Kinetic Modelling of Lactic Acid Production from Whey

By

Duygu ALTIOK

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We approve the thesis of **Duygu ALTIOK**

Date of Signature

....

30.07.2004

Assist. Prof. Figen TOKATLI
Supervisor
Department of Food Engineering

....

30.07.2004

Prof. Dr. Şebnem HARSA
Co-Supervisor
Department of Food Engineering

....

30.07.2004

Assist. Prof. Canan TARI
Department of Food Engineering

....

30.07.2004

Assist. Prof. Sevcan ÜNLÜTÜRK
Department of Food Engineering

....

30.07.2004

Assist. Prof. Oğuz BAYRAKTAR
Department of Chemical Engineering

30.07.2004

Prof. Dr. Şebnem HARSA
Head of Food Engineering Department

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ABSTRACT

Lactic acid is a natural organic acid, which is used in pharmaceuticals, chemical, textile and food industries. Since only L(+) lactic acid is found in normal human metabolism, the microbial production of L(+) lactic acid has great interest in recent years. Use of whey lactose to produce lactic acid by fermentation process is favourable due to the low cost of whey and its high organic matter content. Whey is suitable medium for some fermentations. However, its high lactose content makes it a potential environmental pollutant. The disposal problem of this pollutant could be overcome by utilization of whey lactose in the lactic acid production.

The aim of this study was to develop a kinetic model for lactic acid production from whey by *Lactobacillus casei*, which is a homofermentative lactic acid bacteria and capable of producing L(+) lactic acid. Within this context, several batch fermentation experiments in fermenter were performed at 37 $^{\circ}$C and pH 5.5. Seed culture that was produced in shake flask fermentations was used as the inoculum for the fermenter.

Before the fermentation experiments, some of the proteins in whey were denatured by heat treatment and separated by centrifugation. This treatment decreased the protein amount from 11.15 % to 5.2 % in whey powder. The lactic acid production was associated with the biomass growth up to a certain time, but then a non-growth associated lactic acid production was observed in most of the fermentations except for the 9.0 g l^{-1} initial substrate fermentation run, where all the substrate was utilized when the stationary phase was attained. The maximum theoretical productivity was obtained as 2.4 g lactic acid l^{-1} h^{-1} in the fermentation with S_0 equals to 35.5 g l^{-1}. The kinetic parameters were obtained from different fermentation runs. μ_{max} and K_S were found as 0.265 h^{-1} and 0.72 g l^{-1}, respectively. The average product yield coefficient, Y_{PS}, was determined as 0.682 g lactic acid (g lactose)$^{-1}$.

The modified form of logistic equation with product inhibition term for biomass growth, Luedeking and Piret equation for product formation and substrate utilization considering the consumption of substrate for product formation and maintenance, described most of the fermentation experiments in this study with high accuracy (SSE range was 0.0804-0.1531). The toxic powers in these inhibition terms, h and f, made the model applicable for the fermentation experiments with low and high initial substrate concentrations. In case of high initial substrate concentration fermentation

(S_0= 95.7 g l^{-1}), the same model explains only the exponential phase of biomass and its product formation eventhough the substrate consumption is predicted very well.

ÖZ

Doğal bir organik asit olan laktik asit farmasütik, kimya, tekstil ve gıda endüstrilerinde önemli bir yere sahiptir. İnsan metabolizmasında sadece L(+) laktik asit izomeri bulunduğundan, bu izomerin mikrobiyal yoldan eldesi son zamanlarda çok ilgi çekmektedir. Laktik asitin fermentasyonla eldesinde kullanılan peynir suyundaki laktoz, peynir suyunun ucuz olması ve organic madde içeriğinin yüksek olmasından dolayı tercih edilir. Peynir suyu bazı fermentasyonlar için uygun bir substrattır ancak yüksek laktoz içerdiğinden dolayı potansiyel bir çevre kirleticisidir. Peynir suyundaki laktozun fermentasyon sırasında tüketimi çok değerli bir ürün olan laktik asitin eldesinin yanısıra bu kirletici maddenin yok edilmesini de sağlar.

Bu çalışmanın amacı, homofermentatif laktik asit bakterisi olan ve L(+) izomerini üretebilen *Lactobacillus casei* ile peynir suyundan laktik asit eldesinin değişik laktoz konsantrasyonlarında kinetik modellemesi ve geliştirilmesidir. Bu çerçevede, 37 ^{o}C ve pH 5.5' de kesikli fermentasyonlar yapılmıştır. Çalkalamalı inkübatörde elde edilen kültür, fermentör için inokulum olarak kullanılmıştır.

Fermentasyon çalışmalarından önce peynir suyundaki proteinlerin bir kısmı ısıl işlemle denetüre edilmiş ve santrifüj ile ayrılmıştır. Bu işlemler peynir suyu tozundaki protein miktarını 11.15 %'den 5.2 %'ye düşürmüştür. Laktik asit belli bir saate kadar biomas artşına bağlı olarak üretilmiş daha sonra büyümeden bağımsız olarak üretilmiştir. Ancak 9.0 g l^{-1} başlangıç substrat konsantrasyonuyla yapılan fermentasyonda durağan faza ulaşmadan önce substratın tamamı tüketilmiştir. Maksimum teorik üretkenlik 35.5 g l^{-1} başlangıç substratlı fermentasyonda 2.4 g laktik asit l^{-1} h^{-1} olarak elde edilmiştir. Kinetik parametreler değişik fermentasyon çalışmalarından elde edilmiştir. μ_{max} ve K_S sırasıyla 0.265 h^{-1} ve 0.72 g l^{-1} olarak bulunmuştur. Ürün verim katsayısı ortalama değeri 0.682 g laktik asit (g laktoz)$^{-1}$ olarak saptanmıştır. Mikrobiyal büyüme için lojistik denklemin ürün inhibisyon terimi ile modifiye edilmiş formu, ürün oluşumu için Luedeking ve Piret'in belirttiği denklem ve substrat tüketimini ürün oluşumu ve temel metabolik faaliyetleri göz önünde bulundurarak açıklayan denklem bu çalışmadaki fermentasyonların çoğunu büyük doğrulukla açıklamıştır. İnhibisyon terimlerindeki toksik kuvvetler, inhibisyon etkisini başlangıç substrat konsantrasyonu düşük olduğunda önemsiz, yüksek olduğu durumlarda ise önemli kılarak modelin değişik fermentasyonlara uygulanabilirliğini arttırmıştır. Yüksek başlangıç substrat konsantrasyonlu fermentasyonda (S_0= 95.7 g l^{-1}),

model substrat tüketimini çok iyi açıklayabilmesine rağmen mikrobiyal büyümeyi ve ürün oluşumunu sadece logaritmik faz için açıklayabilmiştir.

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NOMENCLATURE

f   toxic power for biomass inhibition (dimensionless)

h   toxic power for product inhibition (dimensionless)

K_i substrate inhibition parameter (g l^{-1})

K_P product inhibition parameter (g l^{-1})

K_S Monod constant (g l^{-1})

m_S maintenance coefficient (g lactose (g biomass)$^{-1}$ h^{-1})

P   lactic acid concentration (g l^{-1})

P_f final lactic acid concentration (g l^{-1})

P_{max} maximum lactic acid concentration above which bacteria do not grow (g l^{-1})

P_0 initial lactic acid concentration (g l^{-1})

q_P specific production rate (g lactic acid (g biomass)$^{-1}$ h^{-1})

S   substrate concentration (g l^{-1})

S_f final substrate concentration (g l^{-1})

S_0 initial substrate concentration (g l^{-1})

X   biomass concentration (g l^{-1})

X_{max} maximum biomass concentration at stationary phase (g l^{-1})

X_0 initial biomass concentration (g l^{-1})

Y_{PS} product yield coefficient (g lactic acid (g lactose)$^{-1}$)

Y_{XS} biomass yield coefficient (g biomass (g lactose)$^{-1}$)

t   time (h)

α   growth-associated product form. coefficient (g lactic acid (g biomass)$^{-1}$)

β   nongrowth-associated product form. coefficient (g lactic acid (g biomass)$^{-1}$ h^{-1})

μ   specific growth rate (h^{-1})

$μ_{max}$ maximum specific growth rate (h^{-1})

CHAPTER 1

INTRODUCTION

Lactic acid is a natural organic acid, which is an industrially important product. It is used in pharmaceuticals, cosmetics, and chemical, textile and food industries. It exists in two optically active forms, D(-) and L(+) lactic acid. Lactic acid can be produced industrially by chemical synthesis and microbial fermentation process. It is synthetically produced by the hydrolyses of lactonitrile. Chemical synthesis yields only the racemic (DL) lactic acid. However, L(+) lactic acid is the preferred isomer. Therefore, there is a continued interest in the microbial production of L(+) lactic acid in recent years.

Lactose in some organic compounds can be converted to lactic acid by some lactic acid bacteria. Whey is one of these organic compounds used in lactic acid production, which is a clean, abundant food-grade material and a potential environmental pollutant due to its high lactose content. One of the promising ways to use lactose in whey is to use it as a low cost carbon source for the production of organic acids by fermentation. Disposal of whey and doing it profitably continues to be a problem which confronts the entire cheese industry. Pollution of the environment and loss of valuable nutrients are considerations, which militate against traditional disposal methods. Use of a fermentation process to upgrade the nutritional quality of whey or to produce a product with improved palatability was taught as an alternative procedure. In short, the utilization of lactose in whey by fermentation overcomes the disposal problem of this pollutant while it produces value-added end products such as lactic acid.

Lactobacillus casei is found in many food products as well as in the human body and other natural environments. So, it is generally regarded as safe (GRAS) organism. It is capable of fermenting whey lactose to L(+) lactic acid only, which is the lactic acid isomer found in normal human metabolism and has wide applications in industry. Therefore, highly pure preparations of L-lactic acid are in demand as a raw material for the production of biodegradable lactide polymers used in the biomaterial manufacturing. *Lactobacillus casei* produces about 97.5 % of the lactic acid in the L(+) form (Vaccari et al. 1993). Most of the other lactic acid bacteria produce DL and D lactic acids.

Lactic acid can be obtained from whey by batch fermentation. In batch fermentation system, the microorganism is inoculated after the sterilization of reactor and fermentation media. During fermentation, the culture goes through different growth phases. Different products are produced during these phases. To understand the behaviour of the system, modelling is required. Models are used to define the biological, chemical and physical basis of the process. A kinetic model is a set of relationships between biomass growth, substrate utilization and product formation. Kinetic models predict how fast the microorganisms can grow and use substrates or make products. In practice, kinetic data are collected with respect to time in small-scale reactors and then used to scale-up the process along the mass transfer data.

The aim of this study is to develop the kinetic model to explain the lactic acid production from whey by *Lactobacillus casei*. With in this context, batch fermentation experiments with seven different initial substrate concentrations in a range of [9.0 g lactose l^{-1} – 95.7 g lactose l^{-1}] were performed to determine kinetic parameters such as maximum growth rate (μ_{max}), Monod constant (K_s), yield coefficients (Y_{XS} and Y_{PS}).

CHAPTER 2

LACTIC ACID

2.1. History and Applications of Lactic Acid

Lactic acid, 2-hydroxypropionic acid ($CH_3CHOHCOOH$), was isolated and identified by Scheele in 1780. Charles E. Avery set up the first commercial lactic acid fermentation plant in Littleton, MA, in 1881. Worldwide consumption of lactic acid is about 45,000 ton $(year)^{-1}$, of which roughly 40 % belongs to the United States. Lactic acid is used in many food and nonfood applications. In 1989, 7,600 ton were used for the manufacture of emulsifiers, 4,400 ton in food additives, 1,700 ton for pharmaceutical and cosmetics applications, and the balance for industrial and miscellaneous uses (Vick Roy 1985). It is a natural organic acid and industrially important product with a large market due to its attractive properties. For example, the acid and salts are preferred to other acids in the food industry because they do not dominate other flavors and also act as preservatives where it is used as an acidulant and a flavour enhancer in many kinds of foods or beverages, such as beer, jellies, cheese, and dried egg whites. The major use of synthetic and heat-stable lactic acid is in the manufacture of sodium and calcium stearoyl lactylate and other lactylated emulsifiers. Stearoyl lactylate is used in the baking industry as a dough conditioner. Furthermore, the possibility of directly converting lactic acid to acrylic acid has also turned lactic acid into an important raw material for the chemical industry (Hui and Khachatourians 1995). Recently, new applications for lactic acid such as biodegredable plastics (polylactide polymers, polyhydroxybutryate) have accelerated research on its production as a bulk raw material. Lactic acid is also used in pharmaceuticals, cosmetics, and textile industries (Roukas and Kotzekidou 1998).

Lactic acid from cheese whey is produced commercially in Slovakia, Italy, and the United States, whereas synthetic production from acetaldehyde or lactonitrile is cheaper, the low cost of whey makes a large-scale production facility competitive. Lactic acid bacteria such as *Lactococcus lactis*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus acidophilus*, *Lactobacillus helveticus*, *Lactobacillus casei*,

and mixed cultures of these organisms have been successfully used to convert lactose to lactic acid (Henning 1998).

2.2. Properties of Lactic Acid

Lactic acid is the simplest 2-hydroxyacid having a chiral centre, and exists as two enantiomers, L(+) lactic acid and D(-) lactic acid. L(+) lactic acid is the enantiomer involved in normal human metabolism (Hui and Khachatourians 1995). Figure 2.1 shows these isomers of lactic acid. Some of the physical properties of lactic acid are given in Table 2.1.

Table 2.1. Physical properties of lactic acid (Vick Roy 1985)

Molecular weight	90.08
Melting Point D(-) or L(+)	52.8-54 ^{o}C
Boiling Point DL	82 ^{o}C at 0.5 mmHg
	122 ^{o}C at 14 mmHg
Dissociation Constant (K_a at 25 ^{o}C)	1.37 x 10^{-4}
Heat of Combustion (ΔH_c)	1361 kJ mol^{-1}
Specific Heat (C_p at 20 ^{o}C)	190 J mol^{-1} ^{o}C^{-1}

```
        CO₂H                           CO₂H
         |                              |
HO ——— C ——— H              H ——— C ——— OH
         |                              |
        CH₃                            CH₃

   L(+) lactic acid               D(-) lactic acid
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Figure 2.1. Isomers of lactic acid. (Vick Roy 1985)

Optically active, high-purity lactic acid can form colourless monoclinic crystals. Lactic acid may form a cyclic dimer (lactide) or form linear polymers with the general formula $H[OCH(CH_3)CO]_nOH$. It may participate both as an organic acid and an organic alcohol in several types of chemical reactions. Lactic acid is soluble in all proportions with water and exhibits a low volatility. In solutions with roughly 20 % or

more lactic acid, self-esterification occurs because of the hydroxyl and carboxyl functional groups (Vick Roy 1985).

2.3. Microbial Production

2.3.1. Microorganisms and Raw Materials

Microorganisms:

Lactobacilli are large group of rod-shaped bacteria, which can vary form long and slender, sometimes curved, rods to short, often coryneform, coccobacilli. Lactic acid bacteria and some fungi of the species *Rhizopus* produce lactic acid. Lactic acid bacteria are gram-positive, non-sporing and usually nonmotile and are categorized as facultative anaerobes, thus making the strict exclusion of air is unnecessary. Lactic acid bacteria have limited biosynthetic capabilities, so that they require many vitamins (especially B vitamins) amino acids, purines, and pyrimidines (Prescott 1996). They are classified into 3 groups based on their metabolism of sugars. Group 1; species are obligate homofermenters of hexoses to lactate and do not ferment pentoses. Group2; species are facultative heterofermenters of hexoses which means that they ferment hexoses by glycolysis to lactate or, under glucose limitation, to lactate, acetate, ethanol, and formate. Also, they ferment pentoses to lactate and acetate by the phosphoketolase pathway. Group 3 species are obligate heterofermenters of sugars.

Lactobacillus casei, is member of Group 2. These organisms ferment sugars homofermentatively to L(+) lactic acid and do not produce NH_3 from arginine. The homofermentative lactic acid bacteria produce only lactic acid. On the other hand, the heterofermentative lactic acid bacteria produce not only lactic acid, but also acetic acid, carbon dioxide, ethanol and glycerol so it is undesirable because of by-product formation. (Vick Roy 1985).

This species, like *L. acidophilus*, is a gram-positive, catalase-negative, rod-shaped bacterium, which is facultative with regard to oxygen requirements. It is a normal inhabitant of the small intestine and is resistant to bile. Although its optimum growth temperature is nearly 37 ^{o}C, unlike *L. acidophilus*, it will grow at 15 ^{o}C (Cogan and Accolas 1996). Lactobacilli are the most acid tolerant of the lactic acid bacteria, preferring to initiate growth at acidic pH (5.5-6.2) (Frank and Hassan 1998).

Lactobacillus casei is found in many food products, as well as in the human body and other natural environments. For this reason, it is a generally regarded as safe (GRAS) organism and it is also commercially used as probiotic. (Arellano and Martinez 2003).
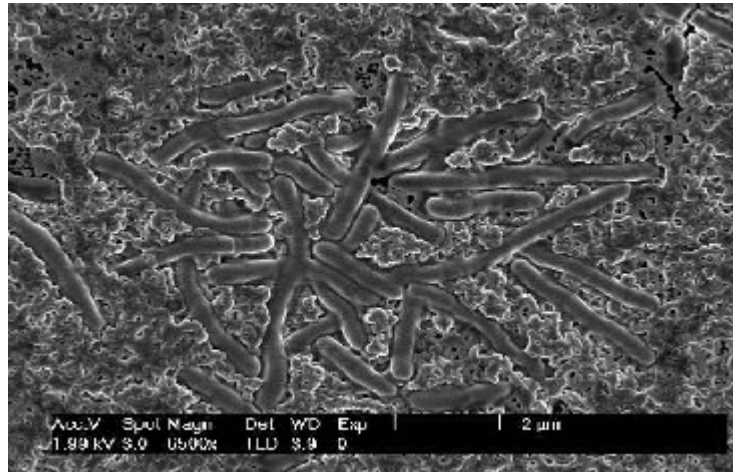
Figure 2.2. SEM picture of *L casei* NRRL B-441 (Polat 2002)

Raw Materials:

Many carbohydrate materials have been used, tested or proposed for the manufacture of lactic acid by fermentation. It is useful to compare these raw materials based on the following desirable qualities:

- low cost,
- low levels of contaminants,
- fast fermentation rate,
- high lactic acid yields,
- little or no by-product formation,
- ability to be fermented with little or no pretreatment,
- year-round availability

Sucrose from cane and beet sugar, whey containing lactose, and maltose and dextrose from hydrolyzed starch are presently used commercially. Although it is expensive, refined sucrose is the most commonly used substrate, followed by dextrose. Molasses are side product of sugar refining that contains most of the material from the

sugar beet or sugar cane, which is not sugar, molasses is one of the cheapest substrate available. Malt extract is made from malted barley by soaking it in water (Bains 1993). Whey is a by-product of the cheese-making process.

Among many carbohydrate materials used for the production of lactic acid, cheese whey lactose deserves special consideration. Cheese whey is a clean, wholesome, abundant food-grade material and a potential environmental pollutant. It is the product separated from milk during cheesemaking and consists of water, lactose, proteins, vitamins, and mineral salts (Roukas and Kotzekidou 1998).

The gross composition of dried and liquid cheese whey is given in Table 2.2.

Table 2.2. Gross composition of liquid and dried whey (Henning 1998)

Component	Liquid whey	Dried whey
Total solids, %	6.35	96.5
Protein, %	0.8	13.1
Lactose, %	4.85	75.0
Fat, %	0.5	0.8
Minerals and Vitamins, %	0.6	1.2
Lactic acid, %	0.05	0.2
Ash, %	0.5	7.3

About 57 billion pounds of liquid whey are produced each year in the U.S. There is an interest in the economic utilization of the large quantities of cheese whey produced by the dairy industry, because of the environmental problem caused by its high organic matter content, essentially due to its lactose content. Disposal of whey and doing it profitably continues to be a problem which confronts the entire cheese industry (Amrane 2001).

There is continued interest in utilizing lactose from cheese whey for the production of value-added end products. Several researchers have utilized anaerobes or facultative anaerobes to ferment lactose to single cell protein, ethanol, biogas, lactic acid and acetate (Tango and Ghaly 1999).

One of the promising ways to use lactose in whey is to use it as a low cost carbon source for the production of organic acids by fermentation. This is not only because of the fact that organic acids are valuable raw materials for the production of

high value end products, but also because of the fact that organic acids such as lactic acid are easily metabolized compared to lactose in many fermentation processes (Fu and Mathews 1999).

Whey is suitable as a medium for only some fermentations. This is true because of its peculiar composition. The composition of whey is variable because cheese making procedures and milk composition are not constant. Data on composition of whey indicates that lactose is the only fermentable carbohydrate in whey. This means that the use of whey is limited to those fermentations that employ microorganisms, which can utilize lactose (Marth 1973).

Nitrogenous sources such as malt sprouts, malt extract, corn-steep liquor, barley, yeast extract or undenatured milk must supplement most carbohydrate sources to give fast and heavy growth. Some growth promoting substances in these nitrogen sources are sensitive to heat. In commercial practice, minimal amounts of substances are used in order to simplify the recovery process.

Additional minerals are occasionally required when the carbohydrate and nitrogenous sources lack sufficient quantities. Calcium carbonate and calcium hydroxide are typically used to neutralize the acid that is formed (Vick Roy 1985).

Numerous methods are available to separate or fractionate whey proteins. An important process for separating proteins from whey is ultrafiltration. Other protein separation processes include heat-acid precipitation, coprecipitation, chemical precipitation, ion-exchange and gel filtration. Maximum protein recovery is obtained by denaturing whey proteins at pH in the range of 6-7 and temperatures greater than $90\ ^{\mathrm{o}}\mathrm{C}$ for 10-30 min, followed by precipitation at pH 4.5-5.5. Heating at neutral pH induces primary aggregation through S-S bridges, which must proceed isoelectric precipitation for maximum protein recovery. The theoretical maximum recovery of crude protein from whey is 55-65 % because the heat stable proteose-peptone fraction constitutes 35-45 % of whey nitrogen. Commercially feasible processes should recover at least 50 % of the crude protein. Recovery of heat-precipitated whey proteins is normally best accomplished by a centrifuge or decanter. For small operations, recovery of heat-precipitation proteins from very sweet whey by filtration might be feasible (Irvine and Hill 1985).

2.3.2. Fermentation and Recovery Processes

Fermentation Process:

Fermentation is the process where the microorganism metabolizes a substrate under aerobic or anaerobic conditions.

The ideal fermentation would serve to;

- upgrade the nutritional properties of substrate
- improve its flavour and appearance so that a palatable product would result
- utilize all substrate without producing wastes that need to be treated
- produce a product which could be sold for more money than the cost of raw materials (Marth 1973).

In batch fermentation system, the reactor is filled with a sterile nutrient substrate and inoculated with microorganism. The culture is allowed to grow until no more of the product is produced after which the reactor is 'harvested' and cleaned out for another run. In batch culture, the culture environment changes continually. The culture goes through lag phase, exponential phase, stationary phase, and death phase. Depending on what the product is, the 'useful' part of the growth cycle can be any one of these four stages, although it is usually the exponential or stationary phase (Bains 1993).

On the other hand, in continuous culture, fresh nutrient medium is continually supplied to a well-stirred culture and products and cells are simultaneously withdrawn. After a certain time, the system usually reaches a steady-state where cell, product, and substrate concentration remain constant.

In fed-batch culture, nutrients are continuously or semicontinuously fed, while effluent is removed discontinuously. Fed-batch culture is usually used to overcome substrate inhibition or catabolite repression (Shuler and Kargi 2002).

Recovery Process:

Lactic acid is sold in three major grades: technical, food, and pharmaceutical. The recovery of lactic acid or lactate salts from the fermentation broth covers the large part of the total manufacture cost. Synthetically made lactic acid may be purified with

less effort and thus in the past has been preferred for uses where heat stability was needed.

The first step in all recovery processes is to raise the fermentation liquor's temperature to 80-100 ^{o}C and increase the pH to 10 or 11. This procedure kills the organisms, coagulates the proteins, solubilizes the calcium lactate, and degrades some of the residual sugars. The liquid is the decanted or filtered. For some purposes, acidification of this liquor yields a useable product; however, for most applications further processing by one of the following methods is required. It should also be noted that use of cheap but impure raw materials must be weighed against higher purification costs. Filtration, carbon treatment and evaporation, calcium lactate crystallization, liquid-liquid extraction, distillation of lactate esters are the basic recovery processes. Lactic acid may also be recovered by the adsorption of lactic acid on solid adsorbents or by the adsorption of lactate on ion-exchange resins (Vick Roy 1985).

CHAPTER 3

MODELLING

Modelling is an essential step in the development of the process under consideration by predicting the behaviour of the system. A model is a set of relationships between the variables of interest in the system being studied. Models are mainly used for defining the biological, chemical, or physical basis of the process, planning the experimental conditions and evaluating the experimental results (Sinclair and Kristiansen 1987). The purpose of fermentation modelling is to design large-scale fermentation processes using data obtained from small-scale fermentations (McNeil and Harvey 1990).

3.1. Model Types

Several different models are used in fermentation engineering:

- Kinetic models predict how fast the microorganisms can grow and use substrates or make products.

- Stoichiometric models predict how much substrate is needed or product is produced given a known amount of biomass or vice versa.

- Transport models predict how fast for example oxygen can be transported to the cells or how fast heat can be removed.

These different models can be put together into a process model, in order to predict the combined effects of the biology and the physics in a fermenter. The overriding factor that propels biotechnology is profit. The maximization of profits is closely linked to optimising product formation by cellular catalysts; i.e. producing the maximum amount of product in the shortest time at the lowest cost.

The large-scale cultivation of cells is central to the production of a large proportion of commercially important biological products. Thus, cell culture system must be described quantitatively. In other words, the kinetic of the process must be known. By determining the kinetics of the system, it is possible to predict yields and reaction times and thus permit the correct sizing of a bioreactor. In practice, kinetic data is obtained in small-scale reactors and then used with mass transfer data to scale-up the

process (Bailey and Ollis 1986). A kinetic model can be very useful for the design of both continuous and batch production systems.

In batch or fed batch fermentation processes, there is no steady state. The control of a fermentation process is based on the measurement of physical, chemical or biochemical properties of the fermentation broth and the manipulation of physical and chemical environmental parameters (Carrillo-Ureta et al. 2001).

The cellular phase of the system was first represented by Arnold Fredrickson and Henry Tsuchiya as in Figure 3.1. They classified approaches to microbial systems according to the number of components used in the cellular representation and the homogeneity of cell culture.

Unstructured	Structured	
Single component, heterogeneous individual cells	**Actual case** Multicomponent description of cell-to-cell heterogeneity	Segregated
Cell population treated as one component solute **Most idealized case**	Multicomponent average cell description	Unsegregated

Figure 3.1. Different perspectives for cell population kinetic representations (Bailey and Ollis 1986)

The complete description of the growth kinetics of a culture would involve recognition of the *structured* nature of each cell and the *segregation* of the culture into individual units (cells) that may differ from each other. A chemically structured model divides the cell mass into components. If the ratio of these components can change in response to disturbances in the extracellular environment, then the model is behaving analogously to a cell changing its composition in response to environmental changes (Shuler and Kargi 2002). However, what can be termed an unstructured mechanism of cell operation is sufficient for many technological purposes. In an unstructured

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mechanism of cellular operation, the microorganism is regarded as a single reacting species, possibly with a fixed chemical composition. Its limitations are improper use of the processes, which occur within the cell, or ability to analyse cells for particular constituents (Sinclair and Kristiansen 1987). Models may be structured and segregated, structured and unsegregated, unstructured and segregated, unstructured and unsegregated. Models containing both structure and segregation are the most realistic, but they are also computationally complex. The degree of realism and complexity required in a model depends on what is being described; the researcher should always choose the simplest model that can adequately describe the system. An unstructured model assumes fixed cell composition, which is equivalent to assuming *balanced growth* (Shuler and Kargi 2002). In this study, the unstructured and unsegregated model was developed.

3.2. Microbial Growth Curve and Microbial Products

Growth is the most essential response of microorganisms to their physiochemical environment. It is a result of both replication and change in cell size.

Microorganisms can grow under different physical and chemical conditions. They require substrates mainly:

- to synthesize new cell material
- to synthesize extracellular products
- to provide the energy necessary to maintain concentrations of materials within the cells which differ from those in the environment and in synthetic reactions

Thus growth, substrate utilisation, maintenance and product formation are all closely related (Sinclair and Kristiansen 1987). When a liquid nutrient medium is inoculated with a seed culture, the organisms selectively take up dissolved nutrients from the medium and convert them into biomass. In a typical batch process, the cell number varies with time and the following phases occur: 1) lag phase, (2) logarithmic or exponential growth phase, (3) deceleration phase, (4) stationary phase, and (5) death phase.

Typical microbial growth curve can be seen in Figure 3.2.
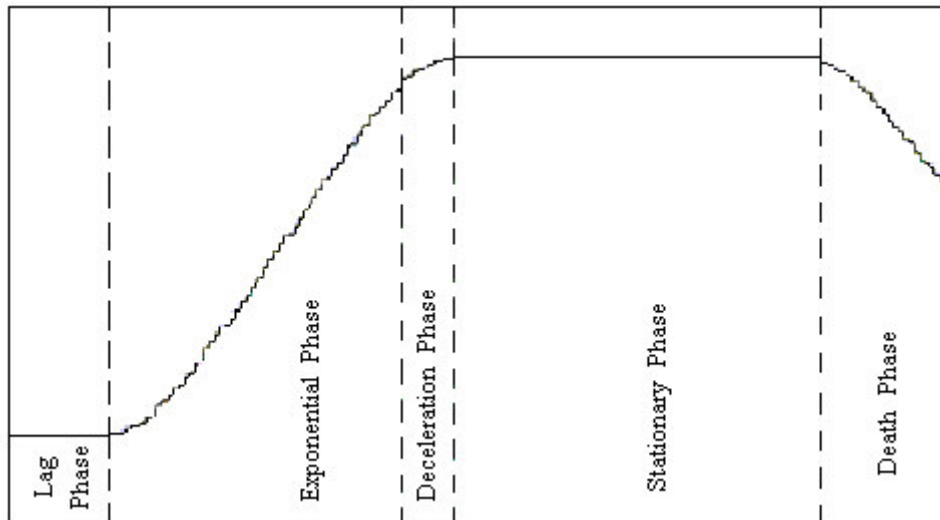
Figure 3.2. Microbial Growth Curve

The *lag phase*, is an adaptation period of cells to a new environment, occurs immediately after inoculation. During this adaptation period, new enzymes are synthesised, and the synthesis of some other enzymes is suppressed. Cell mass may increase, while cell number density remains constant. The lag period is affected from the age and size of the inoculum culture and the nutrient medium. Usually, the lag period increases with the age of the inoculum. To minimize the duration of the lag phase, cells should be young and active, and the inoculum size should be large. The nutrient medium may need to be optimised and certain growth factors can be included in order to minimize the lag phase.

After this adaptation period, the cells adjust to their new environment, start to multiply rapidly and consequently cell mass and cell number density increase exponentially with time. Therefore, this period is named as *exponential* or *logarithmic growth phase.*

The *deceleration growth phase* follows the exponential phase. In this phase, growth decelerates due to either depletion of one or more essential nutrients or the accumulation of toxic by-products of growth. For a typical bacterial culture, these changes occur over a very short period of time.

The *stationary phase* starts at the end of the deceleration phase, when the net growth rate is zero (no cell division) or when the growth rate is equal to the death rate. Even though the net growth rate is zero during the stationary phase, cells are still metabolically active and produce secondary metabolites. *Primary metabolites* are

growth-related products such as lactic acid, ethanol and *secondary metabolites* are nongrowth-related products such as antibiotics. During the stationary phase, the cell catabolizes cellular reserves for new building blocks and for energy-producing monomers. This is called *endogenous metabolism.* The cell must always spend energy to maintain an energised membrane and transport of nutrients and for essential metabolic functions such as motility and repair of damage to cellular structures. This energy expenditure is called *maintenance energy.*

The *death phase* follows the stationary phase. Often, death cells lyse, and a cellular nutrients released into the medium are used by the living organisms during stationary phase. At the end of the stationary phase, because of either nutrient depletion or toxic product accumulation the death phase begins.

Microbial products can be classified in three major categories:

1. Growth-associated products are produced simultaneously with microbial growth. The specific rate of product formation is proportional to the specific rate of growth. The production of a constitutive enzyme is an example of a growth-associated product.

$$q_p = \frac{1}{X}\frac{dP}{dt} = Y_{PX}\,\mu \qquad\qquad (3.1)$$

2. Nongrowth-associated product formation takes place during the stationary phase when the growth rate is zero. The specific rate of product formation is constant. Many secondary metabolites, such as antibiotics (for example, penicillin), are nongrowth-associated products.

$$q_P = \beta = \text{constant} \qquad\qquad (3.2)$$

3. Mixed-growth-associated product formation takes place during the slow growth and stationary phases. In this case, the specific rate of product formation is given by the following equation:

$$q_P = \alpha\mu + \beta \quad \text{(Luedeking-Piret equation)} \qquad\qquad (3.3)$$

Lactic acid fermentation, xanthan gum, and some secondary metabolites from cell culture are examples of mixed-growth-associated products (Shuler and Kargi 2002).

The kinetic pattern, of these types of product formation can be seen in Fig 3.3.
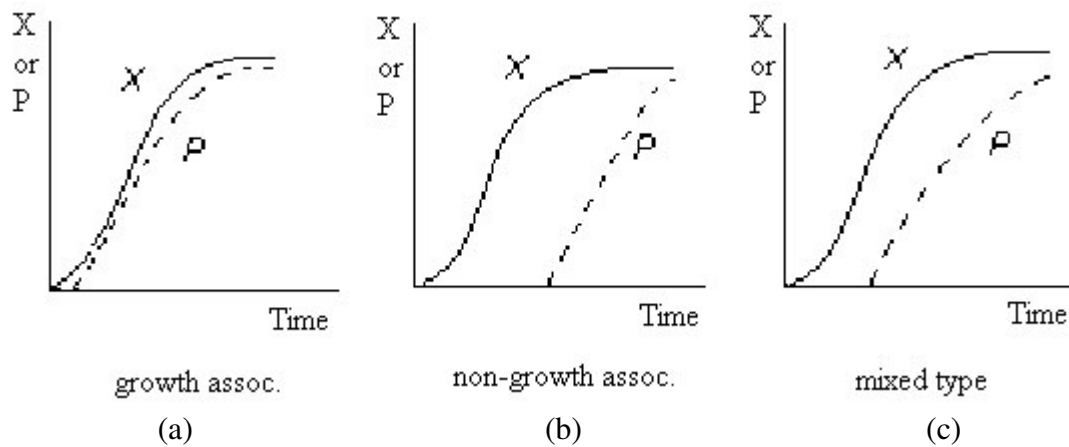
Figure 3.3. Kinetic patterns of product formation in batch fermentations: (a) growth-associated product formation, (b) nongrowth-associated product formation, (c) mixed-growth-associated product formation (Shuler and Kargi 2002).

3.3. Kinetic Models

The majority of kinetic models describing microbial growth use a formal macroapproach to bioprocessing. They are empirical and based on either Monod's equation or on its numerous modifications which take into account the inhibition of microbial growth by a high concentration of product and/or substrate.

The main objective of formulating a fermentation medium is to support good growth and/or high rates of product formation. The amounts of all nutrients in the medium are very critic. For example, excess concentration of a nutrient can inhibit cell growth. Moreover, if the cells grow too extensively, their accumulated metabolic end products will often disrupt the normal biochemical processes of the cells. Consequently, it is common practice to limit total growth by limiting the amount of one nutrient in the medium.
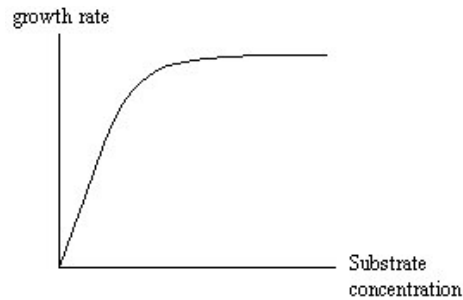
Figure 3.4. The relationship between growth rate and substrate concentration. (Bailey and Ollis 1986)

By changing the concentration of one medium constituent and keeping the others constant, the characteristics of growth rate can be obtained as in Figure 3.4.

Monod proposed a functional relationship between the specific growth rate μ and an essential compound's concentration in 1942. This relationship is similar to the Langmuir adsorption isotherm (1918) and the standard rate equation for enzyme-catalyzed reactions with a single substrate (Henri in 1902 and Michaelis and Menten in 1913). The Monod equation states that

$$\mu = \frac{\mu_{max} S}{K_S + S} \qquad (3.4)$$

where μ_{max} is the maximum achievable growth rate when S>>K_s and the concentrations of all other essential nutrients are constant. The constant K_s is known as the *saturation constant* or *half-velocity constant* and is equal to the concentration of the rate-limiting substance when the specific rate of growth is equal to one-half of its maximum (Bailey and Ollis 1986).

The Monod equation empirically fits a wide range of data satisfactorily and is the most commonly applied unstructured, unsegregated model of microbial growth (Shuler and Kargi 2002).

In many models, the inhibition by product follows a linear, power, exponential, hyperbolic or another non-linear formula. The kinetics of inhibition due to a high substrate concentration depends on the type of substrate and microorganism. It is usually described by an exponential or hyperbolic dependence.

Equations describing the rate of product formation often have a form analogous to that representing the specific growth rate. Mathematically equivalent formula resulting from Herbert's and Pirt's concepts or given by the Luedeking-Piret law are also used. Luedeking and Piret (1959) stated that the mixed-growth associated product formation was as follows:

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \qquad (3.5)$$

divided by X;

$$q_P = \alpha \mu + \beta \qquad (3.3)$$

where q_P is the specific rate of product formation.
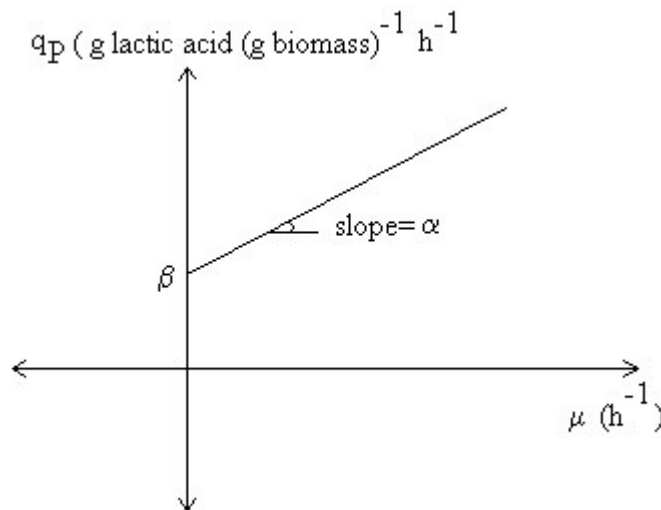
Figure 3.5. Specific rate of product synthesis as a function of the specific rate of bacterial growth during batch fermentations.

From the plot of q_P vs. μ (Figure 3.5), α is calculated from the slope of the graph, and β is found from the intercept of the line with q_P at $\mu=0$ (Luedeking and Piret 1959).

Several models, structured or unstructured, are available in literature. Table 3.1 summarises some of the kinetic modelling studies available in the literature for different lactic acid bacteria. In Table 3.2, the kinetic constant calculations for different lactic acid bacteria in previous studies are presented.

Table 3.1. Previous studies including the kinetic model equations in literature

Reference	Microorganism	Substrate	Product	Biomass growth	Product formation	Substrate utilization
Akerberg et al. (1998)	*L. lactis*	Glucose from wheat flour	Lactic acid	$\dfrac{dX}{dt} = \mu_{max} \dfrac{S}{K_S + S + S^2/K_i}\left(1 - \dfrac{K_P}{P}\right)^h$	$\dfrac{dP}{dt} = \alpha\dfrac{dX}{dt} + \beta X$	$\dfrac{dS}{dt} = -\dfrac{1}{Y_{XS}}\dfrac{dX}{dt} - \dfrac{1}{Y_{PS}}\dfrac{dP}{dt} - m_S X$
Biazar et al. (2003)	*L. helveticus*	Lactose from whey	Lactic acid	$\dfrac{dX}{dt} = \mu X$	$\dfrac{dP}{dt} = \alpha\dfrac{dX}{dt} + \beta X$	$\dfrac{dS}{dt} = -\dfrac{1}{Y_{PS}}\dfrac{dP}{dt} - \left(\dfrac{\mu_{max}}{Y_{XS}} + m_S\right)X$
Dutta et al. (1996)	*L. delbrueckii*	Glucose	Lactic acid	$\dfrac{dX}{dt} = \mu X\left(1 - \dfrac{P}{P_{max}}\right)^h$	$\dfrac{dP}{dt} = \alpha\dfrac{dX}{dt} + \beta X$	$\dfrac{dS}{dt} = -\dfrac{1}{Y_{PS}}\dfrac{dP}{dt} - m_S X$
Fu and Mathews (1999)	*L. plantarum*	Lactose	Lactic acid	$\dfrac{dX}{dt} = \mu X$	$P = Y_{PS}(S_0 - S) + P_0$	$\dfrac{dS}{dt} = -\dfrac{1}{Y_{XS}}\dfrac{dX}{dt}$
Luedeking and Piret (1959)	*L. delbrueckii*	Glucose	Lactic acid	$\dfrac{dX}{dt} = \mu X$	$\dfrac{dP}{dt} = \alpha\dfrac{dX}{dt} + \beta X$	-
Monteagudo et al. (1997)	*L. delbrueckii*	Sucrose from beet molasses	Lactic acid	$\dfrac{dX}{dt} = \mu X\left(1 - \dfrac{P}{P_{max}}\right)$	$\dfrac{dP}{dt} = \left(\alpha\dfrac{dX}{dt} + \beta X\right)\left(1 - \dfrac{P}{P_{max}}\right)$	$\dfrac{dS}{dt} = -\dfrac{1}{Y_{XS}}\dfrac{dX}{dt} - \dfrac{1}{Y_{PS}}\dfrac{dP}{dt} - m_S X$
Starzak et al. (1994)	*S. cerevisiae*	Sucrose	Ethanol	$\dfrac{dX}{dt} = \mu X$ $\dfrac{dX}{dt} = \mu X \exp^{-hP}$	$\dfrac{dP}{dt} = \alpha\dfrac{dX}{dt} + \beta X$	$\dfrac{dS}{dt} = -\dfrac{1}{Y_{XS}}\dfrac{dX}{dt} - m_S X$

Table 3.2. Previous studies including the kinetic parameters in literature

Reference	Microorganism	μ_{max} (h^{-1})	K_s (g/l)	α	β	m_s (h^{-1})	Y_{xs}	Y_{ps}
Schepers et al. (2002)	*L. helveticus*	0.7	0.22	-	-	-	-	-
Amrane and Prigent (1997)	*L.helveticus*	0.76	-	2.69	0.71	-	-	-
Kulozik and Wilde (1999)	*L.helveticus*	-	-	4.26	0.5	-	-	-
Boonmee et al. (2003)	*Lactococcus lactis*	1.1	1.32	0.932	3.02	-	-	0.93
Biazar et al. (2003)	*L.helveticus*	0.25	0.9	4.6	0.23	2.65	0.064	0.61
Kwon et al. (2001)	*L. rhamnosus*	0.633	0.3	6.6	0.33	-	1	1
Amrane (2001)	*L.helveticus*	0.49		2.56	0.76	-	-	0.84
Schepers et al. (2002)	*L.helveticus*	0.82	0.22	4.5	1.62	-	-	0.95
Messens et al. (2002)	*L. curvatus*	-	-	-	-	-	0.23	1
Tango and Ghaly (1999)	*L.helveticus*	0.21	-	-	-	-	0.08	0.71
Luedeking and Piret (1959)	*L.helveticus*	-	-	2.2	0.55	-	-	-
Akarberg et al. (1998)	*Lactococcus lactis*	0.403	0.79	13.2	0.0645	-	-	-
Monteagudo et al. (1997)	*L. delbrueckii*	0.831	-	0.235	0.087	-	0.27	0.91
Dutta et al. (1996)	*L. delbrueckii*	0.0696	0.0967	0.3853	0.0032	0.00014	-	-
Fu and Mathews (1999)	*L. plantarum*	0.364	44.4	-	-	-	-	1.02

CHAPTER 4

MATERIALS AND METHODS

4.1 Materials

The whey powder used in this study was supplied by PINAR Dairy Products, Inc (İzmir,Turkey). The approximate lactose and protein concentrations of whey powder were 72 % and 11.15 % respectively.

Lactobacillus casei NRRL B-441 strain was kindly obtained from United States Department of Agriculture, National Centre for Agricultural Utilization Research. The bacterium was supplied in lyophilised form and activated in the propagation medium, 10%(w/v) sterilised litmus milk.

4.2. Methods

4.2.1. Culture Propagation

20 ml litmus milk suspensions in 25 ml bottles were sterilised for 15 minutes at 121 ^{o} C at 1.1 kg cm^{-2} in the autoclave (Hirayama, Japan). The culture was maintained by transferring 10 % (v/v) culture to sterile litmus milk every 15 days. *L. casei* was incubated at 37 ^{o}C for 24 hours in the incubator (Sanyo) and kept at 4 ^{o}C in the refrigerator. 24 hour old fresh cultures were used as the inoculum for the fermentations.

4.2.2. Pretreatment of Whey

Protein precipitation was induced by heating the whey at 121 ^{o}C for 15 min. Precipitated proteins were removed by centrifugation (Sigma, Germany) at 7,000 rpm for 20 min. The supernatant was used as a substrate for the fermentations.

4.2.3. Lactic Acid Fermentations

Batch fermentation experiments, both in shake flasks and fermenter, were carried out at the best pH and temperature conditions stated by Büyükkileci, 2000.

Since, whey is deficient in some minerals and salts, their addition to the fermentation media is required before each fermentation. The components and their compositions in the fermentation media are listed in Table A.1. in Appendix A. The weight of the chemicals were measured by using Sartorius and AND HM-200 balances. The pretreatment of whey is performed before both fermentations in shake flasks and fermenter.

Lactic Acid Fermentations in Shake Flasks:

Shake flask fermentations were performed in 250 ml erlenmeyer flasks with a working volume of 125 ml. Top of the flasks were closed with cotton and aluminium foil. Whey suspension, yeast extract and the minerals except $MnSO_4$ and $CaCO_3$ were sterilised together at 121 ^{o}C for 15 min in an autoclave. 5 ml of culture was inoculated after the addition of $MnSO_4$ and $CaCO_3$.

Fermentations were carried out in a temperature controlled incubator shaker (Lab-Line, USA) operated at 150 rpm. The shake flasks were inoculated aseptically with 24-hour-old fresh culture propagated in litmus milk with a concentration of 4% (v/v) at 37^{o}C.

Lactic Acid Fermentations in the Fermenter:

Inocula for fermentation were prepared in shake flasks containing pre-sterilized cultivation medium held at 37 ^{o}C for 24 h.

Experimental runs were performed in 5 L laboratory fermenter (Bioengineering, type ALF) with a working volume of 3 L. The batch fermenter was equipped with standard control instrumentation (temperature, pH, and stirrer speed) and a magnetic stirrer for agitation.

Experimental studies involved several batch fermentation runs performed at atmospheric pressure, constant temperature (T=37 ^{o}C) and agitation (n= 200 rev min^{-1}). Experimental runs were performed under pH control at the constant value of 5.5. The

pH level was regulated by 10 N NaOH. The required NaOH was supplied by the peristaltic pump and another peristaltic pump was used to take samples to be analyzed. The heating jacket around the fermentation tank provided the temperature control. The fermentation temperature was kept around 37 ^{o}C.

The fermenter and the fermentation media were sterilized in the autoclave at 121 ^{o}C for 15 min. The shake flask fermentations were used as inocula and they were transferred to the fermenter after sterilization of the equipment. The fermenter was inoculated with 375 ml of inoculum (12.5 % v/v).

4.2.4. Analyses

4.2.4.1. Lactose and Lactic Acid Analyses

Lactose and lactic acid concentrations were determined by HPLC. The HPLC system was composed of Perkin Elmer Series 200 pump, Series 200 refractive index detector, Series 900 interface and a computer. The system was controlled by the software, Turbochrom Navigator. The degassing unit was connected to the Helium gas.

1 ml of fermentation sample was taken every hour throughout the fermentation and then centrifuged at 14,000 rpm for 10 minutes in Hettich EBA 12R centrifuge in order to separate the cell mass and other insoluble materials. Supernatants were diluted 10 times to get more precise results from high-pressure liquid chromatography (HPLC). All the standard solutions were prepared and dilutions were done with 5 mM H_2SO_4 which was the mobile phase used in the HPLC.

The column temperature was maintained at 45 ^{o}C with a MetaTherm column oven. The Aminex HPX-87H cation exchange column (Bio-Rad Laboratories) was used for HPLC analyses. The isocratic elution was performed with 5 mM H_2SO_4 at a flow rate of 0.6 ml min^{-1} for 15 minutes. The retention times for lactose and lactic acid were around 7.6 and 12.4 minutes, respectively. The calibration curves for lactose and lactic acid given as Figure B.1 and Figure B.2 in Appendix B were obtained with the analytical grade standards.

The properties and operating conditions of HPLC system are given in Table 4.1.

Table 4.1. The properties and operating conditions of HPLC system

Properties	Specifications
Column	Aminex HPX 87H ion exclusion column
Column length	300 mm
Column diameter	7.8 mm
Particle size	9 μm
Guard cartridge	Micro-Guard cation-H cartridge (30 x 4.6 mm)
Column cleaning solvent	5% CH_3CN in 5mM H_2SO_4, 30% CH_3CN in 5mM H_2SO_4
Mobile phase	5 mM H_2SO_4
Flow rate	0.6 ml min^{-1}
Temperature	45 ^oC
Detector	Refractive index
Elution type	Isocratic elution

4.2.4.2. Determination of Biomass Concentration

The optical densities of biomass were measured with the UV-Visible Spectrophotometer (Varian, Cary 100) at 610 nm. Each sampling time, around 10 ml of sample was taken and centrifuged at 6,000 rpm for 15 minutes in the Hettich EBA 12R centrifuge. In the analysis of the sample, in UV-Visible spectrophotometer, the supernatant was used as a blank. To convert cell densities to the biomass concentration, the calibration curve for optical density vs. dry cell weight was used. Calibration curve for biomass concentration is given in Figure B.3 in Appendix B.

In the preparation of calibration curve, the sample was taken from the stationary phase in the fermentation. Several dilutions of that sample were prepared for optical density and dry cell weight measurements. For each dilution, 2 ml of sample was used to obtain optical densities at 610 nm wavelength and 15 ml of sample was filtered with a pre-weighed cellulose acetate membrane filter having a pore size of 0.45 μm using a vacuum pump. The biomass collected on the filters was washed with 15 ml of water and the filters were dried at 100 ^oC for approximately 24 h until constant weight was observed.

4.2.4.3. Determination of Protein Concentration of Whey

In the protein determination of untreated and treated whey, very sensitive Lowry method was used. The samples before and after treatment were analyzed and compared to see the effect of pretreatment on the protein concentration of whey.

The Lowry method initially involves complexing the protein with Cu^{2+} in an alkaline solution. Then, the cupper catalyses the reduction of the phosphomolybdate / phosphotungstane anions in the Folin phenol reagent by the tyrosine and tryptophan residues. This reaction leads to a blue color, which can be measured at A_{550} for the range of 30-200 μg protein.

The bovine serum albumin (BSA) was used as a standard for calibration curve. The calibration curve for protein concentration is shown in Figure C.1 in Appendix C.2. Also, the reagents and method are given in Appendix C.1.

4.3. Kinetic Equations & Parameter Estimation

The kinetic model in this study was based on three rate equations: biomass growth, substrate utilization and product formation. Model described the rate of increase in biomass as a function of the biomass only. Thus,

$$\frac{dX}{dt} = f(X) \tag{4.1}$$

where f(X) = μX

The specific growth rate, μ, was expressed as a function of the limiting substrate concentration, S, by a Monod equation

$$\mu = \frac{\mu_{max} S}{K_S + S} \tag{3.4}$$

So that, the first rate equation was obtained as:

$$\frac{dX}{dt} = \frac{\mu_{max} S}{K_S + S} X \tag{4.2}$$

The classic study of Luedeking and Piret (Luedeking and Piret 1959) on the lactic acid fermentation by *L. delbrueckii* indicated that the product formation kinetics combined both growth and non-growth-associated contributions:

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \qquad (3.5)$$

Finally, substrate utilization kinetics may be expressed as:

$$\frac{dS}{dt} = -\frac{1}{Y_{XS}} \frac{dX}{dt} - \frac{1}{Y_{PS}} \frac{dP}{dt} - m_S X \qquad (4.3)$$

which considers substrate consumption for biomass, product and cellular maintenance.

The substrate requirement to provide energy for maintenance is usually assumed to be negligible. The rate equations stated in previous section included many variables and parameters. Variables can be classified as state and operating variables. The state variables were the biomass concentration (X), the limiting substrate concentration (S), and the product concentration (P). The operating variables were the inlet concentrations of the biomass, substrate and product, X_0, S_0, P_0, respectively.

4.3.1. Kinetic and Stoichiometric Parameters

Parameters could be divided into two groups: Kinetic parameters and stoichiometric parameters. The kinetic parameters were the Monod parameters (μ_{max} and K_s) and Luedeking-Piret equation parameters (α and β). The stoichiometric coefficients were the yield coefficients for biomass and product on substrate (Y_{XS} and Y_{PS}).

As mentioned in Section 3.2, the Monod parameter μ_{max} was the maximum growth rate achievable when the concentration of growth limiting nutrient is not limiting. Also, the Monod constant K_S was the concentration of the growth limiting nutrient at which the specific growth rate was half the maximum value. It represents an affinity of the organism for the nutrient. K_S and μ_{max} were dependent on the organism, the growth limiting nutrient, fermentation medium and environmental factors such as pH and temperature.

By changing the initial substrate concentration and keeping the other fermentation media constituents constant, the characteristics of growth rate was obtained as in Figure 4.1.
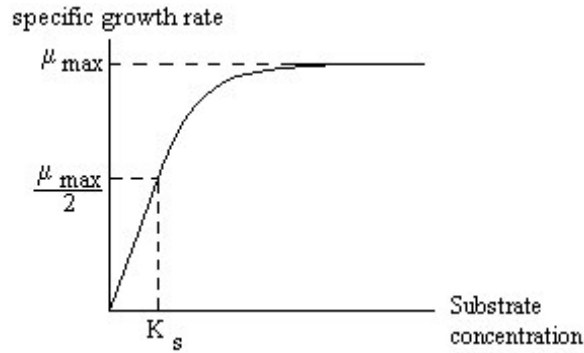

Figure 4.1. Monod kinetics

The specific growth rate was calculated by solving the first order exponential growth rate differential equation:

$$\frac{dX}{dt} = \mu X \qquad (4.4)$$

Integration of Eq (4.4) yields

$$\mu = \frac{\ln X - \ln X_0}{t - t_0} \qquad (4.5)$$

where X and X_0 were biomass concentrations at time t and t=0, respectively.

Maximum specific growth rates were obtained from the exponential phase of batch fermentations with different initial substrate concentrations. The maximum specific growth rates calculated from these fermentations were drawn with respect to initial substrate concentrations.

As it was mentioned before, K_S (Monod constant) was equal to the concentration of the rate limiting substance when the specific rate of growth was equal to one-half of its maximum. The determination of K_S value with high precision was difficult. The Monod equation is;

$$\mu = \frac{\mu_{max} S}{K_s + S} \tag{3.4}$$

This equation was linearized in double- reciprocal form;

$$\frac{1}{\mu} = \frac{1}{\mu_{max}} + \frac{K_s}{\mu_{max}} \frac{1}{S} \tag{4.6}$$

A plot of $1/\mu$ versus $1/S$, which is also known as a Lineweaver- Burk plot, yields a linear line with a slope of K_s/μ_{max} and y-axis intercept of $1/\mu_{max}$ as depicted in Figure 4.2 (Bailey and Ollis 1986).
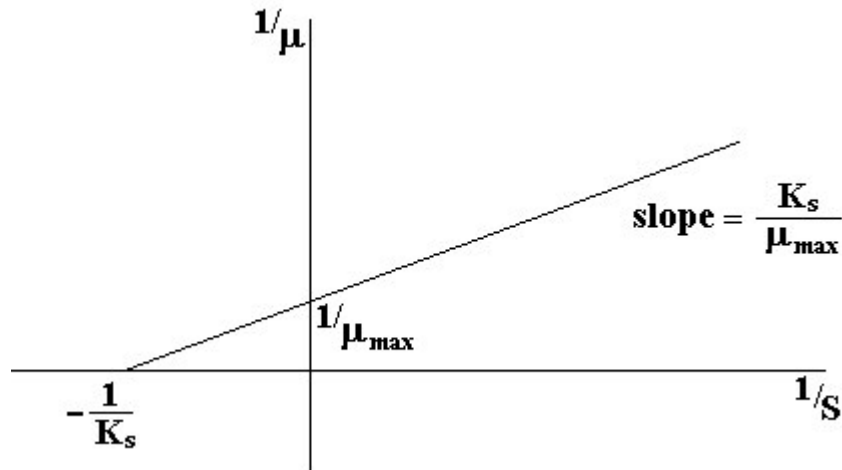

Figure 4.2. Double reciprocal (Lineweaver-Burk) plot

The biomass and product yield coefficients on substrate were defined as the stoichiometric coefficients.

Biomass yield was calculated as the ratio of the weight of biomass produced per weight of substrate utilized and showed as:

$$Y_{XS} = \frac{X_f - X_0}{S_0 - S_f} \tag{4.7}$$

Product yield was also defined as the weight of product produced per weight of substrate utilized and the equation was:

$$Y_{PS} = \frac{P_f - P_0}{S_0 - S_f}$$ (4.8)

The differential equations based on biomass growth, substrate utilization and product formation were solved numerically by second and third order Runga-Kutta method. Initial biomass, lactic acid and lactose concentrations within defined time span were initiate the solution of these differential equations. The initial biomass, substrate and product concentrations for the fermentation run were given in Table.E.1 in Appendix E.

The best values of the parameters of the models were adjusted by minimizing the objective function given as;

$$SSE = \sum_{i=1}^{N} \left\{ \left[\frac{X_{i\exp} - X_{ical}}{X_{\max}} \right]^2 + \left[\frac{P_{i\exp} - P_{ical}}{P_{\max}} \right]^2 + \left[\frac{S_{i\exp} - S_{ical}}{S_{\max}} \right]^2 \right\}$$ (4.9)

Equation (4.9) is the sum of squares of errors of the model. N is the number of observations in a single fermentation, and $X_{\max}$, $P_{\max}$, and $S_{\max}$ are the maximum biomass, product and substrate concentrations, respectively (Starzak et al. 1994). 'exp' subscript is used for the experimental data, 'cal' subscript is used for the simulation results.

CHAPTER 5

RESULTS AND DISCUSSION

5.1. Fermentation Experiments

In the kinetic modelling studies of lactic acid production from whey by *L. casei*, seven different fermentation experiments with different initial substrate concentrations (S_0) were performed. These fermentations, with 9.0, 21.4, 35.5, 48.0, 61.2, 77.1, and 95.7 g l^{-1} initial substrate concentrations, were used to determine kinetic and stoichiometric parameters such as μ_{max}, α, K_S, Y_{PS}, Y_{XS}.

Before each fermentation experiment, whey powder was dissolved in deionized water to get required concentration. However, using the whey powder solution in the fermenter directly causes difficulties in the biomass measurements in the UV spectrophotometer. Therefore, it is necessary to go through a pretreatment procedure of whey solution before the fermentation starts. The heat treatment can be used to denature the proteins of whey, which cause the turbidity during the measurements. For this reason, whey solution was autoclaved at $121^{o}C$ for 15 min. At high temperature some of the whey proteins were denatured and formed small particles. Recovery of heat-precipitated whey proteins was accomplished by a centrifuge. This treatment decreased the protein amount from 11.15 % to 5.2 % in whey powder. The lactose concentration of solution was detected before and after pretreatment. The results showed that there is not any lactose loss with pretreatment.

In these fermentations, the stationary phase was occurred at around 10-12 hours except the fermentations with 9.0 and 21.4 g l^{-1} initial substrate concentration. Also, most of the initial lactic acid came from the inoculum, which was produced during the shake flask fermentation to be used for the fermenter. The rest of the initial lactic acid came from the whey used as substrate in the fermenter.

The fermentation with 9.0 g l^{-1} initial substrate concentration was completed within 4 hours (Figure 5.1). The biomass concentration was reached to 3.5 g l^{-1} at the end of the fermentation. After 4 hours, all lactose was utilized as soon as the stationary phase was attained. However, it was seen that, 60 % of lactose was converted to lactic

acid. The remaining portion of lactose might have been used for cell growth and maintenance.
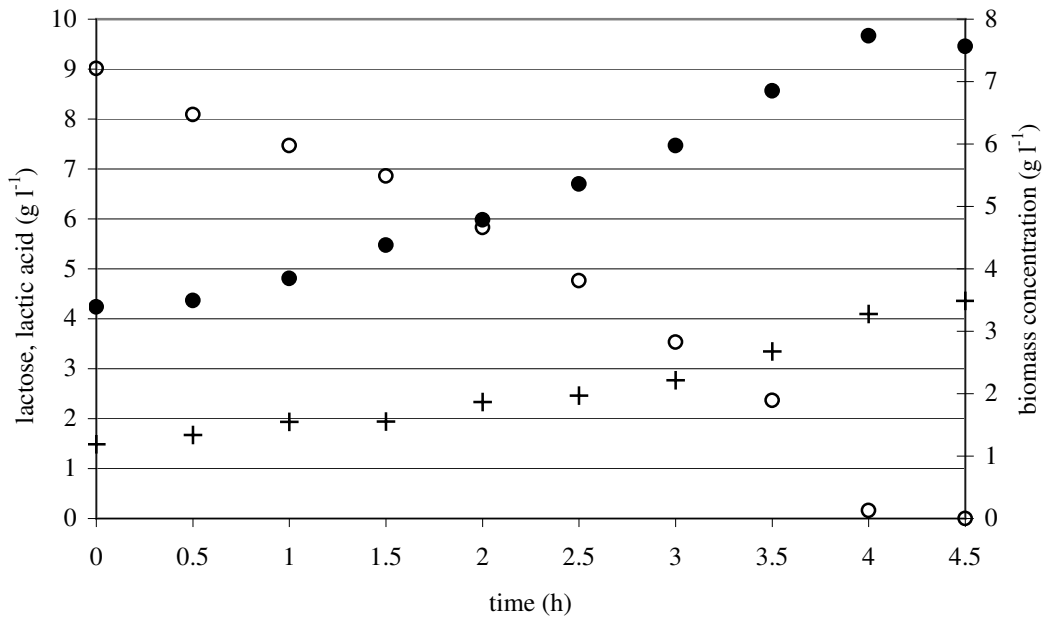

Figure 5.1. Experimental results of fermentation with 9.0 g l^{-1} initial substrate for lactose (o), lactic acid (•) and biomass (+)

Similarly, with the initial substrate concentration of 21.4 g l^{-1}, 60 % conversion of lactose to lactic acid was obtained within 7 hours (Figure 5.2). A similar behaviour as in the initial substrate of 9.0 g l^{-1} was observed in this case, too. Within 7 hours, all the substrate was depleted even the microorganism stopped growing, a small amount of lactic acid, approximately 2 g l^{-1}, was produced. In this fermentation, the biomass concentration was approximately 5 g l^{-1} at the end of the experiment.
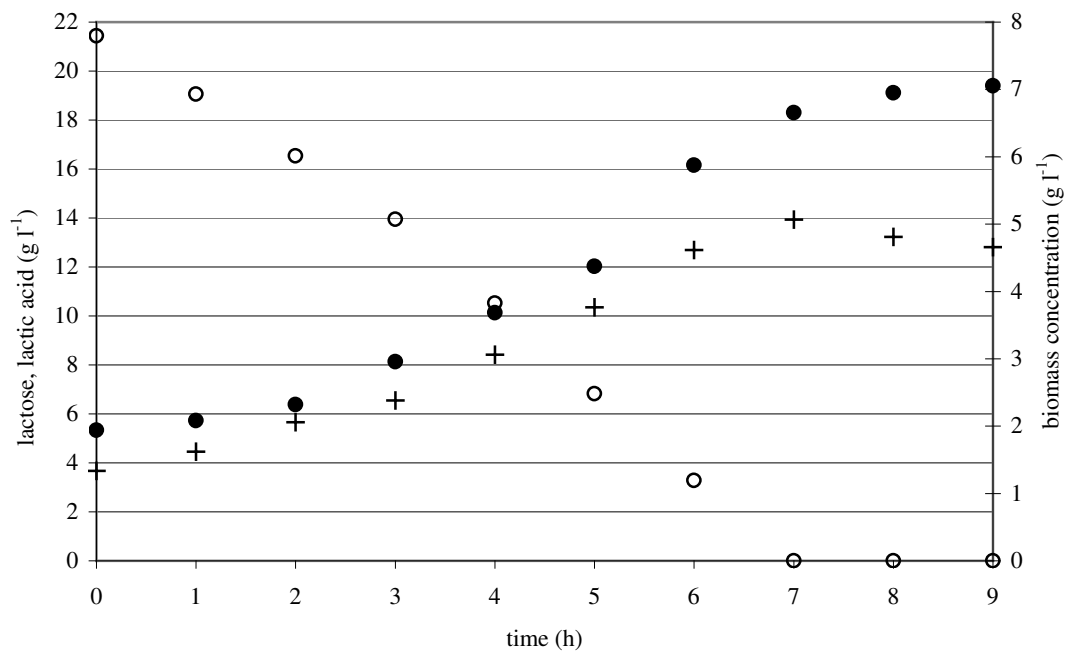
Figure 5.2. Experimental results of fermentation with 21.4 g l^{-1} initial substrate for lactose (o), lactic acid (•) and biomass (+).
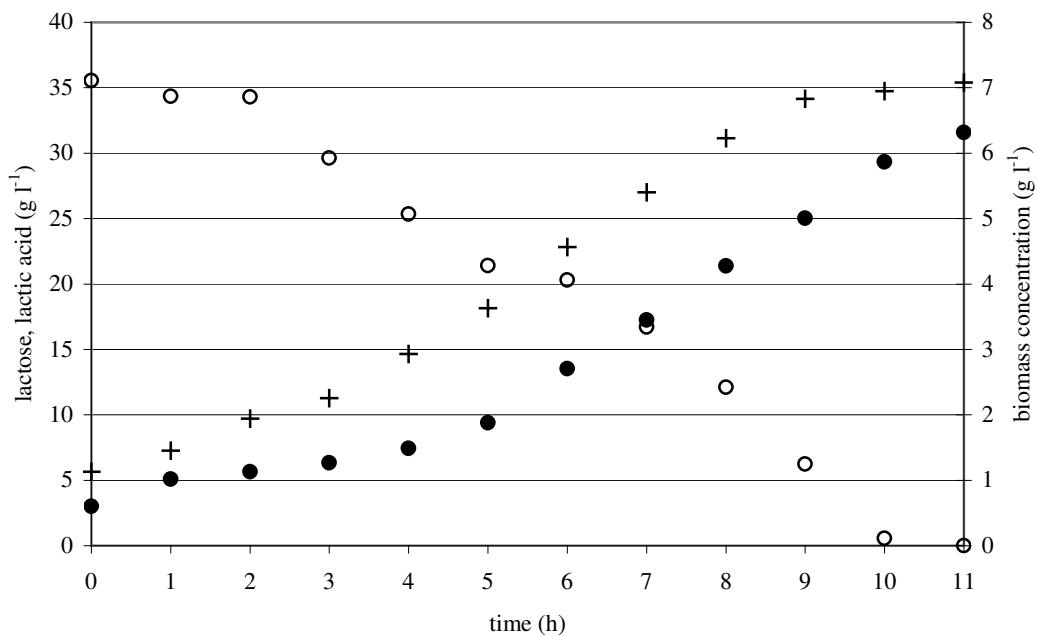

Figure 5.3. Experimental results of fermentation with 35.5 g l^{-1} initial substrate for lactose (o), lactic acid (•) and biomass (+).

Another fermentation experiment was performed with the initial substrate concentration of 35.5 g l^{-1}. In this case, the deceleration and stationary phases were observed at 8 h and 10 h, respectively (Figure 5.3). In stationary phase, 7 g l^{-1} biomass concentration was observed. 53 % of lactose was converted to lactic acid before deceleration phase and 29 % conversion was achieved in deceleration and stationary phases. It was seen that, all substrate was consumed at the end of the tenth hour.

In the fermentation with 48.0 g l^{-1} initial lactose concentration, 50 % of lactose was converted in the exponential phase and 17 % of it was converted in the deceleration and stationary phases (Figure 5.4). Similar to 35 g l^{-1} initial lactose concentration case, the deceleration and stationary phases were observed at 8 h and 11 h, respectively and the biomass concentration was 7 g l^{-1}, too. In this experiment, all lactose was utilized within 14.5 hours.

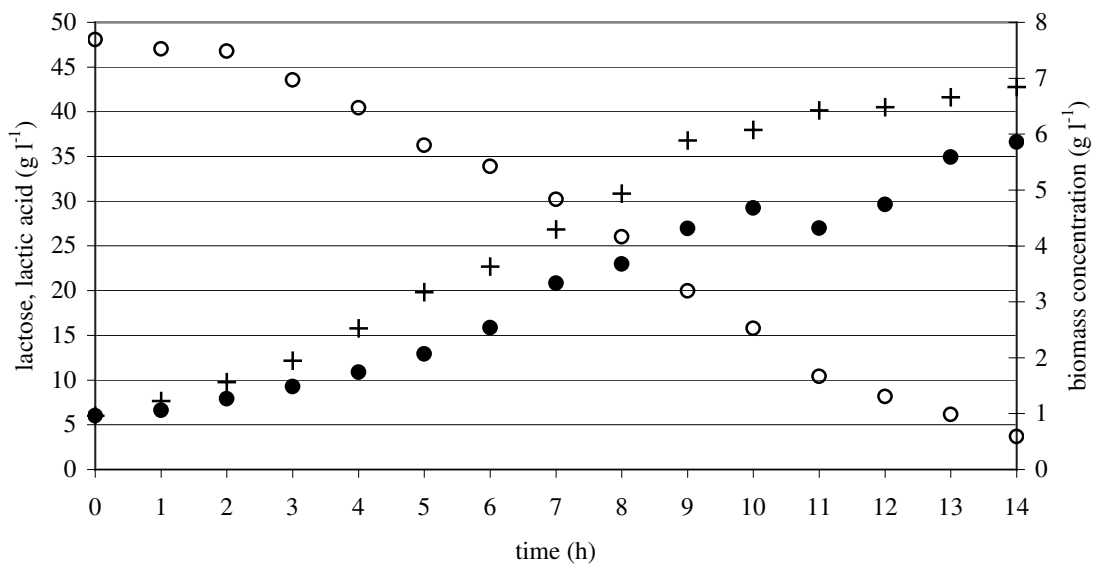Figure 5.4. Experimental results of fermentation with 48.0 g l^{-1} initial substrate for lactose (o), lactic acid (•) and biomass (+).
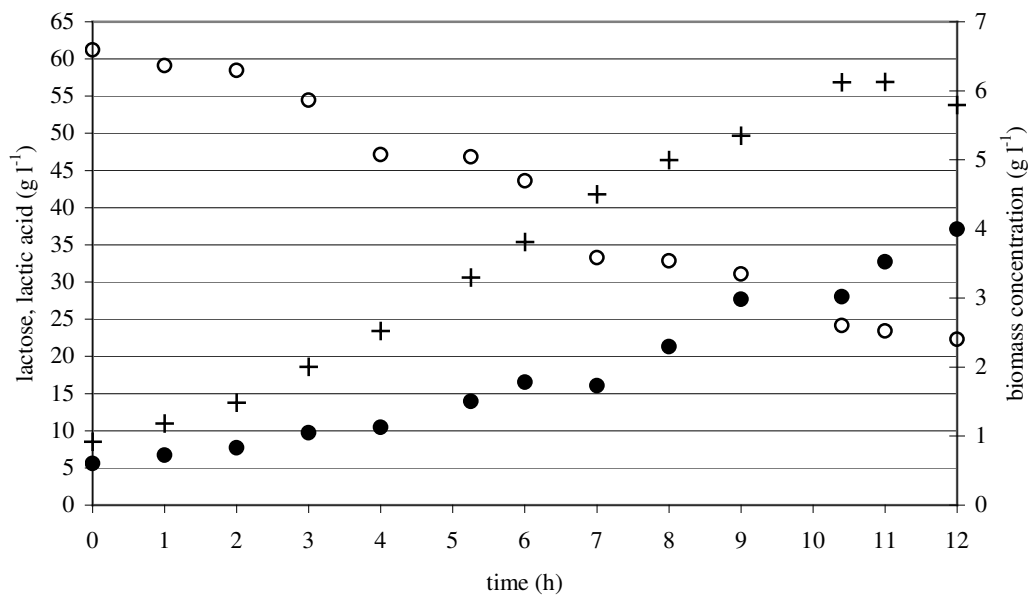
Figure 5.5. Experimental results of fermentation with 61.2 g l^{-1} initial substrate for lactose (o), lactic acid ($\bullet$) and biomass (+).

In another fermentation experiment carried on with 61.2 g l^{-1} initial lactose concentration (Figure 5.5). The biomass concentration was increased approximately from 1 g l^{-1} to 6.2 g l^{-1} during fermentation. Similar to the experiments mentioned before, most of the lactose was converted to lactic acid in exponential phase (36 %). Since the fermentation experiment wasn't completed, it couldn't be possible to predict the final lactic acid and lactose concentrations. However, it was obvious that, the substrate utilization was too slow in the stationary phase. This means that most probably, the complete substrate utilization wouldn't be achieved at the end of the fermentation.

With 77.1 g l^{-1} initial lactose concentration, 36 % conversion of lactose to lactic acid was obtained throughout the exponential phase, which was between 2 and 11 h (Figure 5.6). Similar to the other fermentations mentioned before, the biomass concentration was around 7 g l^{-1} in stationary phase.
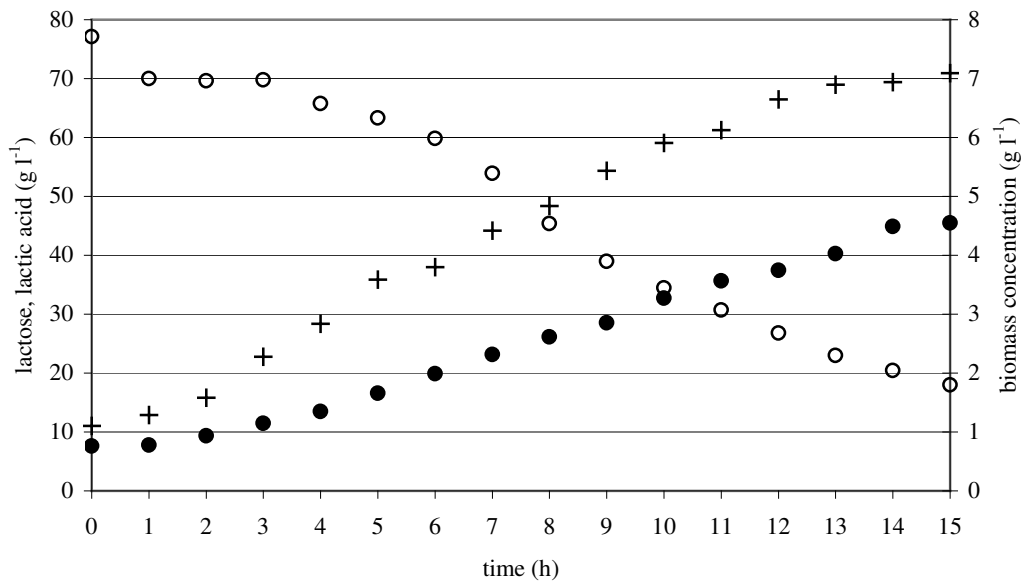
Figure 5.6. Experimental results of fermentation with 77.1 g l⁻¹ initial substrate for lactose (o), lactic acid (•) and biomass (+).

As expected, the concentration of residual lactose decreased during the fermentation with 95.7 g l⁻¹ initial lactose concentration (Figure 5.7). This coincided with an increase in lactic acid production. The concentration of residual lactose fell rapidly during the first 24 hours of fermentation after slow decrease. 31 % conversion of lactose to lactic acid was achieved during exponential phase within 11 hours. Nearly same amount of lactic acid was produced during the deceleration and stationary phases. However, it took more than 22 hours. As a result, the high initial substrate concentrations caused lower yields in very long fermentation time. So, it might be concluded that the fermentations with high initial lactose concentrations are not economical in industrial productions.
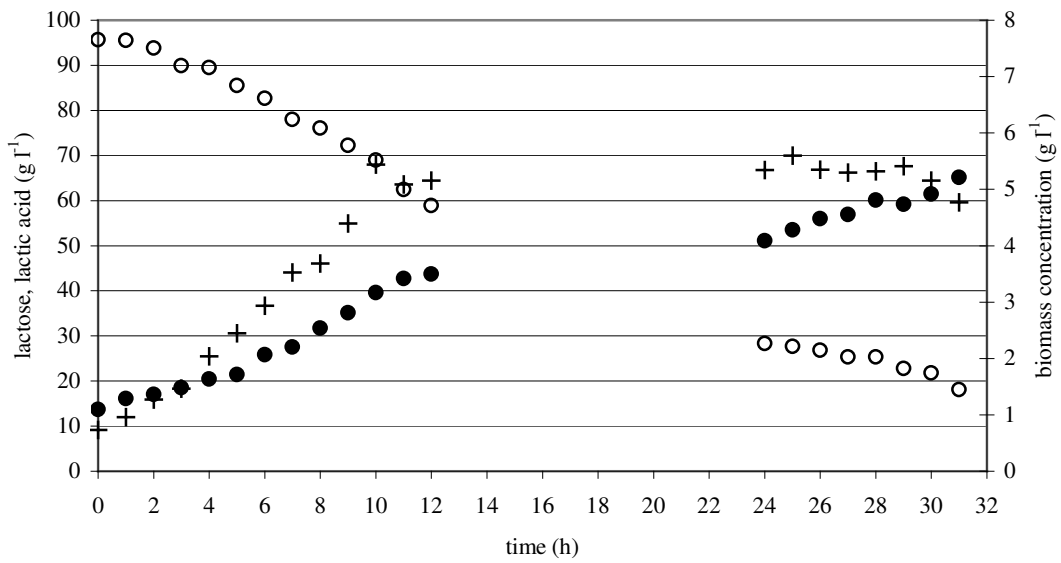
Figure 5.7. Experimental results of fermentation with 95.7 g l^{-1} initial substrate for lactose (o), lactic acid (•) and biomass (+).

5.2. Determination of Kinetic Parameters

Based on the experimental biomass, lactose and lactic acid concentration data, the biomass, and product yield coefficients (Y_{XS} and Y_{PS} respectively) were determined according to the methods given in Section 4.3.1 and presented in Table 5.1.

Table 5.1. Kinetic parameters based on the initial substrate concentration

Fermentations (Initial substrate concentrations (g l^{-1}))	Fermentation Time (h)	Y_{XS}	Y_{PS}	α_{exp}
9.0	4.5	0.255	0.579	3.30
21.4	9	0.155	0.656	3.61
35.5	11	0.168	0.804	3.67
48.0	14	0.133	0.680	4.42
61.2	12	0.138	0.717	4.39
77.1	15	0.101	0.641	5.31
95.7	31	0.064	0.624	5.73

The product yield on substrate, Y_{PS}, changes with different initial substrate concentrations in a narrow range (Figure 5.8). For this reason, the average value of calculated product yield was taken as 0.682 in the model development

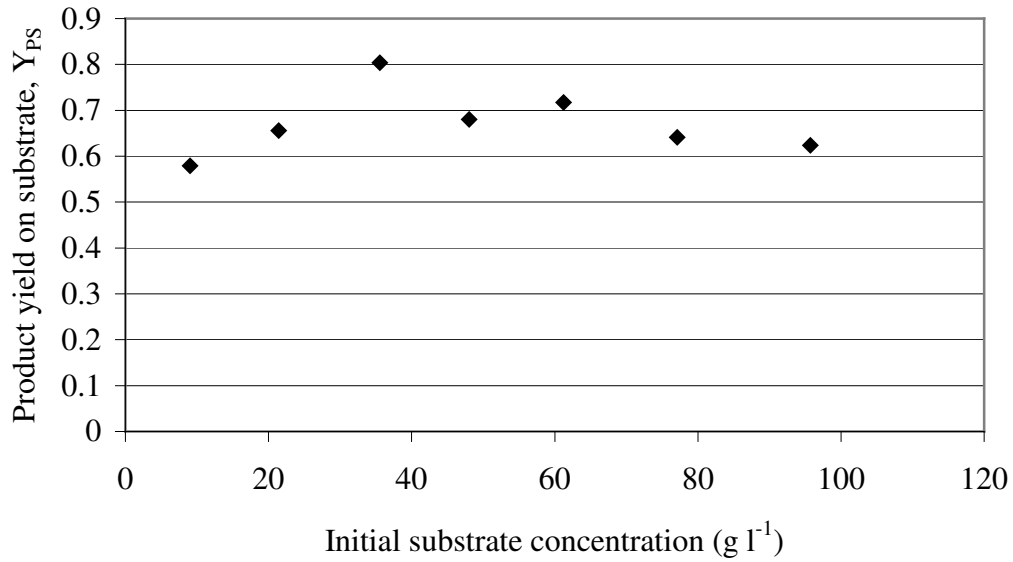.

Figure 5.8. Relation between the product yield coefficient and initial substrate concentration.

However, the biomass yield on substrate, Y_{XS} followed a different trend. The biomass yield decreased with the increase in the initial substrate concentration. The relationship between the biomass yield coefficient and the initial substrate concentration could be clearly seen in Figure 5.9.
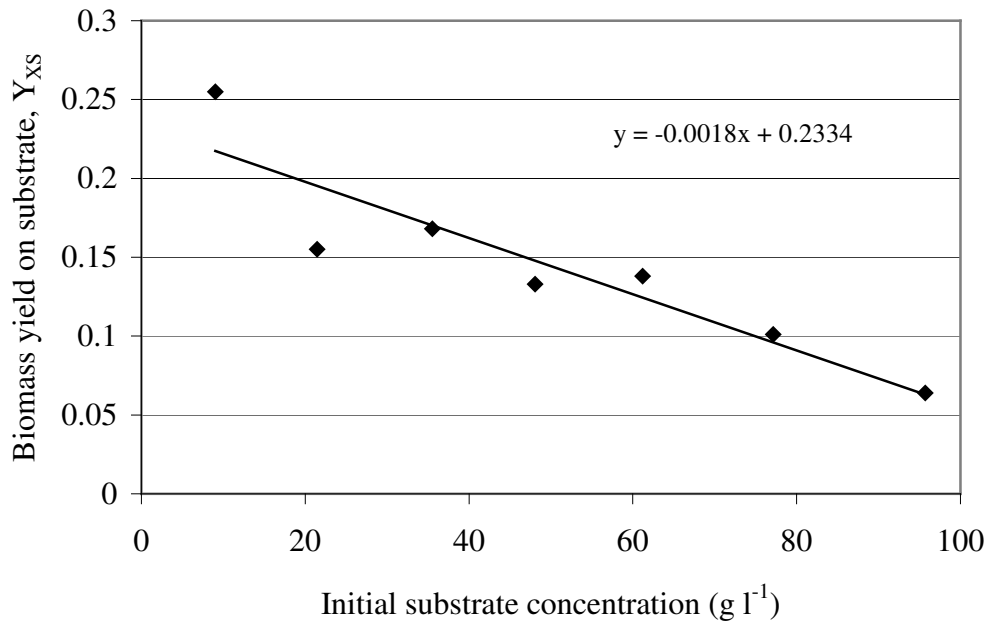
y = -0.0018x + 0.2334

Figure 5.9. Relationship between the biomass yield coefficient, Y_{XS}, and initial substrate concentration, S_0.

Table 5.1 also presents the experimental α values (α_{exp}). α is the growth-associated term parameter in the Leudeking-Piret equation:

$$\frac{dP}{dt} = \alpha\frac{dX}{dt} + \beta X \qquad (3.5)$$

In the study of Amrane and Prigent in 1997, more confident conclusions were reached when the coefficient of Luedeking-Piret equation, α, was calculated from the equation below:

$$(P - P_0) = \alpha(X - X_0) \qquad (5.1)$$

The α values in Table 5.1 were calculated by using the experimental lactic acid and biomass concentration data in Equation (5.1). The results showed that there was a linear relationship between α and the initial lactose concentrations. In Figure 5.10, it is obviously seen that α increased with the increase in initial substrate concentration.

Based on this result, a linear relationship was assumed between α and initial substrate concentration.

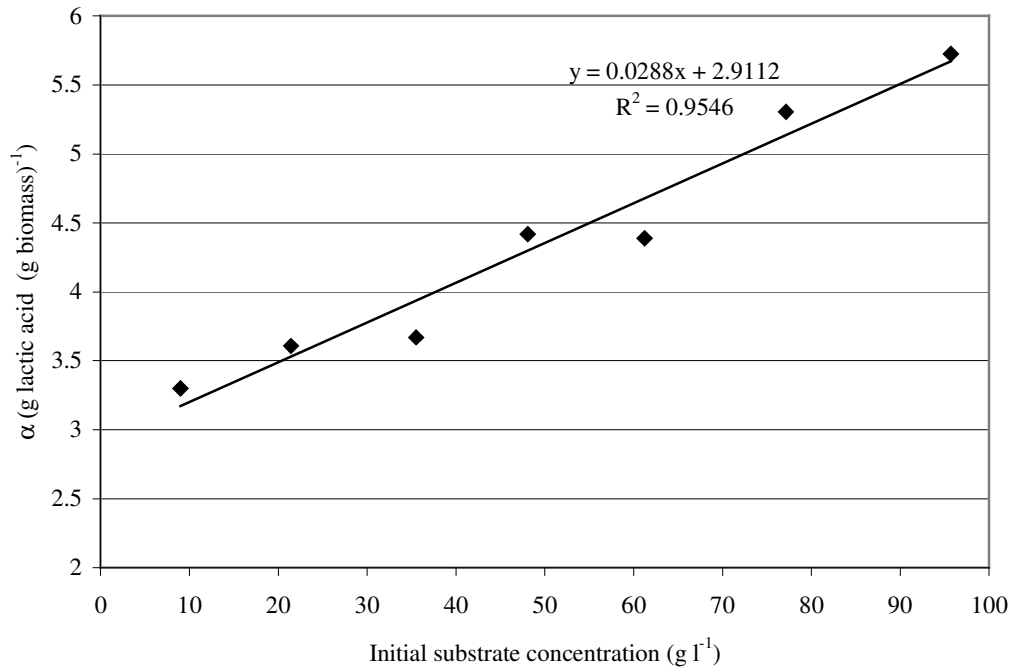Figure 5.10. Relation between experimental α and initial substrate concentrations

The maximum specific growth rate (μ_{max}) was determined from the graph of specific growth rate versus initial substrate concentration. (Figure 5.11) The maximum specific growth rate, μ_{max}, in Figure 5.11 was observed as 0.265 h^{-1}.
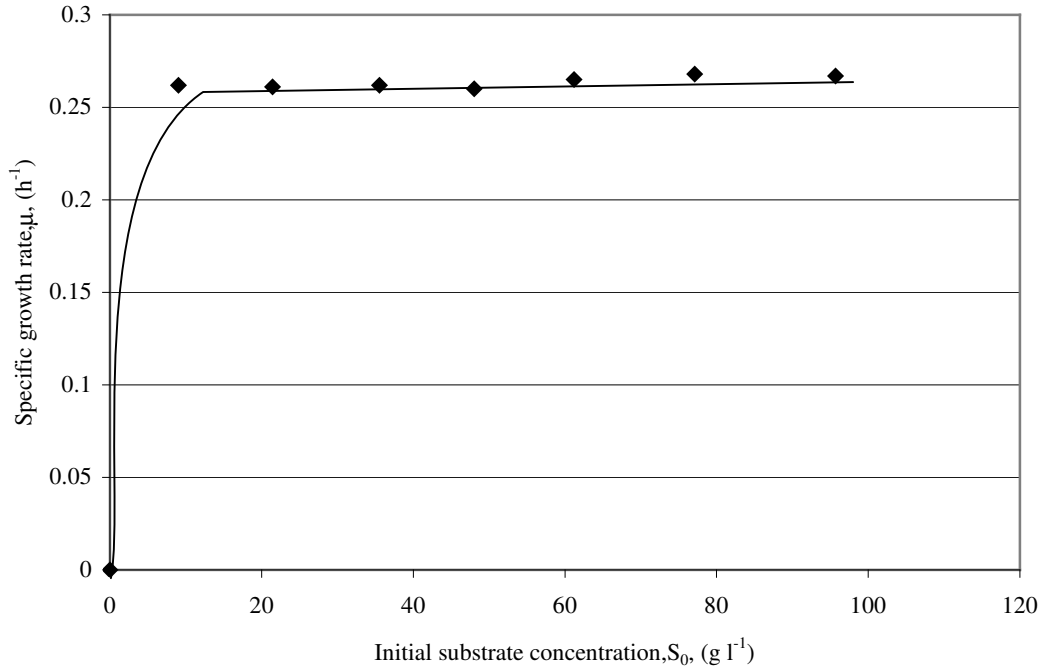
Figure 5.11. Specific growth rates obtained from different fermentation runs

Table 5.2. Specific growth rates, μ obtained from fermentations with different initial substrate concentration

S_0, (g l^{-1})	μ, (h^{-1})
9.0	0.262
21.4	0.261
35.5	0.262
48.0	0.260
61.2	0.265
77.1	0.268
95.7	0.267

K_S is the substrate concentration when μ is equal to $\mu_{max}/2$. After determination of μ_{max} in Figure 5.11, the substrate value in the x-axes corresponding to $\mu_{max}/2$ value in the y-axes gives K_S concentration. However, it is known that K_S calculation by using a graph of μ vs. S_0 is not sensitive enough to be able to read small values of substrate concentrations.

Lineweaver-Burk plot is generated by modifying Monod equation. It is the linearized form of Figure 5.11 (μ vs. S_0). When the reciprocal of both sides of Monod equation is taken, the following equation is obtained:

$$\frac{1}{\mu} = \frac{1}{\mu_{max}} + \frac{K_s}{\mu_{max}} \frac{1}{S} \tag{4.6}$$

In the graphical part, reciprocal experimental μ values ($1/\mu$) versus reciprocal initial substrate values ($1/S_0$) are plotted to generate Figure 5.12.
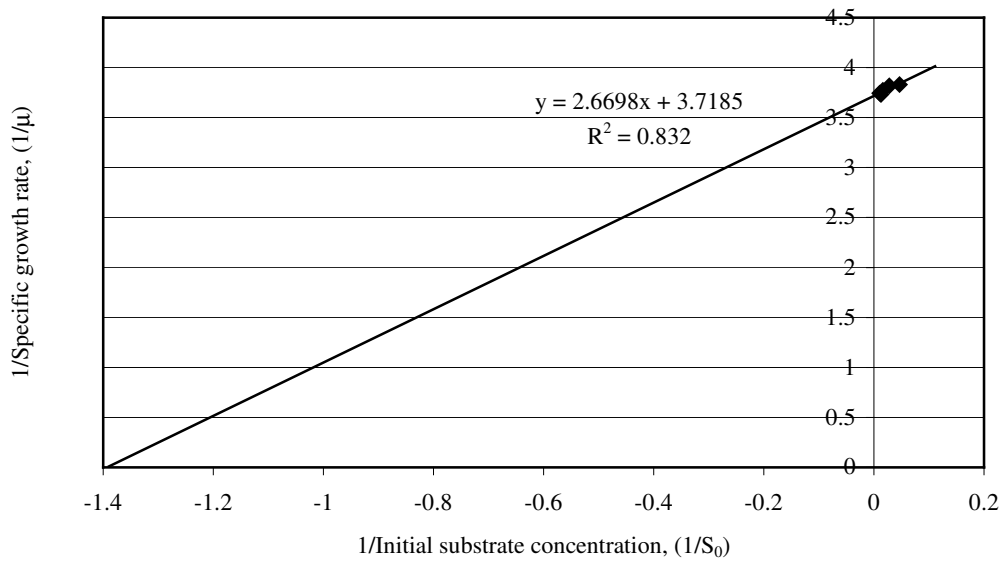
Figure 5.12. Lineweaver-Burk plot

The slope of the linear line is K_S/μ_{max} and the x-axes intercept is $1/K_S$. According to least square equation, K_S is determined as 0.72 g l^{-1}.

During the model development of seven fermentation data, the best α values for each data set were investigated by replacing the experimental α values in the Luedeking-Piret equation in the first attempt. A linear relation between α_{cal} and initial substrate concentration (S_0) was observed (Figure 5.13). As S_0 increases, α value increases, too. The experimental and calculated α values are shown in Table 5.3.
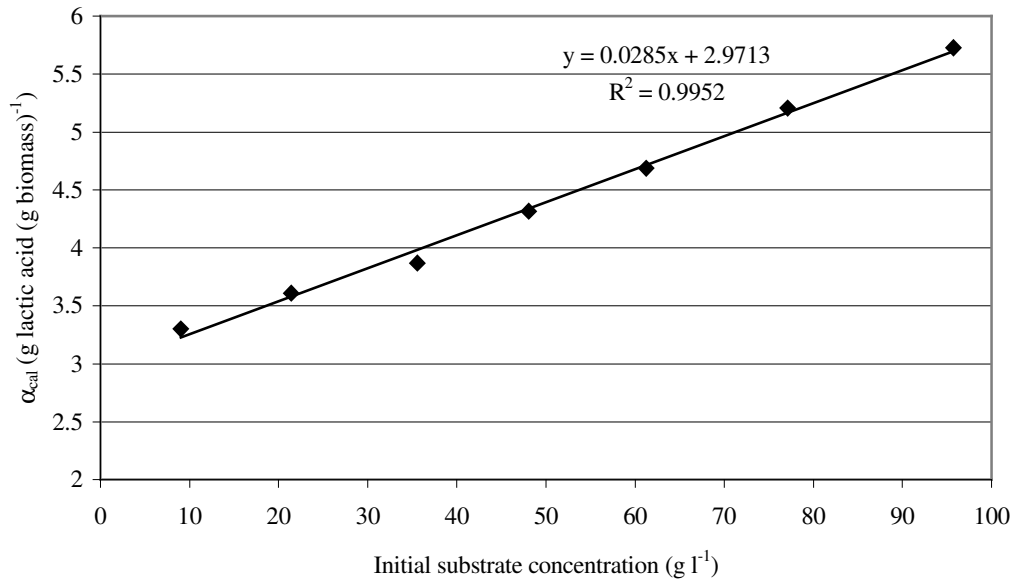
Figure 5.13. Relation between theoretical α_{cal} and initial substrate concentration, S_0

Table 5.3. Experimental and calculated α values

S_0, (g l^{-1})	α_{exp}	α_{cal}
9.0	3.30	3.30
21.4	3.61	3.61
35.5	3.67	3.87
48.0	4.42	4.32
61.2	4.39	4.69
77.1	5.31	5.21
95.7	5.73	5.73

Calculated kinetic and stoichiometric parameters were used in the model development.

5.3. Model Development

The widely used equation of Monod is applicable whenever a single essential nutritional requirement is the growth-limiting factor, and when the toxic powers of metabolic products are assumed to play no role. Monod equation relates the specific growth rate, μ and an essential compound concentration (substrate) and is given by:

$$\mu = \frac{\mu_{max} S}{K_S + S} \qquad (3.4)$$

where μ_{max} is the maximum achievable growth rate when $S \gg K_s$ and the concentrations of all other essential nutrients are not limiting. The constant K_s is the concentration of the rate-limiting substance when the specific rate of growth is equal to one-half of its maximum.

The biomass growth rate equation based on Monod equation (3.4) is;

$$\frac{dX}{dt} = \mu X \qquad (4.4)$$

Under optimal growth conditions and when the inhibitory effect of substrate and product plays no role, the rate of cell growth follows the well known exponential relationship:

$$\frac{dX}{dt} = \frac{\mu_{max} S}{K_S + S} X \qquad (4.2)$$

Equation (4.2) implies that X increases with time regardless of substrate availability. In reality the growth of cell is governed by a hyperbolic relationship and there is a limit to the maximum attainable cell mass concentration. In order to describe such growth kinetics, the logistic equation is introduced:

$$\frac{dX}{dt} = \mu X \left(1 - \frac{X}{X_{max}} \right) \qquad (5.2)$$

where X_{max} was the stationary population size, or the maximum attainable biomass concentration.

Moreover, other forms of Monod equation can be achieved to account different inhibition effects. For the product inhibition effect, Levenspiel proposed a simple generalization form of the Monod equation as follows:

$$\frac{dX}{dt} = \mu X \left(1 - \frac{P}{P_{max}} \right)^h \qquad (5.3)$$

where P_{max} is the maximum product concentration above which bacteria do not grow. The toxic power, h, characterizes the way in which one could approach the upper concentration limit for the inhibitory product P_{max}, above which fermentation ceases. The inhibition effect increases with the increase in toxic power, h (Dutta et al. 1996).

Monod's equation implies that the cells grow at their maximum rate even at the high substrate concentration. But, high concentrations of sugar, salt, acetic acid, etc. could stop cell growth. This is known as substrate inhibition and is modelled by a Monod-type equation with an S^2 term in the denominator as in the following equations:

$$\frac{dX}{dt} = \mu_{max} \frac{S}{K_S + S + K_i S^2} X \qquad (5.4)$$

or

$$\frac{dX}{dt} = \mu_{max} \left(\frac{S}{K_S + S} \right) \left(\frac{K_i}{K_i + S} \right) X \qquad (5.5)$$

where K_i and K_{iS} are the substrate inhibition constants.

Product formation is described by Luedeking and Piret kinetics. The product formation rate depends upon both the instantaneous biomass concentration X and growth rate dX/dt in a linear fashion:

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \qquad (3.5)$$

where α and β are empirical constants that might vary with fermentation conditions.

Many different unstructured models involving microbial growth, substrate utilization and product formation were applied to the experimental data. The models were based on a Monod Equation and Logistic Equation for microbial growth and Luedeking-Piret Equation for product formation. The modified forms of Monod and Logistic Equations were applied to indicate the effects of product, substrate and biomass inhibitions on biomass growth. All the kinetic models applied in the study are presented in Table 5.4.

Table 5.4. Models applied in the study

Model Number	Biomass Growth	Product Formation	Substrate Utilization
Model 1	$\dfrac{dX}{dt} = \mu X$	$\dfrac{dP}{dt} = \alpha \dfrac{dX}{dt} + \beta X$	$\dfrac{dS}{dt} = -\dfrac{1}{Y_{XS}}\dfrac{dX}{dt} - \dfrac{1}{Y_{PS}}\dfrac{dP}{dt} - m_S X$
Model 2	$\dfrac{dX}{dt} = \mu X \left(1 - \dfrac{P}{Pm}\right)^{h}$	$\dfrac{dP}{dt} = \alpha \dfrac{dX}{dt} + \beta X$	$\dfrac{dS}{dt} = -\dfrac{1}{Y_{PS}}\dfrac{dP}{dt} - m_S X$
Model 3	$\dfrac{dX}{dt} = \mu X \exp(-hP)$	$\dfrac{dP}{dt} = \alpha \dfrac{dX}{dt} + \beta X$	$\dfrac{dS}{dt} = -\dfrac{1}{Y_{PS}}\dfrac{dP}{dt} - m_S X$
Model 4	$\dfrac{dX}{dt} = \mu \left(\dfrac{K_i}{K_i + S}\right) X$	$\dfrac{dP}{dt} = \alpha \dfrac{dX}{dt} + \beta X$	$\dfrac{dS}{dt} = -\dfrac{1}{Y_{PS}}\dfrac{dP}{dt} - m_S X$
Model 5	$\dfrac{dX}{dt} = \mu_{max} \dfrac{S}{K_S + S + S^2/K_i} \exp(-P/K_P) X$	$\dfrac{dP}{dt} = \alpha \dfrac{dX}{dt} + \beta X$	$\dfrac{dS}{dt} = -\dfrac{1}{Y_{PS}}\dfrac{dP}{dt} - m_S X$

Table 5.4. *continued…*

Model Number	Biomass Growth	Product Formation	Substrate Utilization
Model 6	$\dfrac{dX}{dt} = \mu\left(1 - \dfrac{X}{X_{max}}\right)X$	$\dfrac{dP}{dt} = \alpha\dfrac{dX}{dt} + \beta X$	$\dfrac{dS}{dt} = -\dfrac{1}{Y_{PS}}\dfrac{dP}{dt} - m_S X$
Model 7	$\dfrac{dX}{dt} = \mu\left(1 - \dfrac{X}{X_{max}}\right)^f X$	$\dfrac{dP}{dt} = \alpha\dfrac{dX}{dt} + \beta X$	$\dfrac{dS}{dt} = -\dfrac{1}{Y_{PS}}\dfrac{dP}{dt} - m_S X$
Model 8	$\dfrac{dX}{dt} = \mu\left(1 - \dfrac{X}{X_{max}}\right)^f \exp(-kP)X$	$\dfrac{dP}{dt} = \alpha\dfrac{dX}{dt} + \beta X$	$\dfrac{dS}{dt} = -\dfrac{1}{Y_{PS}}\dfrac{dP}{dt} - m_S X$
Model 9	$\dfrac{dX}{dt} = \mu\left(1 - \dfrac{X}{X_{max}}\right)^f \left(1 - \dfrac{P}{P_{max}}\right)^h$	$\dfrac{dP}{dt} = \alpha\dfrac{dX}{dt} + \beta X$	$\dfrac{dS}{dt} = -\dfrac{1}{Y_{PS}}\dfrac{dP}{dt} - m_S X$

In these models, the product inhibition was assumed as following the linear or exponential fashion. The kinetics of inhibition due to high substrate concentration depends on the type of substrate and microorganism. It is usually described by an exponential or hyperbolic dependence (Starzak et al. 1994). The hyperbolic substrate inhibition case was applied in this study. In processes involving a high concentration of microbial cells, the biomass itself also inhibits growth and product formation. This was represented by a term proportional to the biomass concentration.

The models have been developed under the assumption that the observed change in cell mass results from the growth process only, neglecting both endogenous metabolism and cell death. Thus, the biomass growth rate was described by the Monod Equation;

$$\frac{dX}{dt} = \mu_{max} \frac{S}{K_S + S} X \tag{4.2}$$

Additionally, the Luedeking- Piret equation (3.5) to explain the product formation was used in the first attempt. In that case, the substrate utilization was expressed as:

$$\frac{dS}{dt} = -\frac{1}{Y_{XS}} \frac{dX}{dt} - \frac{1}{Y_{PS}} \frac{dP}{dt} - m_S X \tag{4.3}$$

A very high value of sum of errors (SSE range is 1.61-3.02) was obtained in Model 1 (Table 5.4). Sudden depletion in substrate concentration promised neither biomass nor product to reach actual values (App. D Figure D.1). This could be caused by a very small value of Y_{XS}, which yields a substrate concentration mostly to biomass growth instead of product formation.

Based on the result of Model 1, the equation of substrate utilization rate was changed by considering the conversion to product and maintenance for biomass growth as Equation (5.6). In the production of lactic acid, the cell dissimilates substrate to lactic acid in order to obtain the energy required to form new bacterial protoplasm. At the same time, it performs its normal metabolic activity irrespective of growth. It might, therefore, be presumed that a portion of substrate is used for maintenance of the cell during fermentation. The substrate utilization kinetics given by equation (5.6), which

was used in the studies of Biazar et al. 2003 and Dutta et al. 1996, considered substrate conversion to product and substrate consumption for maintenance:

$$\frac{dS}{dt} = -\frac{1}{Y_{PS}} \frac{dP}{dt} - m_S X \qquad (5.6)$$

Also, the biomass growth rate equation was modified by the addition of product inhibition term:

$$\frac{dX}{dt} = \mu X \left(1 - \frac{P}{P_{max}}\right)^h \qquad (5.3)$$

where P_{max} is the maximum product concentration above which bacteria do not grow and h is the toxic power. This approach may be linear when h is unity or there is a rapid initial drop in production rate followed by a slow approach to P_{max} when h>1. Luedeking and Piret Equation (3.5) was used to describe lactic acid formation rate in Model 2. The results of Model 2 were seen in Figure D.2 in Appendix D.

In the experiments with high initial substrate concentrations such as 95.696 g l^{-1} and 77.122 g l^{-1}, there wasn't a significant change in sum of squares of errors conforming the important role that product inhibition plays in the process of lactic acid fermentation. However, the product formation of low initial substrate concentration experiments such as 9.014 g l^{-1} and 21.438 g l^{-1}, could be explained better with higher accuracy (SSE is 0.86 and 0.55, respectively).

In Model 3, the product inhibition term was changed to exponential inhibition term (Table 5.4). Similar results were obtained with previous model (App. D Figure D.3). There was not a good agreement in the model simulations and experimental data of the biomass growth in the experiments with low initial substrate concentrations. The reason for this result might be the sudden decrease in the substrate concentration, low increase in product and biomass concentrations. To overcome this problem, the substrate inhibition terms were introduced to Monod Equation and applied as Equation (5.4) and Equation (5.5) (Model 4 and Model 5, respectively). Unexpectedly, the inhibition effect was too strong that all concentrations obtained from the model simulations were lower values than the experimental concentrations. The results of

Model 4 and Model 5 were seen in Figure D.4 and Figure D.5 in Appendix D, respectively.

Monod Equation defines the biomass growth rate as an exponential relationship. In reality the growth of cell is governed by a hyperbolic relationship and there is a limit to the maximum achievable cell mass concentration. The Logistic Equation describes such growth kinetics representing both an exponential and stationary phases. From the point of view of data fitting, Model 6 produced lower value of SSE than the previous models did (App. D Figure D.6). In addition, the Logistic Equation was modified with toxic power (f), to adopt the model to the wide range of initial substrate concentration experiments as given in Equation (5.7) and the results were shown in Figure D.7 in Appendix D (Model 7):

$$\frac{dX}{dt} = \mu\left(1 - \frac{X}{X_{max}}\right)^f X \qquad (5.7)$$

On the other hand, since the effect of product inhibition is considerable for lactic acid fermentation, the modification of Logistic Equation with product inhibition term might give better agreement of model predictions with the experimental data. For this reason, exponential product inhibition term (5.8) and Levenspiel product inhibition term (5.9) were used in models 8 and 9, respectively (Table 5.6).

$$\frac{dX}{dt} = \mu\left(1 - \frac{X}{X_{max}}\right)^f \exp(-kP)X \qquad (5.8)$$

$$\frac{dX}{dt} = \mu\left(1 - \frac{X}{X_{max}}\right)^f \left(1 - \frac{P}{P_{max}}\right)^h \qquad (5.9)$$

The result of Model 8 was seen in Figure D.8 in Appendix D.

The Logistic Equation modified with biomass and product inhibition terms produced lower values of the sum of squares of errors. The use of Equation (5.9) was more applicable to the wide range of initial substrate concentration fermentations since the toxic powers define the power of the inhibition and vary for the extreme cases such as a very low and very high initial substrate concentrations, 9.0 and 96 g l^{-1}, respectively

(Model 9). The good correlations were achieved between the model outputs and experimental data obtained from different fermentation runs by using Model 9. Use of Model 9 is advantageous than the other models used in the study since the inhibition effect can be adjusted by changing the toxic power which enables the model applicable for most of the fermentations. However, there wasn't a good agreement between the model simulation data and the experimental data in 95.7 g l^{-1} fermentation but this model could explain the first 12h of experiment. The results of Model 9 are given in Figures 5.14-5.19, for 9.0, 21.4, 35.5, 48.0, 61.2, 77.1 and 95.7 g l^{-1} of initial substrate concentrations, respectively.
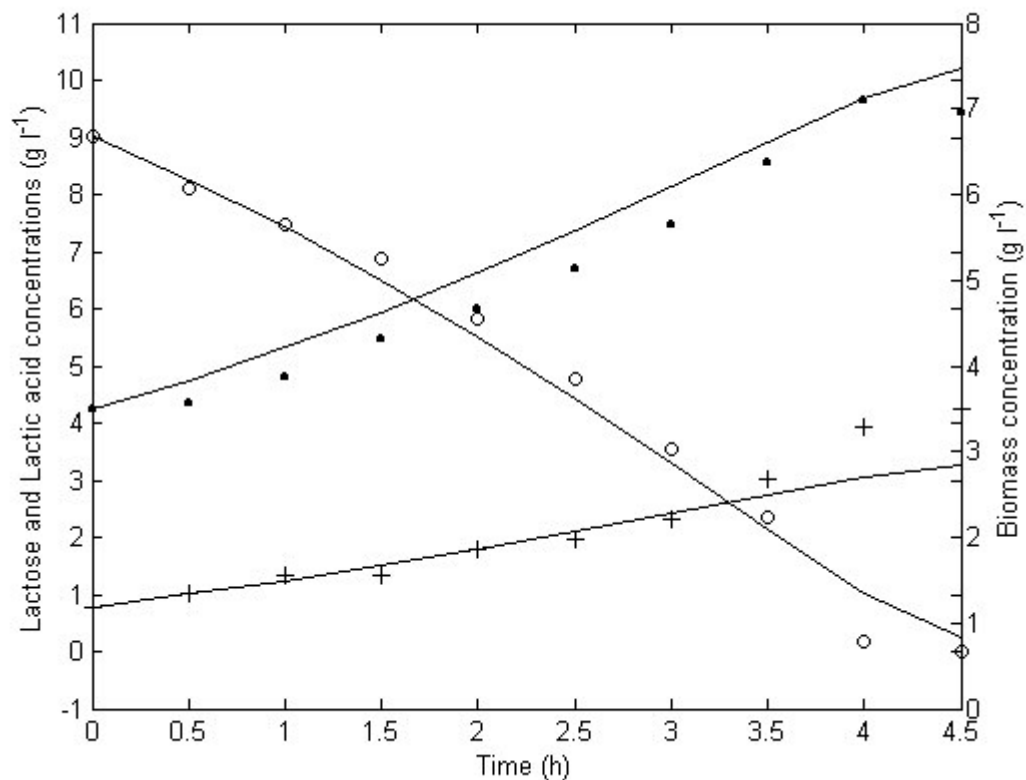
Figure 5.14. Model and experimental data for 9.0 g l^{-1} initial substrate concentration; experimental biomass concentration (+); experimental product formation (•); and experimental substrate utilization (o) and straight lines over them are model outputs
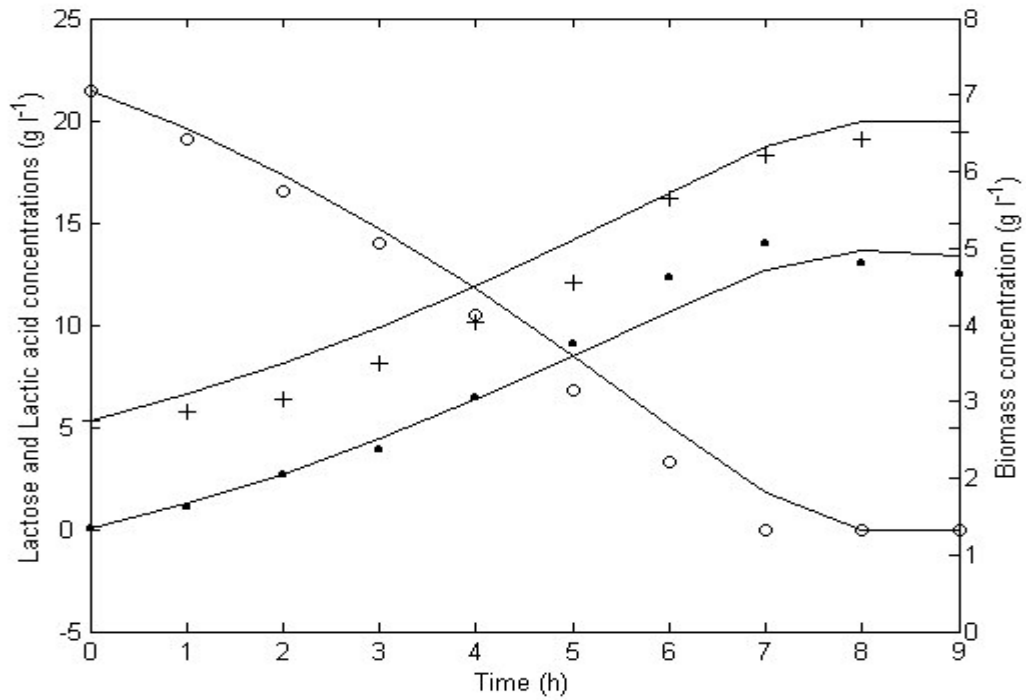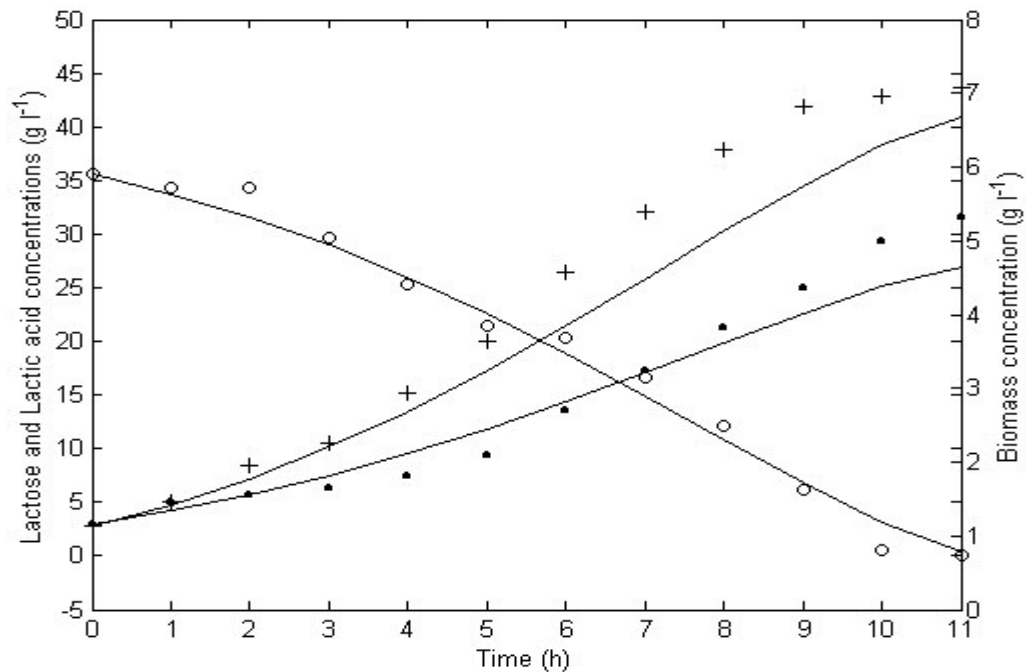
Figure 5.15. Model and experimental data for 21.4 g l^{-1} initial substrate concentration; experimental biomass concentration (+); experimental product formation (•); and experimental substrate utilization (o) and straight lines over them are model outputs
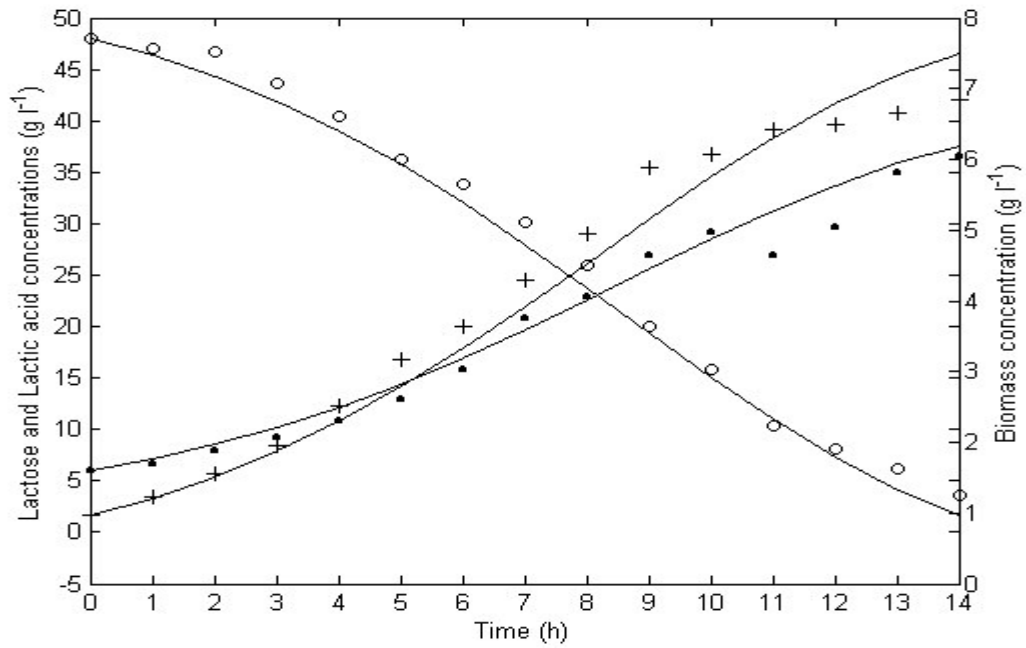

Figure 5.16. Model and experimental data for 35.5 g l^{-1} initial substrate concentration; experimental biomass concentration (+); experimental product formation (•); and experimental substrate utilization (o) and straight lines over them are model outputs

Figure 5.17. Model and experimental data for 48.0 g l⁻¹ initial substrate concentration; experimental biomass concentration (+); experimental product formation (•); and experimental substrate utilization (○) and straight lines over them are model outputs.
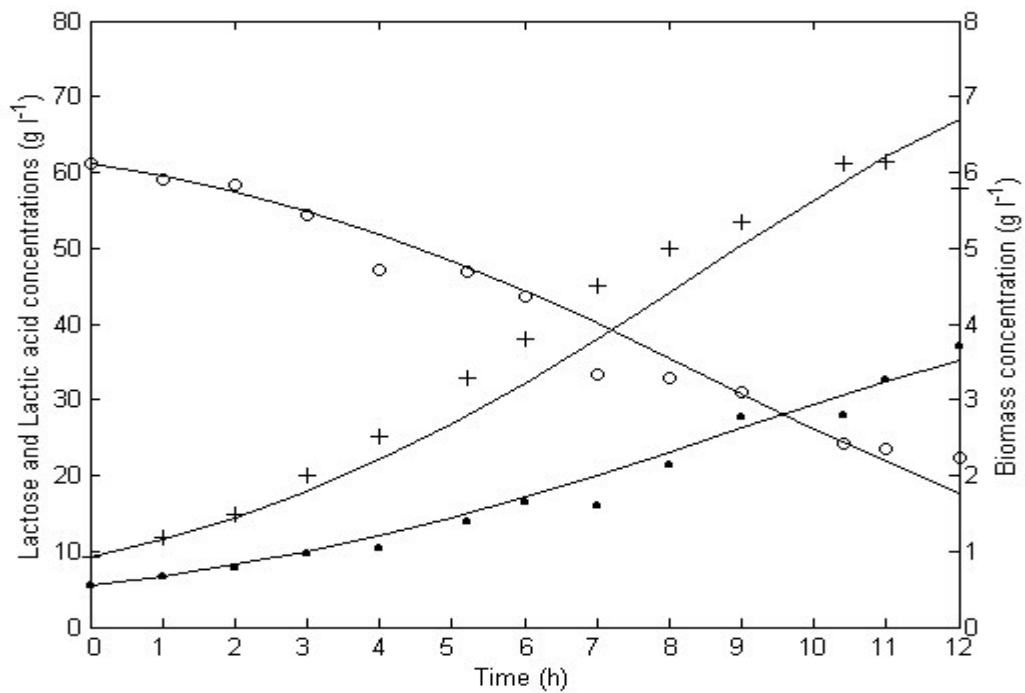

Figure 5.18. Model and experimental data for 61.2 g l⁻¹ initial substrate concentration; experimental biomass concentration (+); experimental product formation (•); and experimental substrate utilization (○) and straight lines over them are model outputs
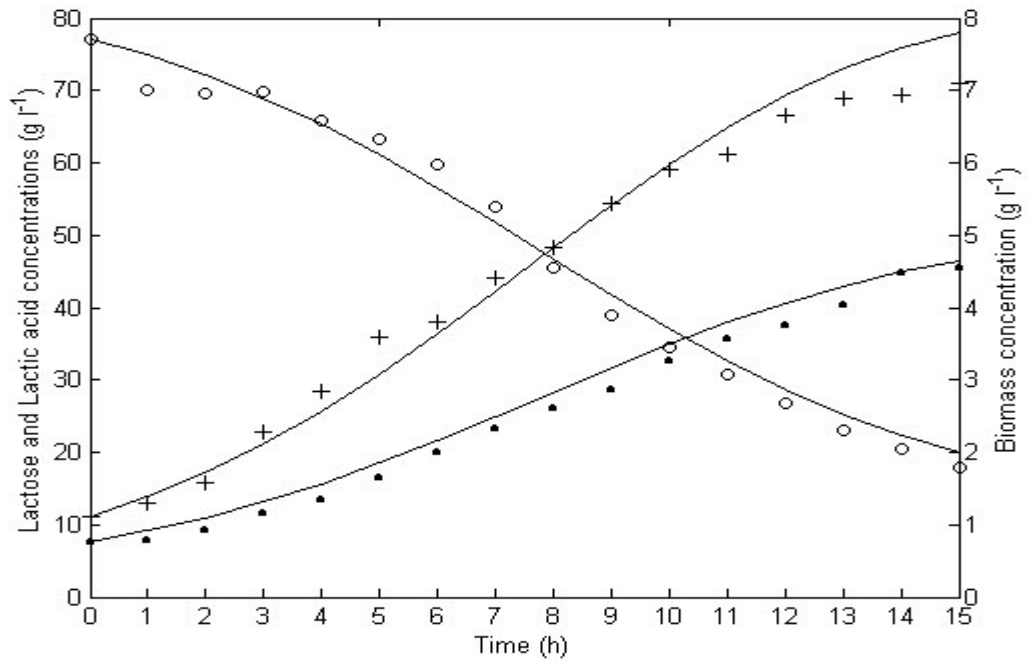
Figure 5.19. Model and experimental data for 77.1 g l^{-1} initial substrate concentration; experimental biomass concentration (+); experimental product formation (•); and experimental substrate utilization (o) and straight lines over them are model outputs

Figure 5.20. Model and experimental data for 95.7 g l^{-1} initial substrate concentration; experimental biomass concentration (+); experimental product formation (•); and experimental substrate utilization (o) and straight lines over them are model outputs
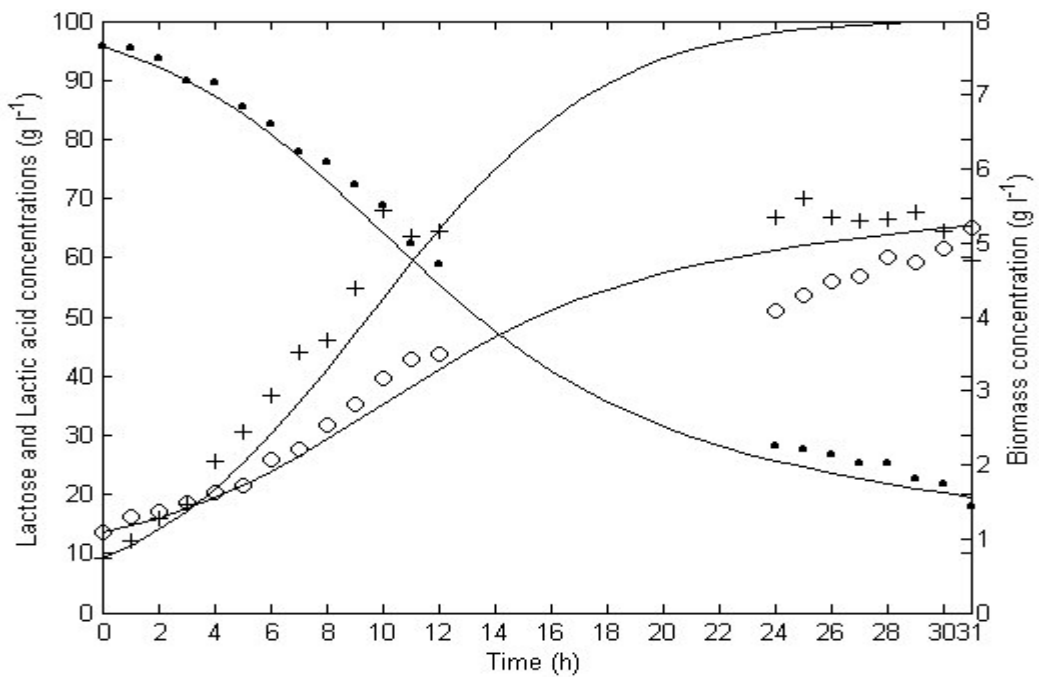
The weighted sum of squares of errors (SSE) and correlation coefficients (R^2) are given in Table 5.5 along with model parameters, α, β, f, and h by using Model 9.

Table 5.5. Model parameters, sum of squares of errors and correlation coefficients for different fermentation runs

S_0, (g l^{-1})	α_{cal}	β_{cal}	f	h	SSE	R^2		
						biomass	product	substrate
9.0	3.3	0.06	0.2	0.3	0.1116	0.9443	0.9839	0.9877
21.4	3.61	0.06	0.5	0.5	0.0873	0.9762	0.9836	0.9919
35.5	3.87	0.06	0.5	0.5	0.1281	0.9861	0.9767	0.9890
48.0	4.32	0.06	0.5	0.5	0.0948	0.9752	0.9784	0.9967
61.2	4.69	0.06	0.5	0.5	0.1258	0.9529	0.9812	0.9636
77.1	5.21	0.06	0.5	0.5	0.0804	0.9879	0.9956	0.9893
95.7*	5.73	0.06	0.6	0.5	0.1531	0.9654	0.9939	0.9948

* The sum of squares of errors and correlation coefficients were calculated for first 13h.

The kinetic parameters μ_{max}, K_S, Y_{PS}, m_S, X_{max} and P_{max} are given in Table 5.6. These values are very close to the parameters in literature, given in Table 3.2.

Table 5.6. Kinetic Parameters

Kinetic Parameters	Experimental Values
μ_{max} (h^{-1})	0.265
K_S (g l^{-1})	0.72
Y_{PS} (g lactic acid (g lactose)$^{-1}$)	0.682
m_S (g lactose (g biomass)$^{-1}$ h^{-1})	0.0015
X_{max} (g biomass l^{-1})	8
P_{max} (g l^{-1})	66

5.4. Productivity of the Fermentation Runs

The productivity values calculated with the experimental data in exponential phase were plotted against initial substrate concentrations as in Figure 5.21. The productivity values were calculated by the equation given below:

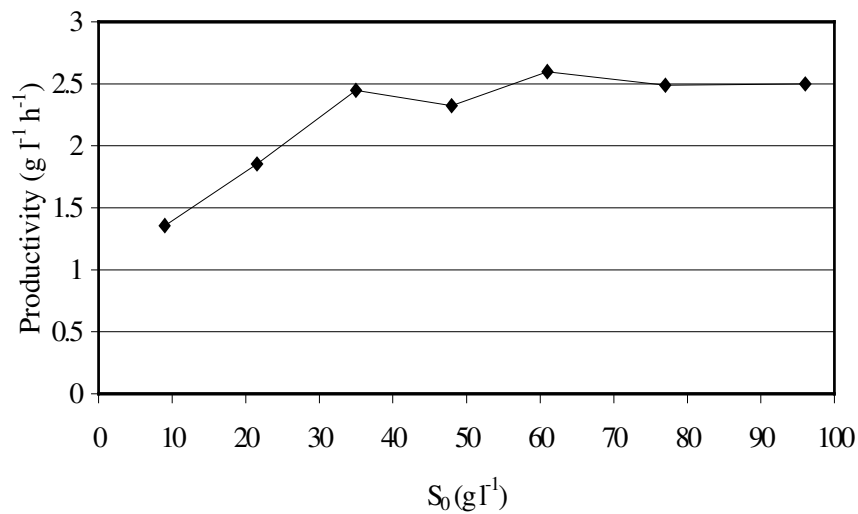$$\text{Productivity} = \frac{P_f - P_0}{t_f - t_0} \tag{5.10}$$

Figure 5.21. Productivity from experimental data in exponential phase

This plot indicates that the productivity remains constant at certain amount of initial substrate concentration. Actually, better explanation for productivity can be achieved by the complete substrate utilization. However, in this study, some of the fermentations were carried out without the utilization of all substrate. Thus, the product values when the substrate is zero were predicted by the model simulations by using Model 9. According to the prediction values of product, the plot of predicted productivity versus initial substrate was obtained as in Figure 5.22.
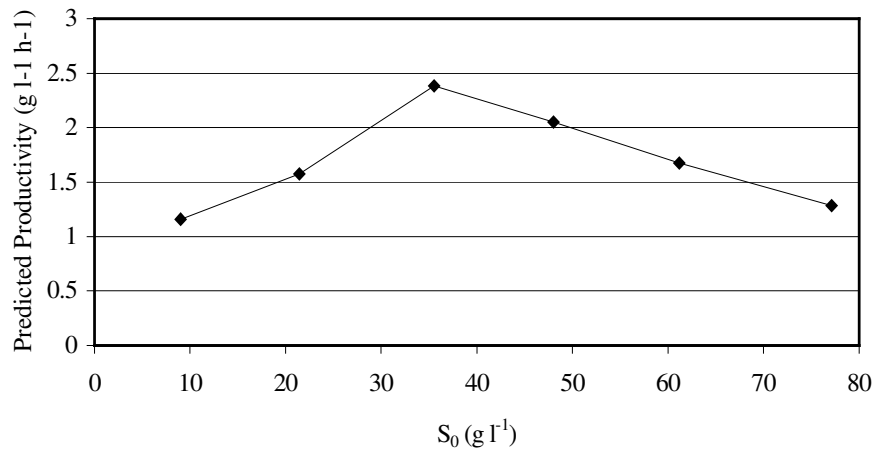
Figure 5.22. Predicted productivity values calculated from the data when the substrate concentration is zero.

It was observed that the maximum productivity was achieved in the fermentation with 35 g l^{-1} initial substrate concentration. This idea is supported by the discussion in Section 5.1. that the maximum conversion of substrate to product was also achieved in that fermentation.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

The fermentation of whey lactose by *Lactobacillus casei* to produce lactic acid, which is a widely used organic acid, was modelled by kinetic equations of biomass growth, product formation and substrate consumption.

The kinetic parameters of lactic acid productions were determined by series of batch fermentation experiments with seven different initial lactose concentrations in a range from 9.0 g l^{-1} to 95.7 g l^{-1}. The maximum specific growth rate, μ_{max}, was obtained as 0.265 h^{-1}. The Monod constant, K_S, was calculated from the Lineweaver-Burk plot as 0.72 g l^{-1}. The product on substrate yield values were very close in different fermentations, so the average value of 0.682 was used in models.

In the model development part, initially different model equations were used such as Monod and logistic equations for biomass growth, and Luedeking and Piret equation for product formation. The substrate utilization rate equation was based on the substrate utilized for product formation, biomass growth and maintenance. Also, the equation for specific growth rate was modified by including a product inhibition and substrate inhibition terms. It was seen that, the addition of biomass and product inhibition terms promised a better fit of the model simulation data to the experimental data. The rate equations including biomass growth, lactic acid production and lactose utilization were solved numerically by Runga-Kutta method.

By using modified logistic equation with product inhibition term for biomass growth, Luedeking and Piret equation for product formation and substrate consumption equation for product formation and maintenance, the most reliable and applicable model was developed. The toxic powers in these inhibition terms made the model applicable for the fermentation experiments with low and high initial substrate concentrations when the inhibition effects became negligible and significant, respectively.

In further studies the effect of pH and temperature on kinetic parameters should be investigated and included in kinetic models. Moreover, the model used in this study (Model 9) should be developed in order to express the fermentations with high substrate concentrations better. Also, if this unstructured model could be gathered with the structured model then the growth kinetics of a culture would be completely described. A

chemically structured model divides the cell mass into components. If the ratio of these components change in response to disturbances in the extracellular environment, then the model behaves analogously to a cell changing its composition in response to environmental changes.

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APPENDICES

APPENDIX A. THE COMPOSITION OF FERMENTATION MEDIA AND THE PRODUCERS OF CHEMICALS

Table A.1. The Composition of Fermentation Media and the Producers of Chemicals

Media Component	Producer	Amount
Litmus Milk Culture	Difco	12.5 % (v/v)
Yeast Extract	Acumedia	1 % (w/v)
Potassium dihydrogen phosphate (KH_2PO_4)	Merck	0.05 % (w/v)
Potassium phosphate (dibasic) (K_2HPO_4)	Sigma	0.05 % (w/v)
Magnesium sulfate ($MgSO_4$)	Sigma	0.002 % (w/v)
Manganese sulfate monohydrate ($MnSO_4.H_2O$)	Merck	0.005 % (w/v)
Sodium hydroxide (NaOH) for fermenter	Merck	10N
Calcium carbonate ($CaCO_3$) for shake flask ferm.	Merck	3g/125ml media

APPENDIX B. CALIBRATION CURVES

B.1. Calibration Curve for Lactose
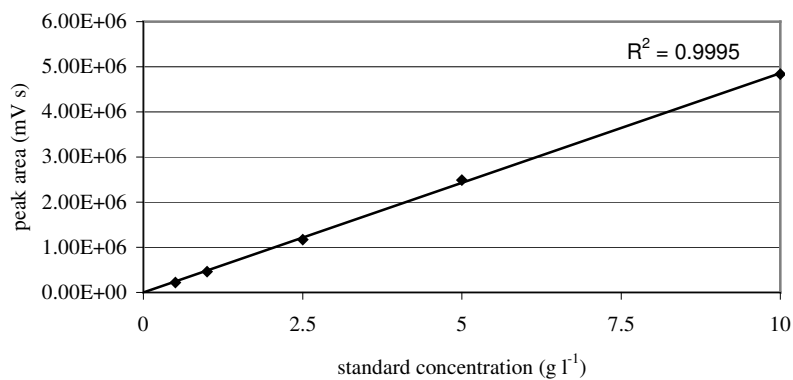
Figure B.1. Calibration curve for Lactose standard

B.2. Calibration Curve for Lactic Acid
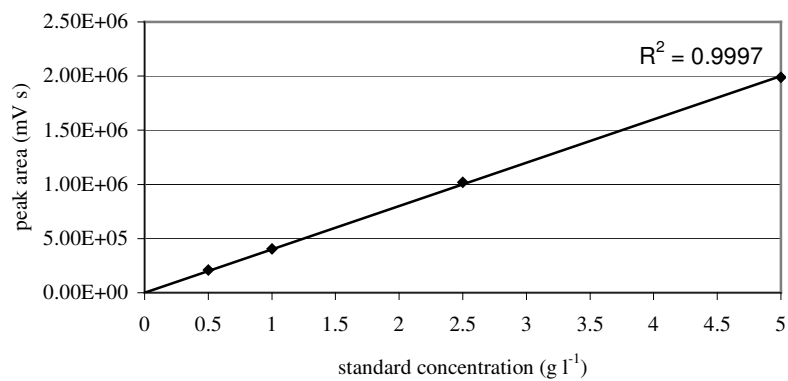
Figure B.2. Calibration curve for Lactic Acid standard

B.3. Calibration Curve for Dry Cell Weigh Measurements
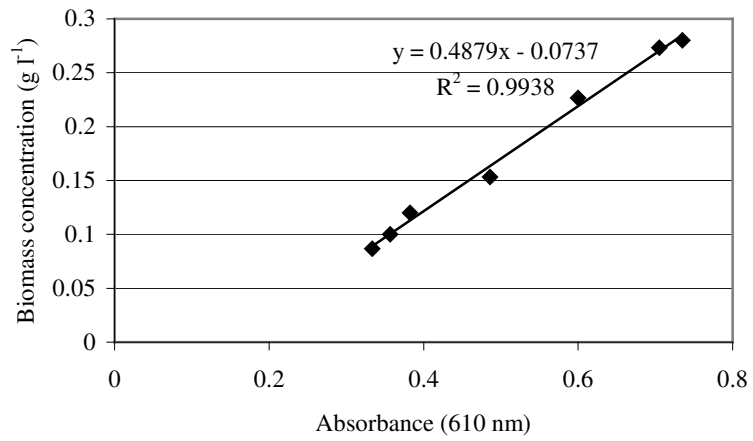
Figure B.3. Calibration curve for dry cell weight measurements

APPENDIX C. LOWRY METHOD (TOTAL PROTEIN ASSAY)

C.1. Procedure

Reagents:

    (a) 1 % $CuSO_4.5H_2O$.

    (b) 1 % NaK tartrate.$4H_2O$.

    (c) 2 % Na_2CO_3 in 0.1 M NaOH.

    (d) Commercial Folin-Ciocalteau reagent (sodium tungstate, sodium molybdate, phosphoric acid, hydrochloric acid).

On the day of use, mix solutions (a) and (b) in a 1:1 ration and then dilute with 98 volumes of (c), to form the diluent. Dilute (d) 1:1 with distilled water to form the color reagent.

Solutions (a), (b) and (c) are stable at room temperature for months. Solution (d) is stable at 4 ^{o}C for months.

Method:

    (i) Prepare a 0.2 ml sample (30-200 μg protein) in water.

    (ii) Add 2.1 of diluent (alkaline copper reagent).

    (iii)Allow to stand for 10 min.

    (iv)Add 0.2 ml of color reagent and mix immediately.

    (v) Allow to stand at room temperature (preferably in a dark place) for 1 h and read the A_{550}, against blank containing water and reagents. The color is stable (in the dark) for several hours.
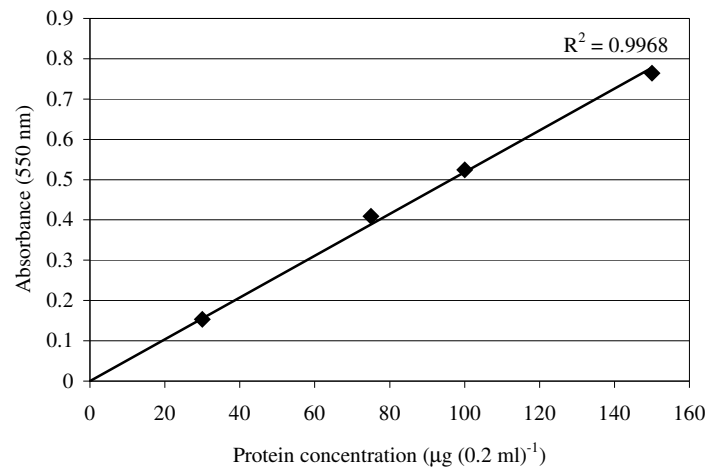
C.2. Calibration Curve for BSA

Figure C.1. Calibration curve for BSA standard

APPENDIX D. THE RESULTS OF DIFFERENT MODELS FOR SAMPLE FERMENTATION, 21.4 g l^{-1} INITIAL SUBSTRATE CONCENTRATION
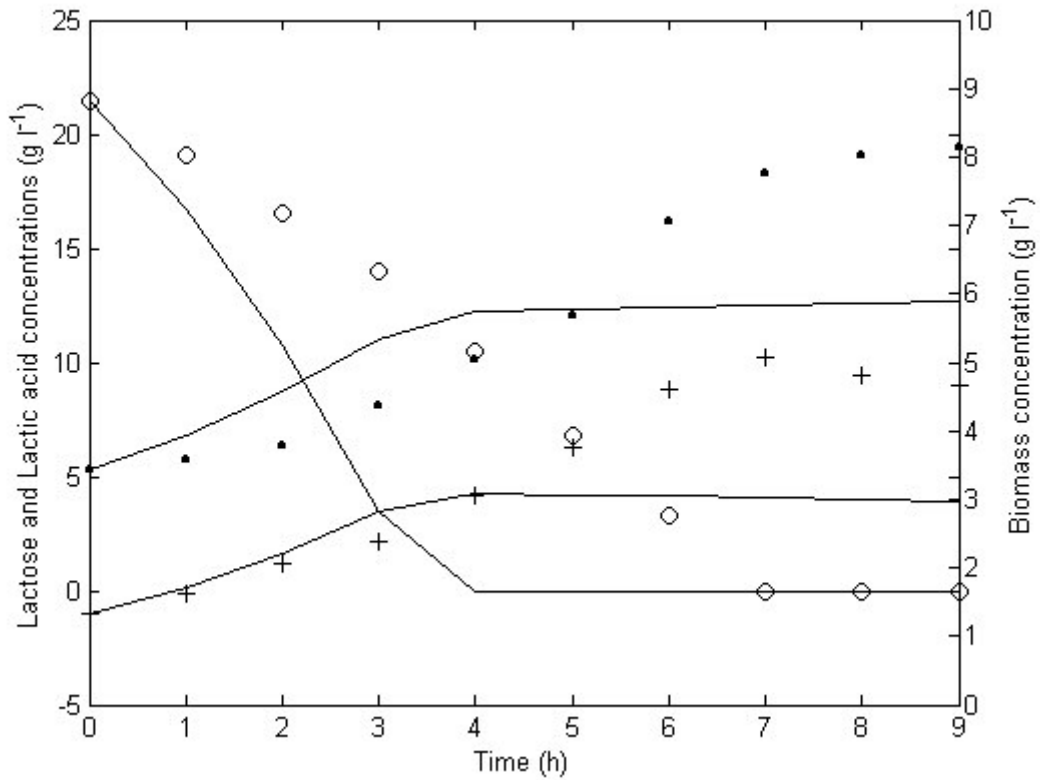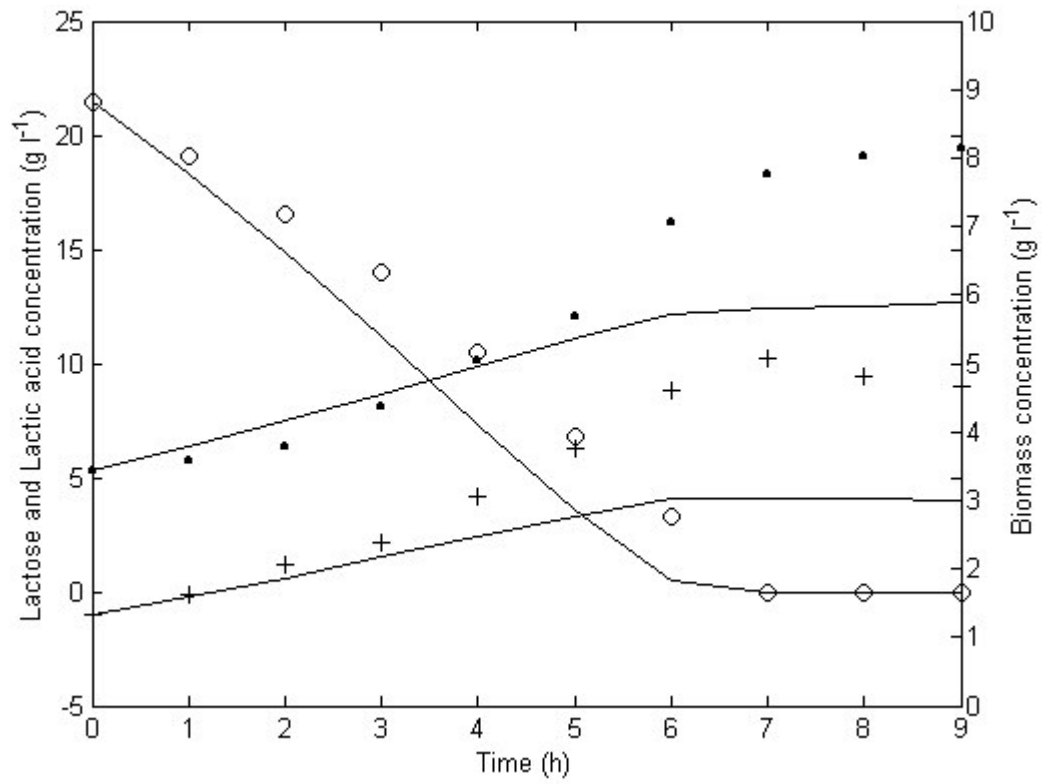
D.1. Result of Model 1

Figure D.1. Model 1 and experimental data for 21.4 g l^{-1} initial substrate concentration; experimental biomass concentration (+); experimental product formation (•); and experimental substrate utilization (o) and straight lines over them are model outputs
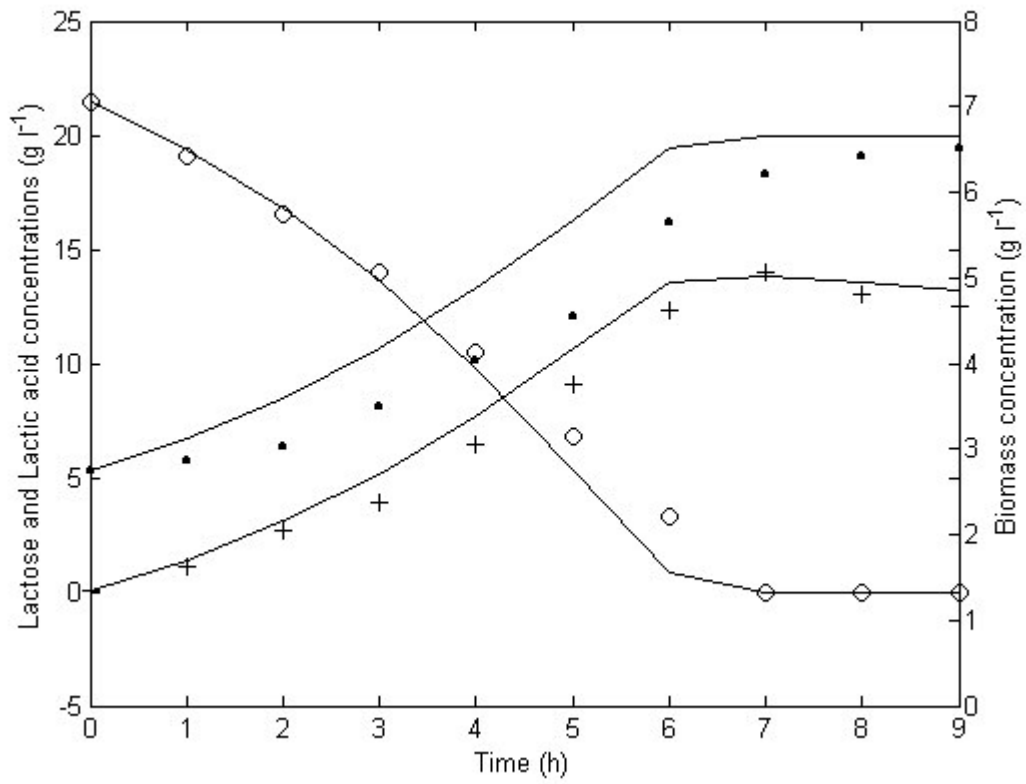
D.2. Result of Model 2

Figure D.2. Model 2 and experimental data for 21.4 g l^{-1} initial substrate concentration; experimental biomass concentration (+); experimental product formation (•); and experimental substrate utilization (o) and straight lines over them are model outputs
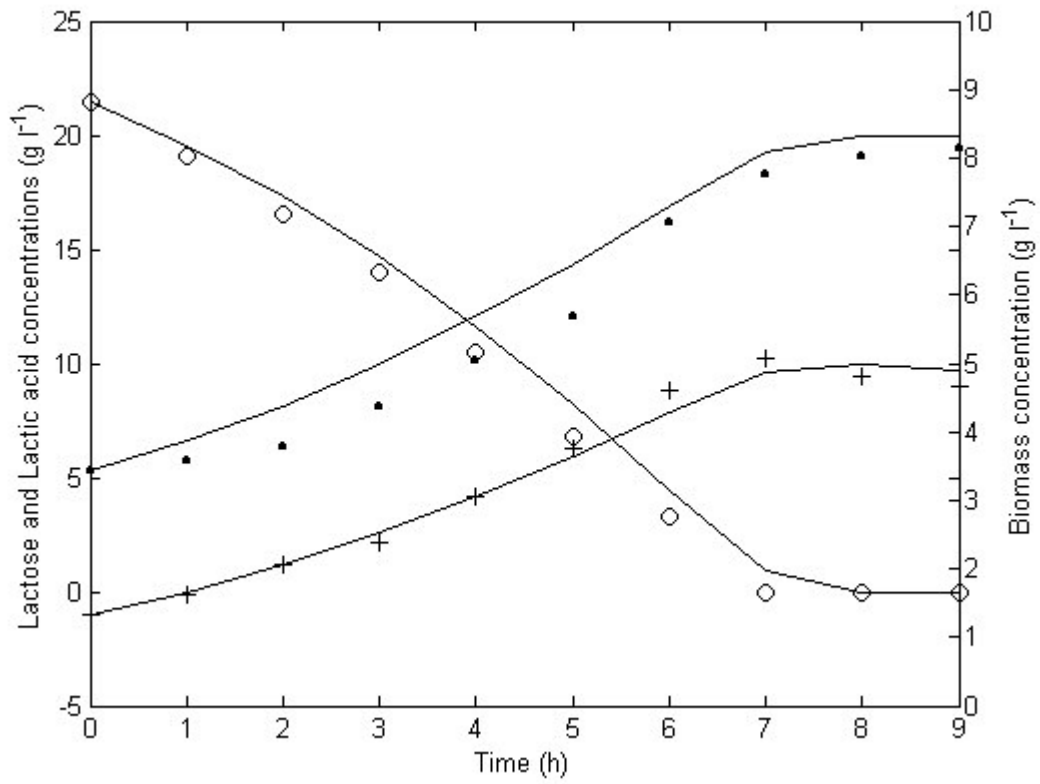
D.3. Result of Model 3

Figure D.3. Model 3 and experimental data for 21.4 g l^{-1} initial substrate concentration; experimental biomass concentration (+); experimental product formation (•); and experimental substrate utilization (o) and straight lines over them are model outputs
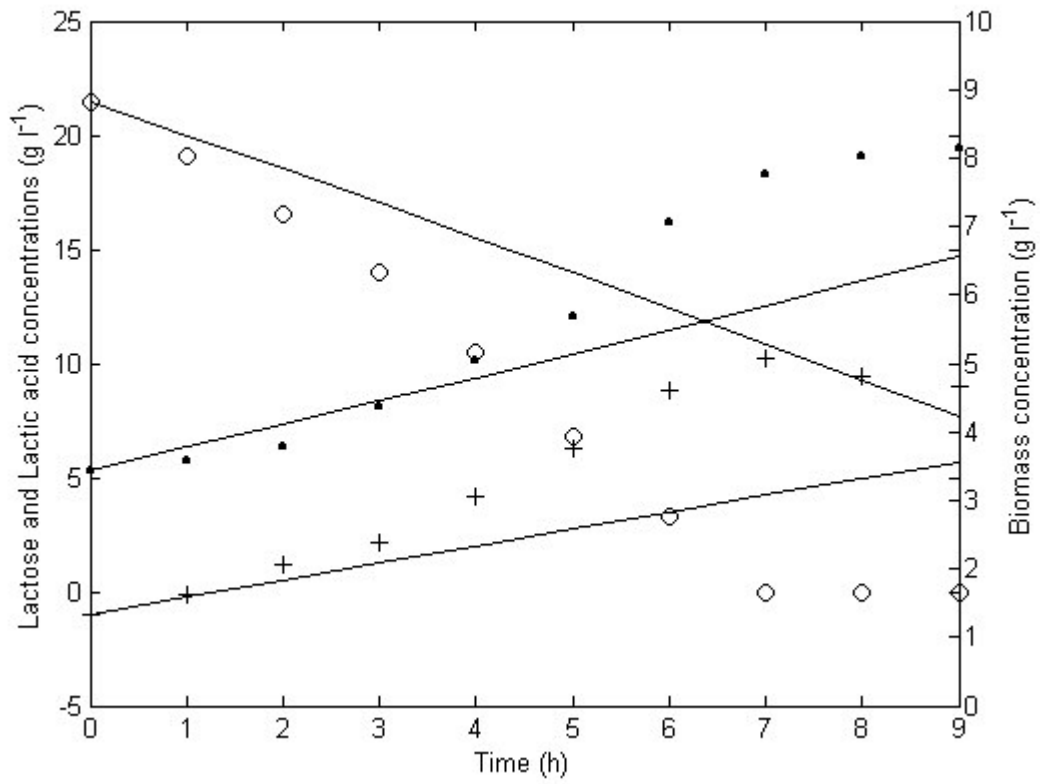
D.4. Result of Model 4

Figure D.4. Model 4 and experimental data for 21.4 g l⁻¹ initial substrate concentration; experimental biomass concentration (+); experimental product formation (•); and experimental substrate utilization (o) and straight lines over them are model outputs

D.5. Result of Model 5

Figure D.5. Model 5 and experimental data for 21.4 g l^{-1} initial substrate concentration; experimental biomass concentration (+); experimental product formation (•); and experimental substrate utilization (o) and straight lines over them are model outputs
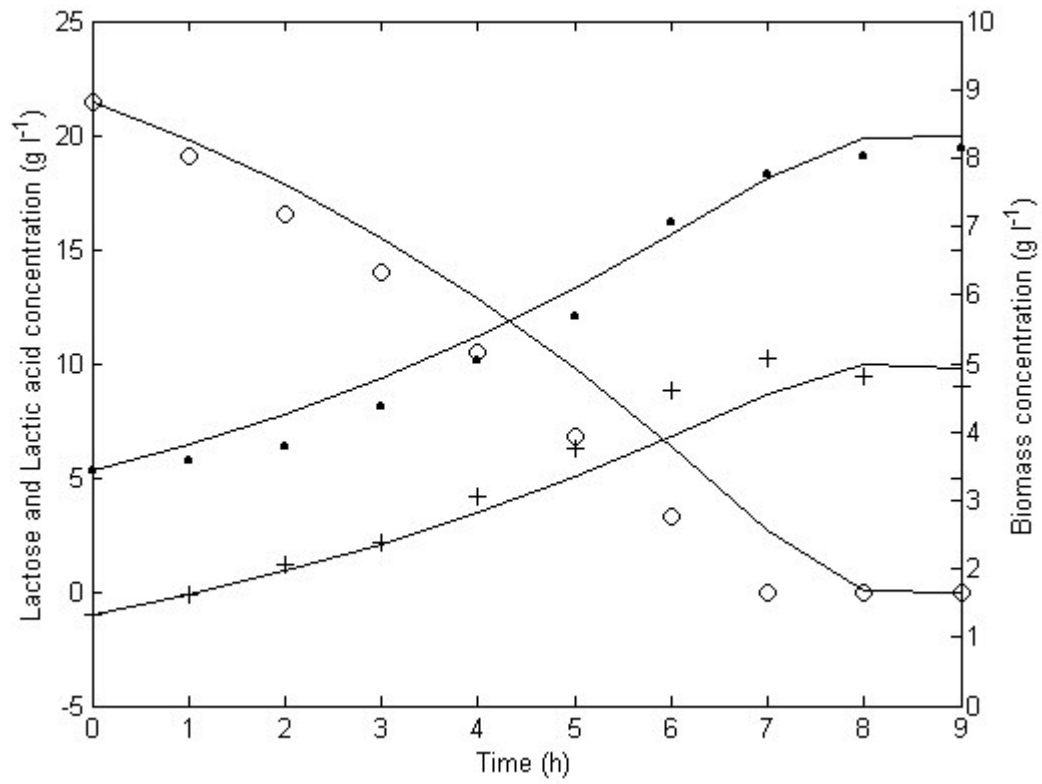
D.6. Result of Model 6

Figure D.6. Model 6 and experimental data for 21.4 g l^{-1} initial substrate concentration; experimental biomass concentration (+); experimental product formation (•); and experimental substrate utilization (o) and straight lines over them are model outputs
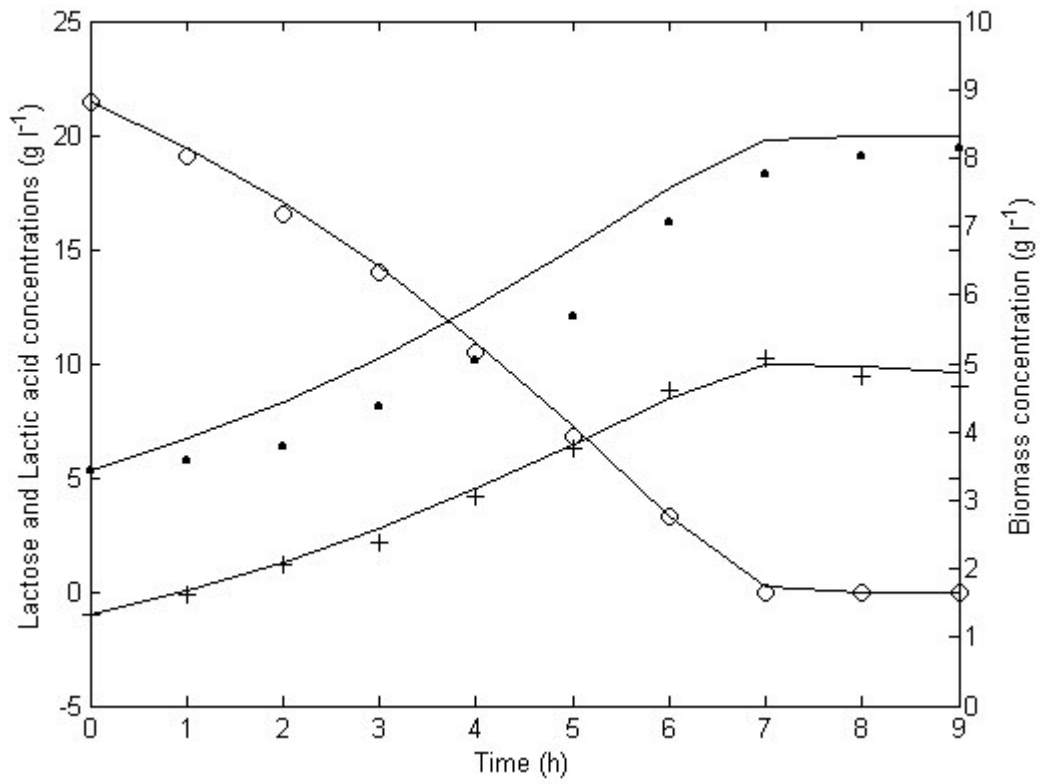
D.7. Result of Model 7

Figure D.7. Model 7 and experimental data for 21.4 g l^{-1} initial substrate concentration; experimental biomass concentration (+); experimental product formation (•); and experimental substrate utilization (o) and straight lines over them are model outputs
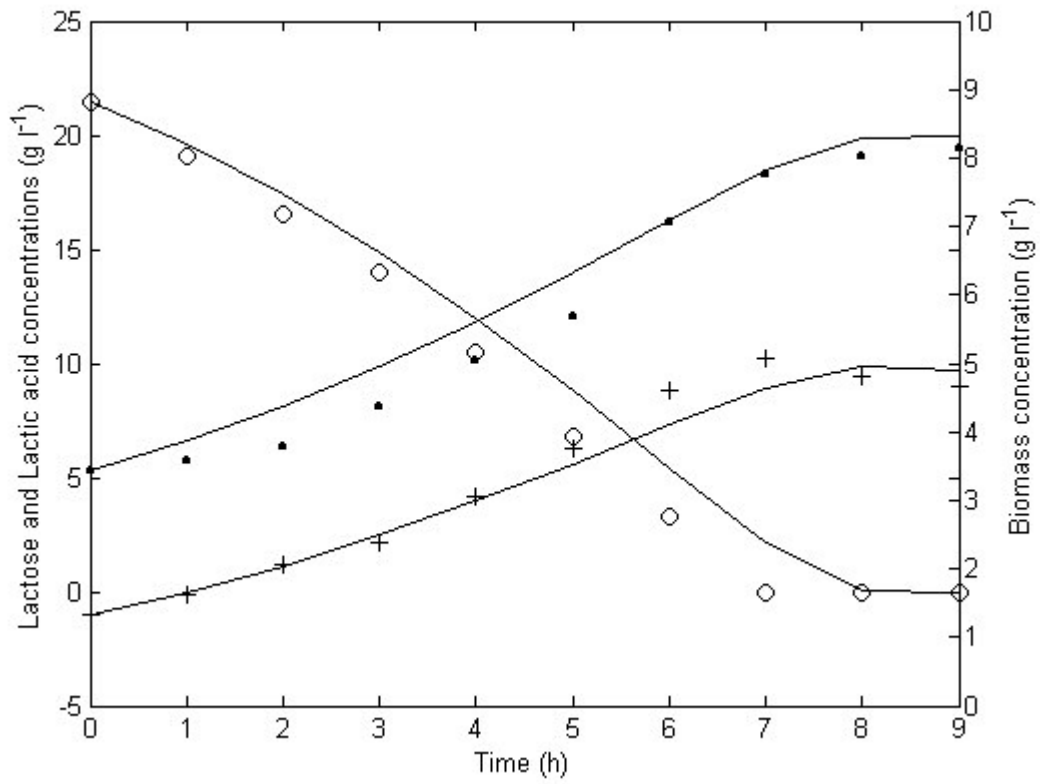
D.8. Result of Model 8

Figure D.8. Model 8 and experimental data for 21.4 g l^{-1} initial substrate concentration; experimental biomass concentration (+); experimental product formation (•); and experimental substrate utilization (o) and straight lines over them are model outputs

APPENDIX E. THE INITIAL BIOMASS, SUBSTRATE AND PRODUCT CONCENTRATIONS FOR FERMENTATION RUNS

Table E.1. The Initial Biomass, Substrate and Product Concentrations for Fermentation Runs

Initial lactose conc. (g l^{-1})	Initial biomass conc. (g l^{-1})	Initial lactic acid conc. (g l^{-1})	Ferm time(h)
9.0	1.2	4.2	4.5
21.4	1.3	5.3	9
35.5	1.1	3.0	11
48.0	1.0	6.0	14
61.2	0.9	5.6	12
77.1	1.1	7.6	15
95.7	0.7	13.7	31