on a similar application of Pirt's model to batch culture of LAB, an empirical equation predicting m_S for *Lactobacillus curvatus* LTH 1174 as a

function of pH and temperature was developed (Messens et al. 2003). With this equation the maintenance coefficient at pH 6 and temperature of 30°C would be approximately 0.84 g glucose/g DCW-h in a glucose-limited system. It will be demonstrated shortly that a maintenance coefficient above 1.0 g glucose/g DCW-h is reasonable. Maintenance energy is utilized for a variety of functions, for example, to maintain an energized membrane (i.e. a proton-motive force), to transport nutrients, and perform essential metabolic functions such as motility and repair damage of cellular structures (Shuler and Kargi 2002). If the maximum biomass yield on glucose remains constant while the apparent biomass yield decreases then it appears that glucose must be increasingly directed to processes that are not growth related; thus, the observed increase in the maintenance coefficient appears to be logical, as the specific rate of glucose consumption must increase to account for the drop in the apparent biomass yield. However, the implications of energy-sufficiency on substrate consumption kinetics must be addressed.

Previously, it was demonstrated that the medium composition utilized results in an energy-sufficient system (Figure 5.2). For a glucose-limited (energy-limited) system, a constant maximum yield and maintenance coefficient is expected based on the physiological interpretation of Pirt`s model for substrate consumption. However, Pirt`s model fails to predict substrate consumption kinetics of energy-sufficient systems (Abbott et al. 1974; Marsh et al. 1985; Neijssel and Tempest 1976; Shah and Coulman 1978; Stouthamer 1977; Stouthamer 1979; Tempest and Neijssel 1984). Modifications to Pirt`s model were proposed, but without physiological justification (Neijssel and Tempest 1976; Pirt 1982). Tsai et al. (1989) have developed a mechanistic model for substrate consumption in energy-sufficient continuous systems that can physiologically rationalize

the anomalies observed when attempting to apply Pirt's model for substrate consumption in energy-sufficient systems. Energy-sufficiency has profound implications on cell metabolism. Under energy-sufficient conditions there exists a third mechanism by which substrate may be consumed known as energy spilling. It is well established that cell growth and maintenance account for the total substrate consumption in energy (substrate) limited systems, as Pirt predicted; in which case, the uptake of substrate by the cell results in the production and utilization of intracellular energy (ATP) and metabolites, which defines a tightly coupled relationship between catabolism and anabolism. However, energy-sufficiency results in the uncoupling of catabolism from anabolism resulting in the phenomenon of energy-spilling. In continuous and batch culture this typically results in lowered apparent growth yields and higher specific substrate consumption rates, much like the results obtained in this work. Physiologically, energy-spilling manifests itself in a variety of ways. For example, the cell can reduce the efficiency of ATP generation by the deletion of oxidative phosphorylation sites, the branching of the respiratory chain, and the shifting of metabolic pathways. Cells can also dispose of intracellular energy by dissipation of membrane potential, ATP hydrolysis, and futile cycles. In continuous systems, the energy-spilling associated consumption of substrate is directly related to the residual substrate concentration within the chemostat. Furthermore, energy spilling associated substrate consumption has been shown to increase as residual substrate concentration increases above a critical value, and a variety of semi-empirical models along with the proposed mechanistic model (Tsai and Lee 1990) have been developed to account for such behaviour (Liu 1998; Liu and Tay 2000). For energy-sufficient batch cultures, an analogous relationship has been shown to exist

between the ratio of initial substrate concentration to initial biomass concentration (S_0/X_0) and energy spilling (Chudoba et al. 1992; Liu 1996; Liu 1998; Liu et al. 1999; Simkins and Alexander 1984). As inoculum size (X_0) was maintained constant throughout the batches run in this study, a direct relationship between S_0 and substrate consumption kinetics based on Pirt's model of substrate consumption thus becomes apparent. The model derived herein does not include a term to account for energy spilling, but a definitive decrease in the apparent growth yield corresponding to an increase in the maintenance coefficient is observed. Thus, it appears that the energy-spilling associated consumption of substrate has been convoluted with the maintenance associated consumption of substrate, causing an increase in maintenance coefficient with S_0. Presumably, the "true" maintenance coefficient would be that achieved under energy-limited conditions. Nonetheless, this does serve to explain the observed variation in the parameters quantifying substrate consumption.

5.1.4 Implications of energy-sufficiency on product formation kinetics

Table 5.3 illustrates the effect that the initial glucose concentration has on product formation. It is apparent that the amount of growth-associated lactate formation increases as initial glucose concentration increases from 1.9 g lactic acid/g DCW at 60 g/L to 2.8 g lactic acid/g DCW at 90 g/L. However, the amount of non-growth-associated lactate formed shows no observable trend as initial glucose concentration is increased. The β-value showed no notable change when the initial glucose concentration increased from 60 to 90 g/L, varying between 0.75 and 0.82 g lactic acid/g DCW-h. The increase in the contribution of growth-associated lactate formation could possibly be due to the glycolytic flux through the respirative pathway being saturated, as a consequence, the "excess" glucose is being diverted to conversion into lactate via LDH. When the flux through glycolysis is high, the ratio of $NADH/NAD^+$ will also be high, favouring LDH activity (Even et al. 1999; Garrigues et al. 1997), which could explain the higher lactic acid yield on biomass. However, it should be noted that this behaviour was observed under anaerobic conditions, and the effect that the shift to respirative conditions has upon *L. lactis* metabolism could be substantially different from that which was observed in those studies. Congruent to this discussion, is the influence that energy-sufficiency has on product formation kinetics. The increase in both apparent and maximum lactic acid yield on biomass can potentially be related to the concept of energy-spilling that was used to explain the behaviour of substrate consumption kinetics. There potentially could be a substantial amount of product formed owing to energy spilling reactions, and this could account for the increase in both the α-value and the apparent product yield. Studies have

shown that both the apparent and maximum product yield on biomass increase as residual substrate concentration in the chemostat increases (Brooke et al. 1990; Mulder et al. 1986). A semi-empirical model to predict product formation kinetics in energy-sufficient systems has been proposed (Liu and Tay 2000). It predicts that both the maximum product yield on biomass (α) and the apparent yield ($Y^{APP}_{P/X}$) will increase with residual substrate concentration within the chemostat. The extension of this model to batch fermentation systems in predicting product formation kinetics is reasonable based on the analogous extension made for substrate consumption kinetics. Thus, conceivably a relationship between S_0/X_0 ratio and the product yield is definable, and would explain the increase yield that is observed with S_0.

Table 5.3 – The effect of initial glucose concentration on the parameters for product formation kinetics. The α-value and β-value were determined from Equation 11.

S_0 (g/L)	α (g lactic acid/g DCW)	β (g lactic acid/g DCW-h)	$Y^{APP}_{P/X}$ (g lactic acid/g DCW)
60	1.936	0.747	5.35
80	2.251	0.842	6.35
90	2.847	0.816	7.44

5.1.5 Comparison between energy-limited and energy-sufficient cultivation

Upon moving from a glucose-sufficient medium to a glucose-limiting medium, it was discovered that both substrate and product formation kinetics were affected, as illustrated in Table 5.4. The maximum yield coefficient appears to be unaffected by the shift from glucose-sufficient to glucose-limiting conditions, as the $Y^{M}_{X/S}$ was 0.18 g DCW/g glucose for both medium compositions. The maintenance energy requirements decreased when moving to the glucose limiting medium. There was a much more profound influence on characterization of product formation when the medium was made to be glucose-limiting. The amount of growth-associated lactate formation increased from 1.94 to 2.55 g lactic acid/g DCW, while the amount of non-growth-associated lactate formed decreased from 0.747 to 0.467 g lactic acid/g DCW-h.

Table 5.4 – Effect of moving from a glucose-sufficient to a glucose-limiting medium on cell growth, substrate consumption, and product formation as determined by Equations 4, 8, and 11, respectively.

Parameter	Glucose-Sufficient	Glucose-Limiting
k (h^{-1})	1.00	1.20
$Y^{APP}_{X/S}$ (g DCW/g glucose)	0.084	0.11
$Y^{M}_{X/S}$ (g DCW/g glucose)	0.18	0.18
m_S (g glucose/g DCW-h)	1.56	1.23
$Y^{APP}_{P/X}$ (g lactic acid/g DCW)	5.35	3.88
α (g lactic acid/g DCW)	1.94	2.55
β (g lactic acid/g DCW-h)	0.75	0.47

Previously, the effect of using an energy-sufficient medium was discussed in terms of the influence that the ratio S_0/X_0 had on substrate consumption kinetics, as it relates to a phenomenon known as energy-spilling due to the presence of an energy surplus. This energy spilling is used to explain the lower apparent biomass yield of an energy-sufficient versus energy-limited system, which is observed in this study. Furthermore, it was also presumed that this energy-surplus was represented by an apparent maintenance coefficient that is higher than the true maintenance coefficient. Again, this is also illustrated in Table 5.4, as the maintenance coefficient under glucose-limited conditions is lower than that observed in glucose-sufficient (or energy-sufficient) system.

The influence of using a glucose-limiting versus glucose-sufficient medium on product formation is also shown in Table 5.4. The apparent yield of lactic acid on biomass decreased. This occurred because the same amount of lactic acid was produced regardless of the medium composition utilized, while the glucose-limited medium produced more biomass, resulting in a lower apparent lactic acid yield on biomass. Curiously, the α-value increased in the glucose-limited medium, and the β-value decreased. Again, the observed differences are most likely related to excess energy of the energy-sufficient system. However, any explanation for the observed behaviour would at best be speculative. Further experimentation would have to be performed using glucose-limited batch cultures with different initial glucose concentration to possibly explain the variation in the characterization of product formation.

5.2 Improved Medium for Growth of *L. lactis* and LAB

5.2.1 Effect of GP Concentration

LAB have a pH optima at around pH 6.0 and will stop growing when pH is lower than 4.5 (Luedeking and Piret 1959). Furthermore, the low pH has been proven to be harmful to LAB, causing the loss of acid production capacity and viability, as well as, inhibition of nisin accumulation (Cabo et al. 2001), recombinant proteins, and the development of bacteriophage plaques. Since the sugar catabolism of LAB produces large amounts of organic acids such as lactic and acetic acid, control of pH is of paramount importance and very challenging. For large-scale fermentations pH-control can be effectively accomplished by an active pH control strategy such as feed-back controlled pumping system to add acid or base solution in response to real-time changes in the pH of the medium. However, this methodology is not feasible for cultivations performed in smaller culture vessels (e.g. culture bottles, flasks or test tubes) or on solid media (e.g. agar plates and slants), which do not lend themselves to such additions. As a compromise, buffering agents are often included in the media to mitigate the change in pH associated with cell growth, which is referred to as a passive pH control strategy.

Numerous studies have been performed evaluating the effect of pH on the growth of lactic acid bacteria (Akerberg et al. 1998; Bibal et al. 1988). However, only a few attempts have been made to evaluate the effect of buffer reagents and their concentrations on growth (Bermudez-Humaran et al. 2002; De Vuyst and Vandamme 1993; Guerra et al. 2001). Furthermore, all those studies utilized KH_2PO_4, which was found to be the best phosphorus source for nisin production (De Vuyst and Vandamme 1993). However, a

significant drawback of the use of inorganic phosphate salts is their tendency to form insoluble salts with multivalent metal ions such as Fe^{++} and Mg^{++}, which function as co-factors for a variety of enzymes essential to cell growth and product formation. High inorganic phosphate concentrations results in the depletion of such metal ions. Consequently, it was not surprising to find that, as reported by De Vuyst and Vandamme (1993) that both cell growth and nisin production deteriorated significantly when KH_2PO_4 concentration was higher than 5%. Conversely, GP, an organic phosphate buffering reagent, has demonstrated no such precipitate formation, while at the same time maintaining the necessary buffering capacity required by the medium. It has been proven to be an excellent pH buffering agent of media formulated for LAB growth (Terzaghi and Sandine 1975). As shown in Figure 5.4, when the total GP concentration is above 49 g/L slight inhibition is observed as indicated by the slightly slower cell growth. However, higher final biomass is achievable with increasing GP concentration in the range of 59 - 79 g/l, even though significant inhibition to cell growth is evident. The high biomass concentration was achieved due to the longer growth phase rendered by the large pH buffering capacity of the medium with higher GP concentration.

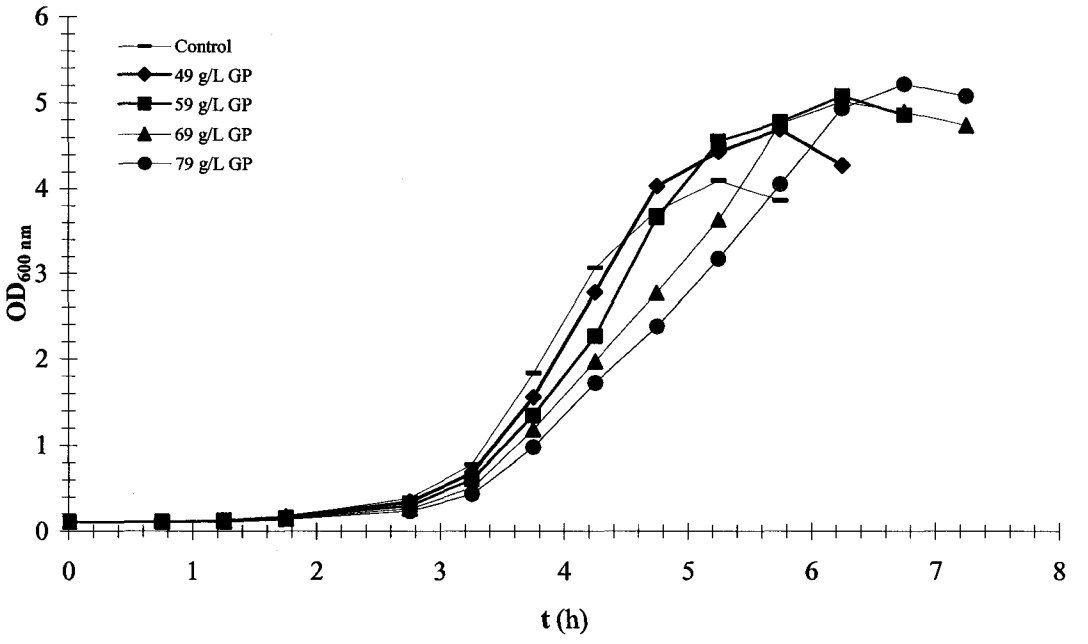

Figure 5.4 - Effects of disodium glycerophosphate pentahydrate (GP) on culture pH. Experiments were carried out with base medium containing 10 g/l glucose and 0.5 mg/l hemin at 30°C at 150 rpm in 500 ml flasks containing 250 ml broth

Figure 5.5 shows the pH change in the course of cell growth with medium containing different GP concentrations. Apparently, the medium containing 79 g/l GP was the only sample to maintain a final pH of above 5.1. This indicates that at the given glucose concentration the pH buffering capacities of all other media was exhausted. Experiments evaluating the influence of glucose on cell growth and broth pH need to be performed to find the balance between the optimal glucose concentration for cell growth and the necessary buffer concentration to ensure an acceptable pH.

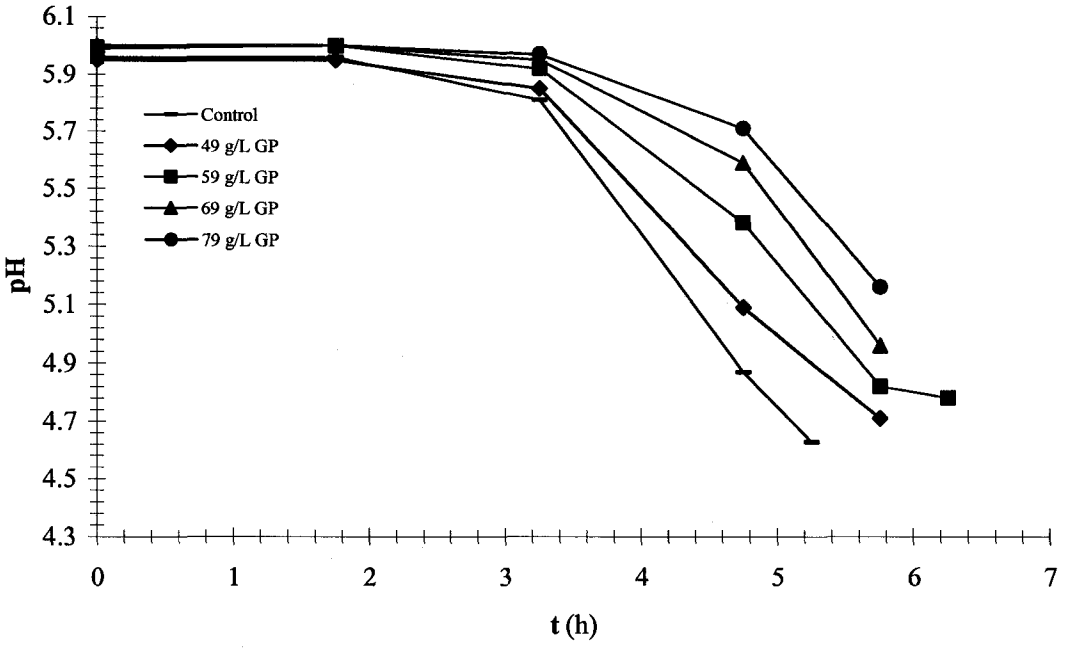

Figure 5.5 - The effect of disodium glycerophosphate pentahydrate (GP) concentration upon cell growth of *L. lactis* IL 1403. Experiments were carried out with the base medium supplemented with 10 g/l glucose and 0.5 mg/l hemin at 30°C at 150 rpm in 500 ml flasks containing 250 ml broth.

5.2.2 Effect of Glucose Concentration

As previously mentioned, elevated substrate levels will result in larger biomass yields; however, it has long been known that at elevated concentrations substrate inhibition will become a factor (GonÃ§alves et al. 1991; Lees and Jago 1976; Tango and Ghaly 1999; Van Niel et al. 2003). A dominating effect of sugar concentration to cell growth and product formation of LAB is that large amounts of organic acids are accumulated during sugar catabolism, high sugar concentration accessing the buffering capacity of the medium will certainly lead the broth pH to drop to a point where it is inhibitive to cell growth. It is therefore important to determine the optimal glucose concentration in a pre-determined optimal buffering reagent concentration.

The beneficial effect of hemin-stimulated respiration to cell growth of *L. lactis* has been well established in previous studies in both flask experiments and by our group in tightly controlled bench top bioreactors (Duwat et al. 2001; Lan et al. 2006). It would seem logical that the inclusion of hemin into a medium with not only be beneficial for the growth of *L. lactis*, but also for maintaining a more desirable pH since the presence of hemin dramatically reduces the amount of lactic acid formed. This also has implications as it relates to the amount of buffering agent required to maintain the pH at an optimal level. It is clear that any medium for the growth of LAB has a specific requirement for a balance between the carbon source and buffering agent, and in the case of *L. lactis* hemin concentration.

The effect of glucose concentration on the maximum specific growth rate, $\square_m$, maximum biomass concentration, X_m, and biomass yield on substrate, $Y_{X/S}$, was evaluated by performing shake-flask experiments with five different initial glucose concentrations,

i.e., 5.0, 7.5, 10.0, and 12.5 g/L. The results are summarized in Table 5.5 and the respective growth curves are shown in Figure 5.6.

The glucose concentration in the test range, i.e. 5.0 – 12.5 g/l, had no effect on the maximum cell growth rate and cell yield, indicating that no substrate inhibition took place in the tested glucose concentration range. However, the final pH varied significantly as the initial glucose concentration changed. The highest pH was observed at 6.2 with 5.0 g/l glucose, followed by 5.84 with 7.5 g/l glucose, and the lowest 4.5 with 12.5 g/l glucose. Apparently, when the glucose concentration is 10.0 g/l or higher, it has exceeded the pH buffering capacity provided by the 59 g/l GP. This argument is confirmed by the glucose consumption data. It seems to be obvious that the glucose consumption can be supported by the pH buffering capacity is approximately 8.1 g/l. Considering both the maximum biomass concentration and final pH obtained in each glucose concentration, the optimal glucose concentration is determined to be 7.5 g/l.

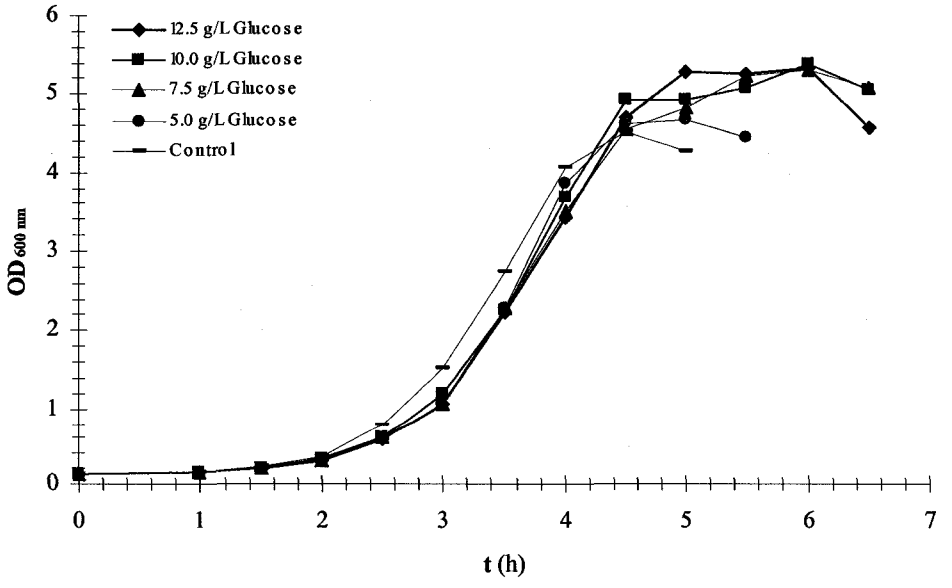

Figure 5.6 - The effect of initial glucose concentration upon cell growth of *L. lactis* IL 1403. Experiments were carried out with the base medium supplemented with 59 g/l GP and 0.5 mg/l hemin at 30°C at 150 rpm in 500 ml flasks containing 250 ml broth.

Table 5.5 - Effect of glucose concentration on cell growth of *L. lactis*

S_0 (g/L)	OD_{600}	μ_m (h^{-1})	pH_{Final}	$Y^{APP}_{X/S}$ (g/g)	S_f (g/l)	ΔS (g/l)
12.5	5.30	1.32	4.31	0.526	4.3	8.1
10	5.31	1.31	4.79	0.564	1.8	8.2
7.5	5.30	1.32	5.81	0.573	0.0	7.5
5.0	4.63	1.30	6.2	0.574	0.0	5.0

5.2.3 Comparison of the performance of the optimized media with the commercial medium M17

Experiments were carried out to compare the performance of different optimized media with that of the most popular commercial medium for LAB cultivation, M17. M17G is a medium of M17 supplied with 10 g/l glucose; M18 was a medium having the same composition as M17 supplemented with 7.5 g/l glucose and that the GP concentration was increased from 19 to 69 g/l; finally, M18H is medium M18 supplemented with 5 ppm hemin. Figures 5.7 and 5.8 show the cell growth kinetics and pH change time courses of the four different media respectively. Experiments were carried out with duplicates at 30°C and 150 rpm. Apparently, the cell growth of different media follows the order of M17 < M17G < M18 < M18H. Significantly, the maximum biomass concentration of medium M18H is more than 4 times as much as that obtained with M17. On the other hand, the M17G medium produced the lowest final pH of 4.43, which is an inhibitive pH that is harmful to the survival of LAB cells. The final pH of M18 is similar to that of M17 and that of M18H is at an even higher value of pH 6.29. In conclusion, M18H is superior to M17 in terms of both cell growth and final pH while M18 supports a better cell growth but maintains a similar final pH as that of M17.

Table 5.6 - A comparison of the performance of different media

Medium	OD_{600nm}	μ_m (h^{-1})	pH_{Final}	$Y^{APP}_{X/S}$ (g/g)	S_f (g/l)
M17	1.3	0.90	5.58	-	0
M17G	3.1	1.3	4.43	0.140	2.1
M18	4.7	1.5	5.51	0.501	0
M18H	5.3	1.5	6.29	0.531	0

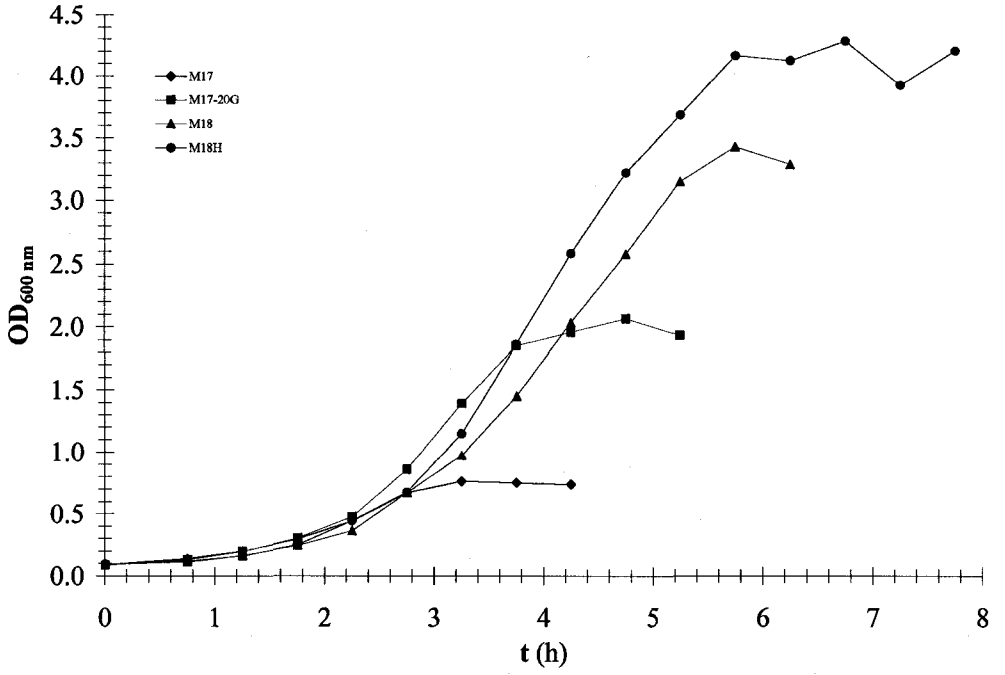

Figure 5.7 - The effect of initial glucose concentration upon cell growth of *L. lactis* IL 1403 with the four different media. Experiments were carried out with the base medium supplemented at 30°C at 150 rpm in 500 ml flasks containing 250 ml broth.

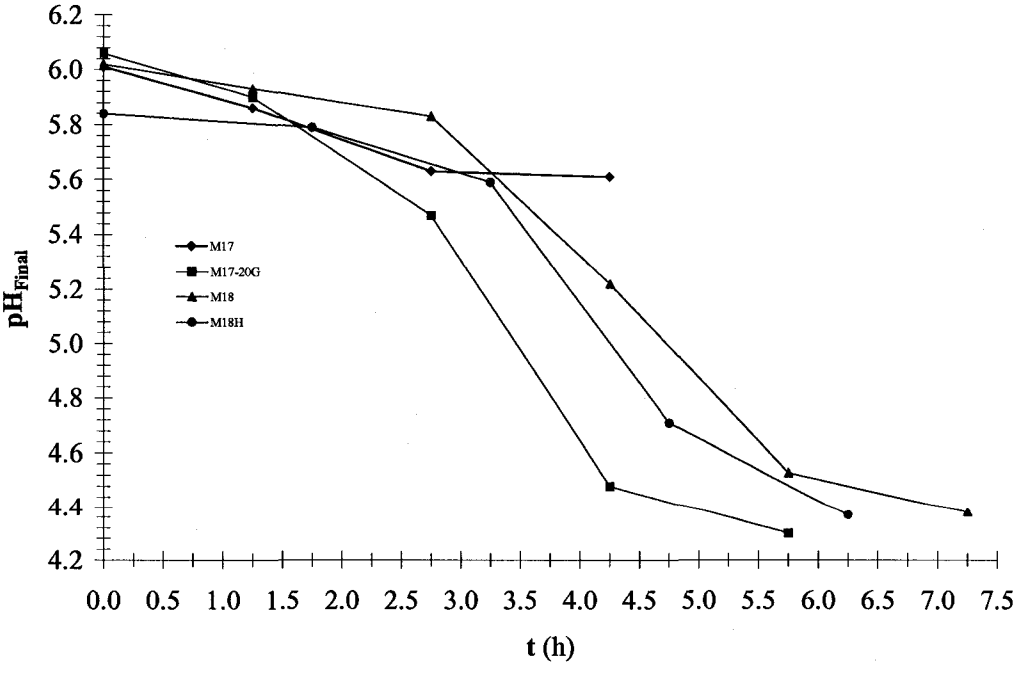

Figure 5.8 - The effect of initial glucose concentration upon broth pH of *L. lactis* IL 1403 with the four different media. Experiments were carried out with the base medium supplemented at 30°C at 150 rpm in 500 ml flasks containing 250 ml broth.

6 Conclusions and Recommendations

The study of batch fermentation kinetics of *Lactococcus lactis* IL1403 at elevated initial substrate concentrations revealed the strain can grow well at high sugar concentrations when it conducts hemin-stimulated respirative growth. It was shown that there was no significant inhibition to cell growth when the initial glucose concentration was as high as 90 g/L. However, initial glucose concentration did show profound influences upon substrate consumption and product formation kinetics dictated by the energy-sufficiency of the medium utilized. As aforementioned, no evidence of substrate inhibition upon cell growth was visible, as the specific growth rate, the maximum biomass yield coefficient, and final biomass concentration were unaffected by the initial glucose concentration utilized. Furthermore, the basic medium was an energy-sufficient system, the cell growth in which was limited by nutrients other than glucose, the energy and carbon source. Modification of the medium resulted in a substantial increase in the biomass yield on substrate. Energy-sufficiency was shown to have a profound influence upon both substrate consumption and product formation kinetics. The maximum yield coefficient ($Y^{M}_{X/S}$) was unaffected, but a noticeable increase in the maintenance requirements was observed. The increase in maintenance requirements was attributed to the energy-spilling associated consumption of substrate, which is a commonly reported phenomenon in energy-sufficient systems. Energy-spilling represents a mechanism separate from growth and maintenance for substrate consumption. As Pirt's original model does not account for energy-spilling substrate consumption, the observed increase in the maintenance coefficient can be viewed as a measure of the energy-spilling associated substrate consumption. Furthermore, in batch systems, it has been shown that

there is a strong dependence of substrate consumption via energy-spilling upon the ratio of initial substrate concentration to initial cell concentration, as demonstrated in this study. Product formation kinetics was also affected by initial glucose concentration, as both the apparent and maximum lactic acid yield increased with initial glucose concentration; again, this increase in product yield was shown to be related to energy-sufficiency. Energy-sufficiency results in a third mechanism by which glucose was consumed and product was formed accounting for the observed changes in the kinetics parameters associated with Pirt's model for substrate consumption and Luedeking-Piret model for product formation. Based upon Pirt's model for substrate consumption, the Luedeking-Piret model for product formation, and the logistic equation for population growth, a novel, analytical model was developed to predict batch fermentation kinetics. The equations derived represent a non-linear system of equations that allow the prediction of cell growth, substrate consumption, and product formation for energy-limited systems.

A medium for improved growth of *L. lactis* was developed by the systematic study of the influence of glucose concentration, the substrate, and sodium glycerophosphate, a buffering agent, based on the concept of balanced carbon sources and buffering capacities. By varying glucose and GP concentration, the medium ensured maximum cell growth while at the same time maintaining an acceptable final broth pH. Cell growth was shown to increase by a factor of 4 while maintaining a pH value of 5.1, well above the range that is inhibitive to *L. lactis* cell growth.

Much work remains to be done for commercial utilization of *L. lactis* as a food-grade expression platform with respect to recombinant proteins for the biopharmaceutical

industry. Numerous examples of this have been realized at a research level, but no commercial product has been produced owing to the poor growth of *L. lactis*. The realization of the beneficial effects of hemin in this regards have been established, and this studied revealed the influence that substrate concentration has on cell growth in the presence of heme. For example, the influence hemin has on the expression of heterologous protein products in tightly controlled fermentors must be performed.

The model developed herein must be tested against a variety of different microbial systems to determine its robustness and overall applicability when modeling batch fermentation processes.

The modeling of *L. lactis* cell growth must also be further developed. The work in this study deals primarily with the primary growth phase of *L. lactis* utilizing the initial substrate as the model substrate. However, it has clearly been established that *L. lactis* displays diauxic growth, and upon the exhaustion of sugar in the broth, the consumption of the lactic acid that has accumulated in the early stage initiates a secondary growth phase, enabling a prolonged growth period. The kinetics of growth and substrate consumption in the secondary growth phase has yet to be quantified with confidence.

The new medium discussed must also be tested with other popular lactic acid bacteria currently used in microbiological applications. Secondly, application of the medium for Lactococcal bacteriophage propagation is under investigation.

8 Nomenclature

List of Variables

$(\Delta q_{obs})_m$	Maximum observed specific substrate consumption rate under substrate-limited conditions	g substrate/g DCW-h
$(\Delta q_w)_{max}$	Maximum energy-spilling-associated substrate consumption rate	G substrate/g DCW-h
$(S_0/X_0)_{min}$	S_0/X_0-ratio for substrate-limited growth	g substrate/g DCW
A	Exponential constant in the Moser equation	Dimensionless
Δq_w	Specific rate of substrate consumption due to energy spilling	g substrate/g DCW-h
I	Toxic inhibitor concentration	G inhibitor/L
K	Rate constant for logistic growth correlation	h^{-1}
k_d	Rate of cell death	h^{-1}
K_I	Substrate inhibition constant	g substrate/L
K_i	Toxic compound inhibition constant	g inhibitor/L
K_m	Enzyme Affinity constant	Arbitrary units
K_P	Product inhibition constant	g product/L
K_S	Substrate saturation constant	g/L
$K_{S/X}$	S_0/X_0-ratio-related saturation constant,	
K_{S0}	Saturation constant for a modified version of the Monod equation	dimensionless
K_{SX}	Saturation constant for the Contois equation, biomass dependent	g substrate/g DCW
m_S	Maintenance coefficient (Substrate consumption associated with processes other than growth)	g glucose/g DCW-h
N	Exponential inhibition constant for logistic correlation	Dimensionless
P	lactic acid concentration	g/L
P_0	Initial lactic acid concentration	g/L
P_m	Concentration at which inhibiting fermentation production causes growth to stop	g/L
q_g	Specific rate of substrate consumption for growth	g substrate/g DCW-h
$q_{g,max}$	Maximum specific rate of substrate consumption for growth	g substrate/g DCW-h
q_{obs}	Observed specific substrate consumption rate for an energy-sufficient system	g substrate/g DCW-h
S	Substrate concentration	g/L
S_0	Initial glucose concentration	g/L
T	Fermentation time	h
v_m	Rate of enzymatic reaction	Arbitrary units
X_0	Initial biomass concentration	G DCW/L
X_m	Maximum achievable biomass concentration	g DCW/L

$Y^{APP}_{X/S}$	Apparent yield coefficient for biomass on substrate	g DCW/g glucose
$Y^{M}_{X/S}$	Maximum yield coefficient for biomass on substrate	g DCW/g glucose

List of Greek Symbols

μ_{net}	Net specific growth rate	h^{-1}
μ_m	Maximum specific growth rate	h^{-1}
μ_g	Specific growth rate	h^{-1}
α	Growth-associated product formation constant	g lactic acid/g DCW
β	Non-growth-associated product formation constant	g lactic acid/g DCW-h

8 References

Abbott BJ, Laskin AI, McCoy CJ. 1974. Effect of growth rate and nutrient limitation on the composition and biomass yield of *Acinetobacter calcoaceticus*. Appl Microbiol 28(1):58-63.

Akerberg C, Hofvendahl K, Hahn-Hagerdal B, Zacchi G. 1998. Modelling the influence of pH, temperature, glucose and lactic acid concentrations on the kinetics of lactic acid production by *Lactococcus lactis ssp. lactis* ATCC 19435 in whole-wheat flour. Applied Microbiology and Biotechnology 49(6):682-690.

Amiali MN, Lacroix C, Simard RE. 1998. High nisin Z production by *Lactococcus lactis* UL719 in whey permeate with aeration. World Journal of Microbiology and Biotechnology 14(6):887-894.

Audouy SAL, van Roosmalen ML, Neef J, Kanninga R, Post E, van Deemter M, Metselaar H, van Selm S, Robillard GT, Leenhouts KJ and others. 2006. *Lactococcus lactis* GEM particles displaying pneumococcal antigens induce local and systemic immune responses following intranasal immunization. Vaccine 24(26):5434-5441.

Audouy SAL, van Selm S, van Roosmalen ML, Post E, Kanninga R, Neef J, Estevao S, Nieuwenhuis EES, Adrian PV, Leenhouts K and others. 2007. Development of lactococcal GEM-based pneumococcal vaccines. Vaccine 25(13):2497-2506.

Bermudez-Humaran L, Langella P. 2006. Bacteria to prevent and treat cervical cancer induced by human papillomavirus type 16. Medecine Sciences 22(5):472-473.

Bermudez-Humaran LG, Cortes-Perez NG, Le Loir Y, Alcocer-Gonzalez JM, Tamez-Guerra RS, Montes De Oca-Luna R, Langella P. 2004. An inducible surface presentation system improves cellular immunity against human papillomavirus type 16 E7 antigen in mice after nasal administration with recombinant lactococci. Journal of Medical Microbiology 53(5):427-433.

Bermudez-Humaran LG, Cortes-Perez NG, Lefevre F, Guimaraes V, Rabot S, Alcocer-Gonzalez JM, Gratadoux JJ, Rodriguez-Padilla C, Tamez-Guerra RS, Corthier G and others. 2005. A novel mucosal vaccine based on live lactococci expressing E7 antigen and IL-12 induces systemic and mucosal immune responses and protects mice against human papillomavirus type 16-induced tumors. Journal of Immunology 175(11):7297-7302.

Bermudez-Humaran LG, Langella P, Miyoshi A, Gruss A, Tamez Guerra R, Montes de Oca-Luna R, Le Loir Y. 2002. Production of human papillomavirus type 16 E7

protein in *Lactococcus lactis*. Applied and Environmental Microbiology 68(2):917-922.

Bibal B, Goma Gr, Vayssier Y, Pareilleux A. 1988. Influence of pH, lactose and lactic acid on the growth of *Streptococcus cremoris*: a kinetic study. Applied Microbiology and Biotechnology 28(4 - 5):340-344.

Birollo GA, Reinheimer JA, Vinderola CGV. 2000. Viability of lactic acid microflora in different types of yoghurt. Food Research International 33(9):799-805.

Blank LM, Koebmann BJ, Michelsen O, Nielsen LK, Jensen PR. 2001. Hemin reconstitutes proton extrusion in an H(+)-ATPase-negative mutant of *Lactococcus lactis*. J Bacteriol 183(22):6707-9.

Bolotin A, Wincker P, Mauger S, Jaillon O, Malarme K, Weissenbach J, Ehrlich SD, Sorokin A. 2001. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis ssp. lactis IL1403*. Genome Research 11(5):731-753.

Borysowski J, Weber-Dabrowska B, Gorski A, Gorski A. 2006. Current status and perspectives of phage therapy. Advances in Clinical and Experimental Medicine 15(4):575-580.

Boumerdassi H, Monnet C, Desmazeaud M, Corrieu G. 1997. Isolation and properties of Lactococcus lactis subsp. lactis biovar diacetylactis CNRZ 483 mutants producing diacetyl and acetoin from glucose. Appl. Environ. Microbiol. 63(6):2293-2299.

Bromberg R, Moreno I, Delboni RR, Cintra HC, Oliveira PTV. 2005. Characteristics of the bacteriocin produced by *Lactococcus lactis subsp. cremoris* CTC 204 and the effect of this compound on the mesophilic bacteria associated with raw beef. World Journal of Microbiology and Biotechnology 21(3):351-358.

Brooke AG, Attwood MM, Tempest DW. 1990. Metabolic fluxes during the growth of thermotolerant methylotrophic *Bacillus* strains in methanol-sufficient chemostat cultures. Archives of Microbiology 153(6):591-595.

Buccato S, Maione D, Rinaudo CD, Volpini G, Taddei AR, Rosini R, Telford JL, Grandi G, Margarit I. 2006. Use of *Lactococcus lactis* expressing pili from group B *Streptococcus* as a broad-coverage vaccine against streptococcal disease. Journal of Infectious Diseases 194(3):331-340.

Budin-Verneuil A, Pichereau V, Auffray Y, Ehrlich DS, Maguin E. 2005. Proteomic characterization of the acid tolerance response in *Lactococcus lactis* MG1363. Proteomics 5(18):4794-4807.

Burdock GA. 2000. Dietary supplements and lessons to be learned from GRAS. Regulatory Toxicology and Pharmacology 31(1):68-76.

Burdock GA, Carabin IG. 2004. Generally recognized as safe (GRAS): History and description. Toxicology Letters 150(1):3-18.

Cabo ML, Murado MA, Gonzalez MP, Pastoriza L. 2001. Effects of aeration and pH gradient on nisin production. A mathematical model. Enzyme and Microbial Technology 29(4-5):264-273.

Carvalho LJM, Alves FA, Bianco Jr C, Oliveira SG, Zanini GM, Soe S, Druilhe P, Theisen M, Muniz JAPC, Daniel-Ribeiro CT. 2005. Immunization of Saimiri sciureus monkeys with a recombinant hybrid protein derived from the Plasmodium falciparum antigen glutamate-rich protein and merozoite surface protein 3 can induce partial protection with Freund and montanide ISA720 adjuvants. Clinical and Diagnostic Laboratory Immunology 12(2):242-248.

Chudoba P, Capdeville B, Chudoba J. 1992. Explanation of biological meaning of the S(o)/X(o) ratio in batch cultivation. Water Science and Technology 26(3-4):743-751.

Cocaign-Bousquet M, Even S, Lindley ND, Loubiere P. 2003. Anaerobic sugar catabolism in Lactococcus lactis: Genetic regulation and enzyme control over pathway flux. Applied Microbiology and Biotechnology 60(1-2):24-32.

Cocaign-Bousquet M, Garrigues C, Novak L, Lindley ND, Loubiere P. 1995. Rational development of a simple synthetic medium for the sustained growth of Lactococcus lactis. Journal of Applied Bacteriology 79(1):108-116.

Cortes-Perez NG, Azevedo V, Alcocer-Gonza?lez JM, Rodriguez-Padilla C, Tamez-Guerra RS, Corthier G, Gruss A, Langella P, Bermudez-Humaran LG. 2005a. Cell-surface display of E7 antigen from human papillomavirus type-16 in *Lactococcus lactis* and in *Lactobacillus plantarum* using a new cell-wall anchor from lactobacilli. Journal of Drug Targeting 13(2):89-98.

Cortes-Perez NG, Azevedo V, Alcocer-Gonzalez JM, Rodriguez-Padilla C, Tamez-Guerra RS, Corthier G, Gruss A, Langella P, Bermudez-Humaran LG. 2005b. Cell-surface display of E7 antigen from human papillomavirus type-16 in Lactococcus lactis and in *Lactobacillus plantarum* using a new cell-wall anchor from lactobacilli. Journal of Drug Targeting 13(2):89-98.

Cortes-Perez NG, Bermudez-Humaran LG, Le Loir Y, Rodriguez-Padilla C, Gruss A, Saucedo-Cardenas O, Langella P, Montes-De-Oca-Luna R. 2003. Mice immunization with live lactococci displaying a surface anchored HPV-16 E7 oncoprotein. FEMS Microbiology Letters 229(1):37-42.

Crespo A, Suh B. 2001. *Helicobacter pylori* Infection: Epidemiology, Pathophysiology, and Therapy. Archives of Pharmacal Research 24(6):485-498.

De Vuyst L. 1995. Nutritional factors affecting nisin production by *Lactococcus lactis* subsp. lactis NIZO 22186 in a synthetic medium. Journal of Applied Bacteriology 78(1):28-33.

De Vuyst L. 2000. Technology Aspects Related to the Application of Functional Starter Cultures. Food Technology and Biotechnology 38(2):105-112.

De Vuyst L, Vandamme EJ. 1993. Influence of the phosphorus and nitrogen source of nisin production in Lactococcus lactis subsp. lactis batch fermentations using a complex medium. Applied Microbiology and Biotechnology 40(1):17-22.

Derzelle S, Bolotin A, Mistou MY, Rul F. 2005. Proteome analysis of *Streptococcus thermophilus* grown in milk reveals pyruvate formate-lyase as the major upregulated protein. Applied and Environmental Microbiology 71(12):8597-8605.

Dunny GM, McKay LL. 1999. Group II introns and expression of conjugative transfer functions in lactic acid bacteria. Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology 76(1-4):77-88.

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. J Bacteriol 183(15):4509-16.

Even S, Garrigues C, Loubiere P, Lindley ND, Cocaign-Bousquet M. 1999. Pyruvate Metabolism in Lactococcus lactis Is Dependent upon Glyceraldehyde-3-phosphate Dehydrogenase Activity. Metabolic Engineering 1(3):198-205.

Fang H, Elina T, Heikki A, Seppo S. 2000. Modulation of humoral immune response through probiotic intake. FEMS Immunology and Medical Microbiology 29(1):47-52.

Fernandez A, Horn N, Gasson MJ, Dodd HM, Rodriguez JM. 2004. High-level coproduction of the bacteriocins nisin A and lactococcin A by *Lactococcus lactis*. Journal of Dairy Research 71(2):216-221.

Fernandez De Palencia P, Nieto C, Acebo P, Espinosa M, Lopez P. 2000. Expression of green fluorescent protein in *Lactococcus lactis*. FEMS Microbiology Letters 183(2):229-234.

Gaeng S, Scherer S, Neve H, Loessner MJ. 2000. Gene cloning and expression and secretion of Listeria monocytogenes bacteriophage-lyric enzymes in *Lactococcus lactis*. Applied and Environmental Microbiology 66(7):2951-2958.

Garcia Fontan MC, Martinez S, Franco I, Carballo J. 2006. Microbiological and chemical changes during the manufacture of Kefir made from cows' milk, using a commercial starter culture. International Dairy Journal 16(7):762-767.

Gargova S, Sariyska M. 2003. Effect of culture conditions on the biosynthesis of Aspergillus niger phytase and acid phosphatase. Enzyme and Microbial Technology 32(2):231-235.

Garrigues C, Loubiere P, Lindley ND, Cocaign-Bousquet M. 1997. Control of the shift from homolactic acid to mixed-acid fermentation in Lactococcus lactis: Predominant role of the NADH/NAD+ ratio. Journal of Bacteriology 179(17):5282-5287.

Gellissen G. 2005. Production of recombinant proteins : novel microbial and eukaryotic expression systems. Weinheim: Wiley-VCH. 404 p.

Gilbert C, Robinson K, Le Page RWF, Wells JM. 2000. Heterologous expression of an immunogenic pneumococcal type 3 capsular polysaccharide in *Lactococcus lactis*. Infection and Immunity 68(6):3251-3260.

GonÃ§alves LMD, Xavier AMRB, Almeida JS, Carrondo MJT. 1991. Concomitant substrate and product inhibition kinetics in lactic acid production. 13:314-319.

Gram GJ, Fomsgaard A, Thorn M, Madsen SM, Glenting J. 2007. Immunological analysis of a *Lactococcus lactis*-based DNA vaccine expressing HIV gp120. Genetic Vaccines and Therapy 5.

Grangette C, Mu?ller-Alouf H, Geoffroy MC, Goudercourt D, Turneer M, Mercenier A. 2002. Protection against tetanus toxin after intragastric administration of two recombinant lactic acid bacteria: Impact of strain viability and in vivo persistence. Vaccine 20(27-28):3304-3309.

Grangette C, Mu?ller-Alouf H, Hols P, Goudercourt D, Delcour J, Turneer M, Mercenier A. 2004. Enhanced Mucosal Delivery of Antigen with Cell Wall Mutants of Lactic Acid Bacteria. Infection and Immunity 72(5):2731-2737.

Guerra NP, Rua ML, Pastrana L. 2001. Nutritional factors affecting the production of two bacteriocins from lactic acid bacteria on whey. International Journal of Food Microbiology 70(3):267-281.

Guimaraes VD, Gabriel JE, Lefevre F, Cabanes D, Gruss A, Cossart P, Azevedo V, Langella P. 2005. Internalin-expressing *Lactococcus lactis* is able to invade small intestine of guinea pigs and deliver DNA into mammalian epithelial cells. Microbes and Infection 7(5-6):836-844.

Guimaraes VD, Innocentin S, Lefevre F, Azevedo V, Wal JM, Langella P, Chatel JM. 2006. Use of native lactococci as vehicles for delivery of DNA into mammalian epithelial cells. Applied and Environmental Microbiology 72(11):7091-7097.

Haier E, Janzen T, Henriksen C, Rattray F, Brockmann E, Johansen E. 1999. The Production, Application and Action of Lactic Cheese Starter Cultures. In: Law BA, editor. The Technology of Cheesemaking. Sheffield, England: Sheffield Academic Press Ltd. p 99-131.

Hanniffy SB, Carter AT, Hitchin E, Wells JM. 2007. Mucosal delivery of a pneumococcal vaccine using Lactococcus lactis affords protection against respiratory infection. Journal of Infectious Diseases 195(2):185-193.

Hernandez I, Molenaar D, Beekwilder J, Bouwmeester H, Vlieg JETVH. 2007. Expression of plant flavor genes in Lactococcus lactis. Applied and Environmental Microbiology 73(5):1544-1552.

Heuer OE, Hammerum AM, Collignon P, Wegener HC. 2006. Human health hazard from antimicrobial-resistant enterococci in animals and food. Clinical Infectious Diseases 43(7):911-916.

Hols P, Ramos A, Hugenholtz J, Delcour J, de Vos WM, Santos H, Kleerebezem M. 1999. Acetate utilization in Lactococcus lactis deficient in lactate dehydrogenase: a rescue pathway for maintaining redox balance. J Bacteriol 181(17):5521-6.

Hu SY, Wu JL, Huang JH. 2004. Production of tilapia insulin-like growth factor-2 in high cell density cultures of recombinant *Escherichia coli*. Journal of Biotechnology 107(2):161-171.

Ishizaki A, Ueda T. 1995. Growth kinetics and product inhibition of *Lactococcus lactis* IO-1 culture in xylose medium. Journal of Fermentation and Bioengineering 80(3):287-290.

Johansen E. 1999. Genetic engineering (b) Modification of bacteria. In: Robinson R, Batt C, Patel P, editors. Encyclopedia of Food Microbiology. London: Academic Press. p 917-921.

Kim SJ, Jun DY, Yang CH, Kim YH. 2006a. Cloning and expression of hpaA gene of Korean strain helicobacter pylori K51 in oral vaccine delivery vehicle *Lactococcus lactis subsp. lactis* MG1363. Journal of Microbiology and Biotechnology 16(2):318-324.

Kim SJ, Jun DY, Yang CH, Kim YH. 2006b. Expression of Helicobacter pylori cag12 gene in *Lactococcus lactis* MG1363 and its oral administration to induce systemic anti-Cag12 immune response in mice. Applied Microbiology and Biotechnology 72(3):462-470.

Kondo JK, Johansen E. 2002. Product development strategies for foods in the era of molecular biotechnology. Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology 82(1-4):291-302.

Korakli M, Vogel RF. 2006. Structure/function relationship of homopolysaccharide producing glycansucrases and therapeutic potential of their synthesised glycans. Applied Microbiology and Biotechnology 71(6):790-803.

Kucukoner E, Tarakci Z, Sagdic O. 2006. Physicochemical and microbiological characteristics and mineral content of herby cacik, a traditional Turkish dairy product. Journal of the Science of Food and Agriculture 86(2):333-338.

Labrie S, Bart C, Vadeboncoeur C, Moineau S. 2005. Use of an a-galactosidase gene as a food-grade selection marker for Streptococcus thermophilus. Journal of Dairy Science 88(7):2341-2347.

Lan CQ, Oddone G, Mills DA, Block DE. 2006. Kinetics of *Lactococcus lactis* growth and metabolite formation under aerobic and anaerobic conditions in the presence or absence of hemin. Biotechnology and Bioengineering 95(6):1070-1080.

Lapujade P, Cocaign-Bousquet M, Loubiere P. 1998. Glutamate Biosynthesis in *Lactococcus lactis subsp. lactis* NCDO 2118. Appl Environ Microbiol 64(7):2485-9.

Lee MH, Roussel Y, Wilks M, Tabaqchali S. 2001. Expression of *Helicobacter pylori* urease subunit B gene in *Lactococcus lactis* MG1363 and its use as a vaccine delivery system against H. pylori infection in mice. Vaccine 19(28-29):3927-3935.

Leenhouts K, Bolhuis A, Venema G, Kok J. 1998. Construction of a food-grade multiple-copy integration system for *Lactococcus lactis*. Applied Microbiology and Biotechnology 49(4):417-423.

Lees GJ, Jago GR. 1976. Formation of acetaldehyde from threonine by lactic acid bacteria. Journal of Dairy Research 43(1):75-83.

Liu Y. 1996. Bioenergetic interpretation on the S0/X0 ratio in substrate-sufficient batch culture. Water Research 30(11):2766-2770.

Liu Y. 1998. Energy uncoupling in microbial growth under substrate-sufficient conditions. Applied Microbiology and Biotechnology 49(5):500-505.

Liu Y, Chen GH, Rols JL. 1999. A kinetic model incorporating energy spilling for substrate removal in substrate-sufficient batch culture of activated sludge. Applied Microbiology and Biotechnology 52(5):647-651.

Liu Y, Tay JH. 2000. A kinetic model for energy spilling-associated product formation in substrate-sufficient continuous culture. Journal of Applied Microbiology 88(4):663-668.

Lopez De Felipe F, Starrenburg MJC, Hugenholtz J. 1997. The role of NADH-oxidation in acetoin and diacetyl production from glucose in *Lactococcus lactis subsp. lactis* MG1363. FEMS Microbiology Letters 156(1):15-19.

Loubiere P, Novak L, Cocaign-Bousquet M, Lindley ND. 1996. Nutritional requirements of lactic acid bacteria: Interactions between carbon and nitrogen flux. Besoins nutritionnels des bacteries lactiques : Interactions entre flux de carbone et d'azote 76(1-2):5-12.

Luedeking R, Piret EL. 1959. A kinetic study of the lactic acid fermentation. Batch process at controlled pH. J Biochem Microbiol Technol Eng 1:393-421.

MacCormick CA, Griffin HG, Gasson MJ. 1995. Construction of a food-grade host/vector system for *Lactococcus lactis* based on the lactose operon. FEMS Microbiology Letters 127(1-2):105-109.

Marsh PD, McDermid AS, Keevil CW, Ellwood DC. 1985. Environmental regulation of carbohydrate metabolism by *Streptococcus sanguis* NCTC 7865 grown in a chemostat. Journal of General Microbiology 131(10):2505-2514.

Mercenier A, Muller-Alouf H, Grangette C. 2000. Lactic acid bacteria as live vaccines. Current issues in molecular biology 2(1):17-25.

Messens W, Verluyten J, Leroy F, De Vuyst L. 2003. Modelling growth and bacteriocin production by *Lactobacillus curvatus* LTH 1174 in response to temperature and pH values used for European sausage fermentation processes. International Journal of Food Microbiology 81(1):41-52.

Miyoshi A, Bermudez-Humaran LG, Ribeiro LA, Le Loir Y, Oliveira SC, Langella P, Azevedo V. 2006. Heterologous expression of Brucella abortus GroEL heat-shock protein in *Lactococcus lactis*. Microbial Cell Factories 5.

Mor-Mur M, Carretero C, Pla R, Guamis B. 1992. A survey on the microbiological quality of a semi-soft on-farm manufactured goat cheese. Food Microbiology 9(4):345-352.

Morishita T, Tamura N, Makino T, Kudo S. 1999. Production of Menaquinones by Lactic Acid Bacteria. J. Dairy Sci. 82(9):1897-1903.

Mulder MM, Teixeira de Mattos MJ, Postma PW, van Dam K. 1986. Energetic consequences of multiple K+ uptake systems in Escherichia coli. Biochimica et biophysica acta 851(2):223-228.

Neijssel OM, Tempest DW. 1976. Bioenergetic aspects of aerobic growth of *Klebsiella aerogenes* NCTC 418 in carbon-limited and carbon-sufficient chemostat culture. Archives of Microbiology 107(2):215-221.

Neves AR, Pool WA, Kok J, Kuipers OP, Santos H. 2005. Overview on sugar metabolism and its control in *Lactococcus lactis* - The input from in vivo NMR. FEMS Microbiology Reviews 29(3 SPEC. ISS.):531-554.

Norton PM, Brown HWG, Wells JM, Macpherson AM, Wilson PW, Le Page RWF. 1996. Factors affecting the immunogenicity of tetanus toxin fragment C expressed in *Lactococcus lactis*. FEMS Immunology and Medical Microbiology 14(2-3):167-177.

Norton PM, Le Page RW, Wells JM. 1995. Progress in the development of *Lactococcus lactis* as a recombinant mucosal vaccine delivery system. Folia Microbiologica 40(3):225-230.

Norton PM, Wells JM, Brown HWG, Macpherson AM, Le Page RWF. 1997. Protection against tetanus toxin in mice nasally immunized with recombinant *Lactococcus lactis* expressing tetanus toxin fragment C. Vaccine 15(6-7):616-619.

Nouaille S, Ribeiro LA, Miyoshi A, Pontes D, Le Loir Y, Oliveira SC, Langella P, Azevedo V. 2003. Heterologous protein production and delivery systems for *Lactococcus lactis*. Genetics and Molecular Research 2(1):102-111.

Pacheco LGC, Zucconi E, Mati VLT, Garcia RM, Miyoshi A, Oliveira SC, De Melo AL, Azevedo V. 2005. Oral administration of a live Aro attenuated *Salmonella* vaccine strain expressing 14-kDa *Schistosoma mansoni* fatty acid-binding protein induced partial protection against experimental schistosomiasis. Acta Tropica 95(2):132-142.

Parente E, Hill C. 1992. A comparison of factors affecting the production of two bacteriocins from lactic acid bacteria. Journal of Applied Bacteriology 73(4):290-298.

Parisien A, Allain B, Zhang J, Mandeville R and Lan CQ. 2007. Novel Alternatives to Antibiotics: Bacteriophages, Bacterial Cell Wall Hydrolases, and Antimicrobial Peptides. Journal of Applied Microbiology In press.

Piard JC. 1998. Lactic acid bacteria in live vaccine development: designing tools to target antigens to a defined cell compartment: Elsevier Science. 101-102 p.

Pirt SJ. 1965. The maintenance energy of bacteria in growing cultures. Proceeding of the Royal Society B 163:224-231.

Pirt SJ. 1982. Maintenance energy: A general model for energy-limited and energy-sufficient growth. Archives of Microbiology 133(4):300-302.

Ramasamy R, Yasawardena S, Zomer A, Venema G, Kok J, Leenhouts K. 2006. Immunogenicity of a malaria parasite antigen displayed by *Lactococcus lactis* in oral immunisations. Vaccine 24(18):3900-3908.

Rezaiki 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.

Robinson K, Chamberlain LM, Schofield KM, Wells JM, Le Page RWF. 1997. Oral vaccination of mice against tetanus with recombinant *Lactococcus lactis*. Nature Biotechnology 15(7):653-657.

Rong R, Slupska MM, Chiang JH, Miller JH. 2004. Engineering large fragment insertions into the chromosome of *Escherichia coli*. Gene 336(1):73-80.

Roussel Y, Harris A, Lee MH, Wilks M. 2007. Novel methods of quantitative real-time PCR data analysis in a murine *Helicobacter pylori* vaccine model. Vaccine 25(15):2919-2929.

Ruas-Madiedo P, Hugenholtz J, Zoon P. 2002. An overview of the functionality of exopolysaccharides produced by lactic acid bacteria. International Dairy Journal 12(2-3):163-171.

Saito Y, Ishii Y, Sasaki H, Hayashi M, Fujimura T, Imai Y, Nakamura S, Suzuki S, Notani J, Asada T and others. 1994. Production and characterization of a novel tissue-type plasminogen activator derivative in *Escherichia coli*. Biotechnology Progress 10(5):472-479.

Salama MS, Sandine WE, Giovannoni SJ. 1995. A milk-based method for detecting antimicrobial substances produced by lactic acid bacteria. Journal of dairy science 78(6):1219-1223.

Salminen S, Von Wright A, Morelli L, Marteau P, Brassart D, De Vos WM, Fonden R, Saxelin M, Collins K, Mogensen G and others. 1998. Demonstration of safety of probiotics - A review. International Journal of Food Microbiology 44(1-2):93-106.

Seegers JF. 2002. Lactobacilli as live vaccine delivery vectors: progress and prospects. Trends Biotechnol 20(12):508-15.

Shah DB, Coulman GA. 1978. Kinetics of nitrification and denitrification reactions. Biotechnology and Bioengineering 20(1):43-72.

Shuler M, Kargi F. 2002. Bioprocess Engineering, Basic Concepts. Amundson N, editor: PHPTR. 553 p.

Sijpesteijn AK. 1970. Induction of cytochrome formation and stimulation of oxidative dissimilation by hemin in *Streptococcus lactis* and *Leuconostoc mesenteroides*. Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology 36(3):335-348.

Simkins S, Alexander M. 1984. Models for mineralization kinetics with the variables of substrate concentration and population density. Applied and Environmental Microbiology 47(6):1299-1306.

Slos P, Dutot P, Reymund J, Kleinpeter P, Prozzi D, Kieny MP, Delcour J, Mercenier A, Hols P. 1998. Production of cholera toxin B subunit in Lactobacillus. FEMS Microbiology Letters 169(1):29-36.

Sriraman K, Jayaraman G. 2006. Enhancement of recombinant streptokinase production in *Lactococcus lactis* by suppression of acid tolerance response. Applied Microbiology and Biotechnology 72(6):1202-1209.

Steidler L, Robinson K, Chamberlain L, Schofield KM, Remaut E, Le Page RWF, Wells JM. 1998. Mucosal delivery of murine interleukin-2 (IL-2) and IL-6 by recombinant of *Lactococcus lactis* coexpressing antigen and cytokine. Infection and Immunity 66(7):3183-3189.

Stouthamer AH. 1977. Energetic aspects of the growth of microorganisms. Symp. Soc. Gen. Microbiol. 27:285-315.

Stouthamer AH. 1979. The search for correlation between theoretical and experimental growth rates. Int. Rev. Biochem. 21:1-47.

Sutherland IW. 1998. Novel and established applications of microbial polysaccharides. Trends in Biotechnology 16(1):41-46.

Tango MSA, Ghaly AE. 1999. Kinetic modeling of lactic acid production from batch submerged fermentation of cheese whey. Transactions of the American Society of Agricultural Engineers 42(6):1791-1800.

Tempest DW, Neijssel OM. 1984. The Status of YATP and Maintenance Energy as Biologically Interpretable Phenomena. Annual Review of Microbiology 38(1):459-513.

Terzaghi BE, Sandine WE. 1975. Improved medium for lactic streptococci and their bacteriophages. Journal of Applied Microbiology 29(6):807-813.

Teuber M. 2001. Veterinary use and antibiotic resistance. Current Opinion in Microbiology 4(5):493-499.

Theisen M, Soe S, Brunstedt K, Follmann F, Bredmose L, Israelsen H, Madsen SM, Druilhe P. 2004. A Plasmodium falciparum GLURP-MSP3 chimeric protein; expression in *Lactococcus lactis*, immunogenicity and induction of biologically active antibodies. Vaccine 22(9-10):1188-1198.

Tsai SP, Lee YH. 1990. A model for energy-sufficient culture growth. Biotechnology and Bioengineering 35(2):138-145.

Van Niel EWJ, Claassen PAM, Stams AJM. 2003. Substrate and product inhibition of hydrogen production by the extreme thermophile, *Caldicellulosiruptor saccharolyticus*. Biotechnology and Bioengineering 81(3):255-262.

Van Niel EWJ, Hofvendahl K, Hahn-Hagerdal B. 2002. Formation and conversion of oxygen metabolites by *Lactococcus lactis subsp. lactis* ATCC 19435 under different growth conditions. Applied and Environmental Microbiology 68(9):4350-4356.

Velasco S, Arskold E, Paese M, Grage H, Irastorza A, Radstrom P, van Niel EWJ. 2006. Environmental factors influencing growth of and exopolysaccharide formation by *Pediococcus parvulus* 2.6. International Journal of Food Microbiology 111(3):252-258.

Walsh G. 1998. Biopharmaceuticals : biochemistry and biotechnology. Chichester ; New York: John Wiley. 431 p.

Ward PP, Lo JY, Duke M, May GS, Headon DR, Conneely OM. 1992. Production of biologically active recombinant human lactoferrin in *Aspergillus oryzae*. Nature Biotechnology 10(7):784-789.

Wells JM, Wilson PW, Norton PM, Gasson MJ, Le Page RWF. 1993. *Lactococcus lactis*: High-level expression of tetanus toxin fragment C and protection against lethal challenge. Molecular Microbiology 8(6):1155-1162.

Welman AD, Maddox IS. 2003. Exopolysaccharides from lactic acid bacteria: Perspectives and challenges. Trends in Biotechnology 21(6):269-274.

Witte W. 2000. Selective pressure by antibiotic use in livestock. International Journal of Antimicrobial Agents 16.

Wolf F, Traupe B, Takors A, Sauermann G, Hoppe U. 1997. A novel lantibiotic from *Lactococcus lactis* for the treatment of acne vulgaris: Elsevier Science. 657 p.

Wood B, Warner PJ. 2003. Genetics of lactic acid bacteria. New York : : Kluwer Academic / Plenum Publishers. 394 p.

Xin KQ, Hoshino Y, Toda Y, Igimi S, Kojima Y, Jounai N, Ohba K, Kushiro A, Kiwaki M, Hamajima K and others. 2003. Immunogenicity and protective efficacy of orally administered recombinant *Lactococcus lactis* expressing surface-bound HIV Env. Blood 102(1):223-228.

Yang R, Ray B. 1994. Factors influencing production of bacteriocins by lactic acid bacteria. Food Microbiology 11(4):281-292.

Yazawa R, Hirono I, Aoki T. 2006. Transgenic zebrafish expressing chicken lysozyme show resistance against bacterial diseases. Transgenic Research 15(3):385-391.

Zhang Y, Dai L, Sun X. 2003. Kinetic aspect of hepatitis B surface antigen production in recombinant *Saccharomyces cerevisiae* fermentation. Process Biochemistry 38(11):1593-1598.

Zhang ZH, Jiang PH, Li NJ, Shi M, Huang W. 2005. Oral vaccination of mice against rodent malaria with recombinant *Lactococcus lactis* expressing MSP-119. World Journal of Gastroenterology 11(44):6975-6980.

Zhou XX, Li WF, Ma GX, Pan YJ. 2006. The nisin-controlled gene expression system: Construction, application and improvements. Biotechnology Advances 24(3):285-295.

9 Appendix A – Raw Data for Substrate Concentration Study

Table 9.1 – Results from replicate batches run at 60 g/L glucose

Trial 1				Trial 2				Trial 3				Trial 4			
t	X	S	P	t	X	S	P	t	X	S	P	t	X	S	P
0.00	0.021	59.0	0.095	0.0	0.067	60	0.229	0.0	0.057	60.0	0.222	0.0	0.052	60	0.200
0.50	0.035	59.2	0.119	0.75	0.116	60	0.322	0.50	0.069	60.0	0.319	0.5	0.077	60	0.250
1.00	0.063	58.8	0.152	1.25	0.206	60	0.420	1.00	0.115	60.0	0.408	1.0	0.097	60	0.319
1.75	0.149	58	0.266	1.75	0.374	59	0.568	1.50	0.167	60.0	0.477	1.5	0.135	60	0.406
2.50	0.345	56.8	0.501	2.75	1.285	57	1.26	2.50	0.566	59.7	0.899	2.0	0.210	60	0.526
3.00	0.683	56.4	0.754	3.75	3.171	53.8	2.54	3.00	0.886	58.5	1.26	2.5	0.341	58.5	0.703
3.50	1.263	56.0	1.20	4.25	4.501	48.4	4.10	3.50	1.64	57.3	1.78	3.0	0.729	58.5	1.03
4.25	2.219	53.2	1.86	4.75	6.181	46.0	5.66	4.00	2.538	55.1	2.28	3.5	1.26	56.0	1.42
4.75	3.01	50.8	2.71	5.25	7.261	38.8	7.16	4.50	3.728	52.0	3.44	4.0	2.14	55.8	2.02
5.25	4.36	48	4.04	5.75	8.681	34.8	9.96	5.00	4.788	49.6	4.62	4.5	2.87	54.6	2.99
5.75	5.56	46.0	5.46	6.75	11.45	24.8	15.2	5.50	6.288	45.8	5.94	5.5	5.26	49.6	5.38
6.25	6.66	41.8	7.22	7.75	12.23	16.3	19.1	6.00	7.428	40.0	6.82	6.0	6.80	42.4	6.96
6.75	7.78	37.4	9.12	8.25	12.64	11.8	21.2	6.50	8.948	35.6	8.44	6.5	8.37	34.5	8.43
7.25	8.60	32.4	11.6	9.25	12.76	4.28	23.8	7.00	9.408	28.8	9.76	7.0	9.66	29.7	11.4
7.75	10.0	27.2	14.1	-	-	-	-	7.50	11.06	26.0	12.2	7.5	10.8	26.7	13.1
8.25	10.3	22.8	16.0	-	-	-	-	8.00	12.11	23.5	13.7	8.5	12.1	19.3	16.4
8.75	10.8	19.6	18.4	-	-	-	-	8.50	11.72	18.6	14.3	9.5	12.2	10.3	19.2
9.25	11.2	16.3	20.4	-	-	-	-	9.00	12.26	15.5	16.3	10.25	12.6	4.8	21.5
9.75	11.4	13.0	21.7	-	-	-	-	9.50	12.58	11.9	17.7	10.5	12.9	2.7	22.0
10.25	11.6	9.67	23.8	-	-	-	-	10.5	13.06	4.41	20.0	-	-	-	-

Table 9.2 - Results from batch replicates run at 80 g/L glucose

Trial 1				Trial 2				Trial 3			
t	X	S	P	t	X	S	P	t	X	S	P
0.00	0.04	82.0	0.253	0.00	0.005	80.0	0.084	0.00	0.046	81.2	0.256
0.5	0.065	82.0	0.278	0.5	0.006	80.0	0.087	0.75	0.085	80.4	0.309
1.00	0.087	81.0	0.315	1.00	0.007	79.2	0.090	1.50	0.194	80.0	0.455
1.5	0.134	82.0	0.381	1.75	0.014	78.4	0.102	2.25	0.417	80	0.701
2.25	0.29	80	0.551	2.50	0.027	78.0	0.119	3.00	1.278	79.6	1.39
2.75	0.596	80.0	0.820	3.75	0.101	77.4	0.237	3.75	2.698	77.2	2.16
3.25	1.124	81.2	1.26	4.25	0.164	77.1	0.32	4.50	4.064	74.4	3.88
3.75	1.936	79.6	1.70	4.75	0.278	76.8	0.456	5.25	6.474	68.8	6.60
4.25	2.846	79.2	2.33	5.25	0.409	76.4	0.655	6.00	8.874	60.8	9.64
4.75	3.90	76.8	3.58	5.75	0.81	74.8	0.980	6.50	9.87	54.8	11.9
5.25	5.30	72.0	5.00	6.50	1.56	72.4	1.56	7.25	11.4	48.8	16.4
5.75	6.98	65.2	7.36	7.00	2.38	70.5	2.17	7.75	11.6	42.4	18.3
6.25	8.13	60.8	10.1	7.50	3.21	66.5	3.39	8.50	11.7	35.6	20.5
6.75	9.48	53.2	12.5	8.00	4.42	63	4.52	9.50	12.47	30.52	23.92
7.25	10.6	50.0	15.8	9.50	7.74	53.0	11.0	-	-	-	-
8.00	11.4	41.6	19.4	10.00	8.48	48.0	12.8	-	-	-	-
8.75	11.9	37.0	21.9	10.50	8.84	42.0	15.6	-	-	-	-
9.75	12.6	30.0	26.0	11.00	9.20	38.4	17.7	-	-	-	-
10.75	12.8	25.6	27.8	11.50	9.80	36.2	20.2	-	-	-	-
11.75	12.9	21.2	28.8	12.50	10.08	29.4	22.1	-	-	-	-
-	-	-	-	13.50	10.24	24.3	24.1	-	-	-	-

Table 9.3 – Batch run at 60 g/L glucose with 1.5 times the concentration of yeast extract, peptone, and tryptone relative to the base medium composition utilized

t	X	S	P
0.00	0.021	60.0	0.122
0.75	0.041	60	0.158
1.50	0.118	58.8	0.350
2.25	0.291	58.4	0.685
3.25	1.54	55.2	1.63
5.25	8.29	44.6	7.22
6.00	11.4	33.4	11.3
7.50	18.8	10.7	22.4
8.75	19.3	0.0	25.5

Table 9.4 - Results from a batch run at 90 g/L glucose

t	X	S	P
0.00	0.007	90.0	0.095
0.75	0.018	89.6	0.099
1.25	0.035	90	0.125
2.00	0.071	88.0	0.169
2.50	0.122	87.6	0.254
3.50	0.326	86.4	0.549
4.25	0.812	84.8	1.07
4.75	1.536	84.0	1.42
5.25	2.30	83.5	2.20
5.75	3.00	82.8	2.99
6.25	4.18	79.6	4.40
6.75	5.18	78.0	5.85
7.25	6.48	74.6	7.86
7.75	7.38	70.2	10.2
8.25	8.26	64.2	12.6
9.00	9.48	57.2	16.2
10.50	10.2	45.6	20.6
11.50	10.2	40.4	22.7
12.50	10.3	36.7	26.2

10 Appendix B – Raw Data from Medium Optimization Study

Table 10.1 – Experimental data for study of the influence of glycerophosphate concentration on medium performance against M17. All cultivations were supplemented with 10 g/L glucose and 0.5 g/L hemin.

t	OD				
(h)	30 g/L GP	40 g/L GP	50 g/L GP	60 g/L GP	Control
0	0.112	0.112	0.111	0.113	0.11
0.75	0.111	0.11	0.109	0.112	0.114
1.25	0.126	0.127	0.117	0.111	0.132
1.75	0.168	0.156	0.147	0.14	0.171
2.75	0.349	0.305	0.267	0.239	0.393
3.25	0.683	0.603	0.508	0.437	0.783
3.75	1.56	1.347	1.184	0.982	1.838
4.25	2.785	2.267	1.974	1.727	3.073
4.75	4.035	3.657	2.774	2.377	3.743
5.25	4.435	4.557	3.634	3.177	4.103
5.75	4.695	4.777	4.754	4.057	3.863
6.25	4.275	5.077	5.014	4.937	-
6.75	-	4.857	4.894	5.217	-
7.25	-	-	4.734	5.077	-
7.75	-	-	-	-	-

Table 10.2 - Experimental data for study of the influence of glucose concentration on medium performance against M17. All cultivations were supplemented with 40 g/L GP and 0.5 g/L hemin

t	OD			
(h)	12.5 g/L	10 g/L	7.5 g/L	5 g/L
0.0	0.123	0.118	0.115	0.118
1.0	0.147	0.144	0.141	0.162
1.5	0.196	0.197	0.206	0.226
2.0	0.325	0.306	0.293	0.353
2.5	0.611	0.613	0.603	0.8
3.0	1.187	1.073	1.061	1.535
3.5	2.265	2.283	2.283	2.740
4.0	3.675	3.513	3.853	4.060
4.5	4.935	4.553	4.633	4.52
5.0	4.935	4.833	4.673	4.28
5.5	5.095	5.233	4.453	-
6.0	5.395	5.313	-	-
6.5	5.055	5.093	-	-
7.0	-	-	-	-
7.5	-	-	-	-