Intensive propagation of the lactic acid bacterium Lactococcus lactis.

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Intensive Propagation of the lactic acid bacterium Lactococcus Lactis

by

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A Thesis Submitted in Partial Fulfilment of the Requirements for the Degree

Master of Philosophy (M.Phil.)

October 2006

Abstract

L.lactis is an important lactic acid bacterium, widely used in the dairy industry nowadays. The coccus is used as a natural acidifier for the inoculation of bulk quantities of milk in order to produce a variety of fermented products. As such, large quantities of its biomass are necessary. The possibility of producing the cellular biomass of the coccus in mass quantities was investigated through several techniques. Firstly, the bacterium was grown into simple batch cultures without pH control where the physicochemical needs of the coccus were determined. Through the determination of the optimum nutritional conditions for the propagation of the coccus, an optimised medium for growth occurred.

The growth efficiency on the medium was tested on a 2L STR reactor operated batchwise with continuous pH control. The optimum pH conditions for the growth of the coccus were determined as well as parameters such as cellular yield coefficient, substrate and starter inoculum concentration.

The metabolism o the coccus was determined as homofermentative, mainly producing lactic acid through measurements of the organic acids produced at the end of the fermentation process in the STR. In addition carbohydrate consumption rate in the optimum pH values selected was measure and the constant coefficients for substrate consumption end product inhibition effect and a maintenance coefficient term was determined. A simple mathematical model was constructed to describe the growth of the coccus batchwise and a correlation was made between the experimental data obtained from the STR fermentations and the theoretical predictions. An inhibition term was incorporated in the model in order to describe the inhibitory effect of lactic acid over the growth. With the use of the inhibition term a good fit between the experimental data and the model was obtained.

The growth kinetics of *L.lactis* were further investigated by modelling its growth in a continuous system and in a Membrane Bioreactor system. The feasibility and the efficiency in all 3 systems was evaluated in terms of the volumetric cell productivity. (g/l/h). The models were also tested against different substrate concentrations, different starter inoculums, different dilution and flux rates. The MBR system has found to be highly productive especially when operated in the continuous mode of substrate feed , the volumetric cell productivity (g/l/h) (45.94 g/l/h) was over 10 times higher when compared with the volumetric cell productivity (g/l/h) given by the continuous system (1.4 g/l/h)and over 30 times when compared with the batch system(0.45 g/l/h). MBR was proven to be a possibly useful system for the development of high concentrations of cellular biomass but its practical application has to be further investigated.

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I would like to thank deeply Dr. R. W. Lovitt, Department of Chemical Engineering for his continuous professional supervision, guidance and support

I would like to thank Dr. Ilseon Jung, Department of Chemical Engineering, for his guidance and support throughout the experimental procedures of this project.

I would like to thank Dr. M. Jones, Department of Chemical Engineering, for his support on the MBR

I would like to thank Miss Karnika Ratanaponglika for her help throughout this project.

And finally I would really like to thank my aunt, Roi Chroistophilopoulou and all of my friends for their continuous support, love and help throughout his academic year.

TABLE OF CONTENTS

Table of Contents

Table of Contents

Table of Contents

Table of Contents

Table of Contents

Chapter 1: Introduction

Chapter One: Introduction

1. Lactococcus lactis and its industrial importance

Lactococcus Lactis (L.lactis) belongs in the genera of Lactic Acid Bacteria (LAB), microorganisms whose distinctive ability is to produce lactic acid as a major end product of their metabolism. This process is done via fermentation (zymosis, anaerobic catabolism of carbon compounds) of carbohydrates existing into several types of food products.

L.lactis has been graded as a non pathogenic bacterium for humans. (GRAS, Grade One). (Skinner, 1974) This fact enables its wide use in the food industry, as a natural acid–production bioreactor. It is used especially in the dairy industry, for the manufacture of cultured milk products such as cheese, yogurt, and butter and in a variety of similar natural milk-derived products. *L.lactis* is used in the form of a starter culture and it is inoculated in the milk after the pasteurization process.

Its presence is extremely important in order to produce fine quality dairy products. The use of *L.lactis* assures the production of enough lactic acid which will lower the pH point of milk (originally 6.5 to 6.9) to an acidic point (usually between 4.8 to 4.9) (Frazier, 1978).At this point a secondary starter culture of different microbial strains can be added so as to produce the desirable flavor, taste, texture and odor of the dairy product. *L.lactis* strains can also be used as a single starter culture in milk products which do not need long ripening conditions.

The presence of *L.lactis* is also vital because of the production of nisin during metabolism. Lactococcal nisin inhibits the development of other pathogenic microbial strains and also helps in the preservation for longer periods of the dairy products. In this chapter the industrial importance of *L.lactis* will be analyzed.

1.1 Starter Cultures of Lactic Acid Bacteria (LAB)

Starter cultures used in dairy industry are carefully selected and propagated cultures of known strains of bacteria or yeasts in order to produce the suitable type of fermentation (homolactic, heterolactic, citrate etc.) .The starter cultures either consist of one pure strain of bacteria or yeasts or of a combination of strains of different microbial species.

There are three types of starter cultures according to the number of microbial strains used:

- Single strain culture: One strain of one single specie
- Multiple strains culture: Two or more strains of one specie
- Mixed strains culture: One or more strains of two or more species

Another important factor for the selection of the starter culture is the genetical stability and the biomass production of each organism used. Starter cultures should be genetically pure and not producing any off flavor effects or mutants which may affect the quality of the dairy products (Mitsuoka, 1971, Mocquot, 1971).

1.1.1 Basic Functions and Advantages of Usage of Starter Cultures

A high quality starter culture can provide the following functions:

1. In the field of biopreservation and avoidance of spoilage:

- Extended preservation.
- Higher quality and safety standards. The growth of other pathogenic microorganisms is inhibited.

2. In the field of sensory properties a starter culture can provide the food product with the following substances:

- Organic acids such as citric acid, lactic acid
- Carbonyl compounds such as acetaldehyde
- Participation in hydrolysis of proteins and/ or fats

3. In the field of texture properties:

- Gas production (CO_2)
- Color

The proper use of a starter culture can also contribute to the development and preservation of probiotic flora, for example genus *Bifidobacterium*, which has been proved to be of high value over the protection of intestinal tract from various diseases including colorectal cancer .Also many strains of *Lactobacilli* which are used as starter cultures, are in current use for therapeutic purposes (Frazier, 1978).

The main advantages of the use of starter cultures versus the traditional process of souring naturally the milk are the following:

1. Inhibition of undesirable effects of the secondary end products of bacterial metabolism such as bitter flavor due to alcohol production.

2. Stop of economic losses as there is a much smaller possibility for the production of curds to fail.

3. A standard high quality of products can be achieved as the function of starter culture is well known and its activity can be well predictable

4. A starter culture can also serve as a "protective shield" towards bacteriophage attack; coli form bacteria and other pathogenic organisms (Casida, 1968).

1.1.2 Starter Cultures Microorganisms

The basic microorganisms used as starter cultures in the contemporary dairy industry are the following:

Primary Starters	• Genus *Lactococcus* *L.lactis, L.cremoris, L.lactis var diacetylactis* • Genus *Leuconostoc* *L.mesenteroides var cremoris, L.mesenteroides var dextranium* • Genus *Lactobacillus* *Obligatory Homofermentative* *L. delbruckii var lactis, L. delbruckii var bulgaricus, L.helveticus,L.acidophilus, L.gasser, L.johnsonii* *Facultative Heterofermative* *L.casei var casei, L.paracasei var paracasei, L.rhamnosus, L.plantarum* *Obligately Heterofermentative Lactobacilli* *L.brevis, L.fermentum,L.kefir, L.renteri* • Genus *Pediococcus* • Genus *Streptococcus* *S.Salivarius var thermophilus*
Secondary Starters	• Genus *Bifidobacterium* • Genus *Propionobacterium* • Genus *Brevibacterium* • Yeasts • Moulds

. (Frazier, 1978, Fields, 1979)

The primary starter culture is completely responsible for the lactic acid fermentation where the secondary starters are mainly responsible for other functional properties

such as propionic or acetic fermentation or surface ripening (Ayres, Mundt& Sandrine, 1980).

1.1.3 Technological Properties of Starter Cultures

Nowadays, starter cultures are produced into specialized laboratories in a ready to use form. It is highly desirable, to produce large quantities in aspect of biomass of the starters as to be able to inoculate them in a larger volume of milk. Starter cultures can be obtained in the following forms:

- Liquid Starter Cultures
- Dried Starter Cultures

1. Spray- dried concentrated or unconsecrated cultures
2. Freeze-dried or lyophilized concentrated or unconcentrated cultures.

A freeze-dried culture the most commonly used form of starter culture in dairy industry

- Frozen Starter Cultures

1. Deep frozen at -40°C
2. Ultra-low temperature frozen in liquid nitrogen at -196°C

- Immobilized Starter

1. The selected bacterium or fungus in Ca^{2+} alginate beads, suitable for continuous fermentation process.

(Rose, 1978; Fields, 1979,; Nickerson & Sinskey, 1972).

1.2 Use of L.lactis in the Contemporary Dairy Industry

As previously mentioned, *L.lactis* is used as a starter culture in a wide variety, cultured milks, fermented butter creams, creams and other similar products. Their role in the process of cheese manufacture and the role of *L.lactis* will follow. (Table 1.1)

A table of the use of *L.lactis* in the several kinds of cheese is shown in is the following:

Cheese Category	*Example Varieties*	*Moisture Category*	*Starter Composition*	*Starter Function*	*Secondary Flora*
Unripened soft	• Cottage • Mozzarella	>80% >50%	*L.lactis,L.lactis var diediacetylactis,* *Leuconostoc spp.,* *Str.thermophilus,* *L.bulgaricus*	Acid and Diacetyl production	none
Ripened soft /semihard	• Cammambert • Brie • Caerphilly • Limburg	48% 55% 45% 45%	*L.cremonis,* *L.lactis,L.lactis var diacetylactis* *Leuconostoc spp.*	Acid and Diacetyl production	None
Hard	• Cheddar • Gouda	≈40%	*L.cremonis,* *L.lactis,L.lactis var diacetylactis* *Leuconostoc spp.*	Acid production CO2 production	Penicillium caseilolum, Yeast, Lactobacilli, Brevibacterium linens, Pediococcoi, Propionobacteria
Blue vein	• Roquefort • Gorgonzola • Stilton • Blue	40%-45%	*L.cremonis,* *L.lactis,L.lactis var diacetylactis* *Leuconostoc spp.*	Acid production CO2 production	Penicillium Roqueforti, Yeasts Micrococcoi

Table 1.1: Use of L.lactis in Cheese manufacturing (Rose, 1978)

1.2.1 Cheese Manufacture

Cheese is milk derived product and a very important element of human diet. Its high nutritive value and the variety in flavor and taste have contributed to its constantly elevated consumption (Board, 1983).

Milk is mainly consisted of water, proteins, lipids, carbohydrates (mainly lactose), organic compounds, salts, minerals and vitamins. It is a fluid, an emulsion of fat globules and a suspension of casein micelles all suspended in an aqueous phase environment (Ayres, Mundt & Sandrine, 1980). Milk's pH is about 6.6 to 6.9, osmotic pressure at about 700 kpa, ionic strength at 0.08 molar (M) and its water activity (Aw) 0.993(May, 1978). The water quantity and also the amount of its compounds varies according to the species of animals from which is extracted .For example cow milk is consisted of 87% of water, milk fat can range between 1% to 50%, proteins between 1% to 14% and lactose from trace to 7% (Roberts &Skinner, 1983).

The primary carbohydrate in milk is lactose; a disaccharide consisted of two monosaccharides of dextrus form, galactose and glucose, which are bonded together via β-1, 4 glycosidic linkage (Fytou-Pallikari, 1997). Other carbohydrates are also present in milk. Small concentrations of free glucose and galactose have been detected and also other sugars such as amino sugars, sugar phosphates, neutral and acid oligosaccharides and nucleotide sugars have been detected. Lipids in milk are mainly represented by triglycerides (TG).TG are chemical esters of glycerol meaning that they are consisted by three molecules of fatty acids covalently bound to each other and through esteric bonding to a glycerol molecule (White, 2000). The major protein molecule in milk is casein. Casein is a phosphoprotein consisted by a relatively high number of proline peptides .It is a relatively hydrophobic molecule which cannot be easily denaturated due to the absence of disulphide bonds. There are four types of casein in milk, α- casein, β- casein, σ- casein and κ- casein. All these types of casein have similar chemical structure and physical properties. In raw milk casein exists in a multi molecular form of granular structure named casein micelle (Ayres, Mundt & Sandrine 1980, Board, 1983). This micelle also contains water and salts mainly Ca^{2+} and P. Other proteins such as β-lactoglobulin and α-lactalbumin have been also identified. Milk minerals are mainly calcium Ca^{2+} and P and the vitamins contained are A, D, E, K and B (Mandolstan & MacQuillen, 1973, May, 1978).

1.2.1.1 Curdling the Milk

The initial treatment of milk results in the formation of firm curd (gelatinous material consisted mainly of coagulated casein) (Pelczar& Reed, 1974). The curdling of milk is the result of the addition of a starter culture of LAB and of the enzyme rennin. *L.lactis* and *L.cremoris* are usually added and as being mesophilic bacteria, the heat treatment after pasteurization should not exceed 38°C. If the heat treatment is between 20°C to 30°C, a high population development of *Lactococci* will occur within hours. This very rapid increase may lead to gas or off flavor production .On the other hand a heat treatment between 10°C to 20°C will end into slower development of population of LAB within few days usually, and mainly to the production of lactic acid. Starters should multiply from 10^7 cfu/ml to 10^8 CFU/g of curd in order to complete the conversion of milk into acid curd. (Pelczar & Reed, 1972). Rennin attacks κ-casein which functions in milk as a protective colloid to keep the other casein fractions (α-, β-, σ-) in suspension. During cheese making, rennin converts the κ-casein molecule (molecular weight 30.000 d) into to two molecules of molecular weight of 22.000 d and 8.000 d. (Board, 1983). The presence of lactic acid in this point is very important as it causes the precipitation of casein by reducing the pH below the protein's isoelectric point and eliminates the stabilising effect of repelling negative charges on the casein molecules, according to the following chemical reaction:

$$R_{NH_2} - COO + nH^+ \longrightarrow R_{NH_2} - COOH$$
$$pH\ 6.8 \longrightarrow \longrightarrow \longrightarrow \longrightarrow \longrightarrow pH\ 4.7$$

Rennin may be added either at the same time as the starter bacteria or after an initial ripening period of 30 to 60 minutes. (Frazier, 1978). Chemically rennin or chymosin E.C. 3.4.23.4 has a broad specificity to carbohydrates. It clots milk by cleavage of a single 105-ser-phe-met-ala-108 bond in the k-chain of casein. It belongs to peptidase family A1, has a weak proteolysis activity and it is formed from prochymosin. (Fytou-Pallikari, 1997; Drasar, & Hill, 1974).

When the curd is prepared using whole milk it chiefly consists of fats, proteins, minerals, vitamins and lactic acid. If prepared by skim milk in order to form the curd a watery fluid is developed which is called whey. Whey consists approximately at about 93% of water, other dissolved substances and 5% of lactose (Fields, 1979).

After milk curdling, the curd is separated from the whey by several techniques. (Nickerson & Sinskey, 1972; Frobisher, 1968).

1.2.1.3. Salting

NaCl is practically added to all varieties of cheeses, in some stage of production process. NaCl may be mixed with the drained curd or can be added in the surface of pressed form .The salting process contributes to flavor, to the control of moisture (dehydration) and prevents the development of undesirable bacteria. Commonly, a percentage between 2% to 4% of NaCl is added as the majority of LAB cannot withstand a larger amount. (Nickerson & Sinskey, 1972; Leadbetter, & Poindester, 1985).

1.2.1.4. Cheese ripening

Some types of cheese consist essentially of fresh curd eg. cottage cheese. Cream is worked into this type of curd and produces creamed cheese. This type of cheese is referred as unripened or soft cheese and in this form is offered for consumption. Most other types of cheese demand ripening process by bacteria or molds after the curd is pressed into form. Depending, whether a manufacture of hard or semi soft cheese will take place, cheese ripening is divided into 2 types. For hard cheese such as cheddar or swiss cheese the bacteria are distributed within the interior of the volume of the formed curd to be ripened. On the contrary, in soft cheese ripening, the microorganisms are distributed in the surface .Due to this difference; soft cheeses can be made only in small sizes since the enzymes of the bacteria which are responsible for ripening must diffuse from the surface where they are produced by the bacteria, to the interior (Rose, 1978; May, 1978).

During the process of cheese ripening several microbiological changes occur, as cheese ripening involves the growth of a mixed population of bacteria on a complex substrate (Ayres, Mundt & Saudrine, 1980).The main substances contained in the curd which are ripened are lactose, protein and lipids .The majority of lactose is degraded, during the first few days of ripening to lactic acid according to the following chemical reaction:

$$C_{12}H_{22}O_{11} + H_2O \xrightarrow{\text{LACTASE}} 2C_6H_{12}O_6 \xrightarrow{\text{SYSTEMS OF ENZYMES DERIVED BY BACTERIA}} 2CH_3CHOHCOOH$$

LACTOSE — GLUCOSE, GALACTOSE — LACTIC ACID

LAB, which are mainly responsible for the ripening process of hard cheese, do not elaborate potent extra cellular proteinases. But as they grow and die and autolyze small amounts of intracellular proteinases can be liberated. All the characteristics of the cheese referring to aroma, taste and flavor corresponding to each type of ripened cheese develop during this stage. Conditions of ripening which do not adequately favor development of the right kind of microorganisms will be reflected, in a product of inferior flavor and aroma or even of spoilage. (Board, 1983, Casida, 1968). The process of ripening involves also the complete proteolysis and lipolysis of the nitrogenous and fat constituents of the milk. The process of cheese manufacture can be described in tabloid form by the following diagram:

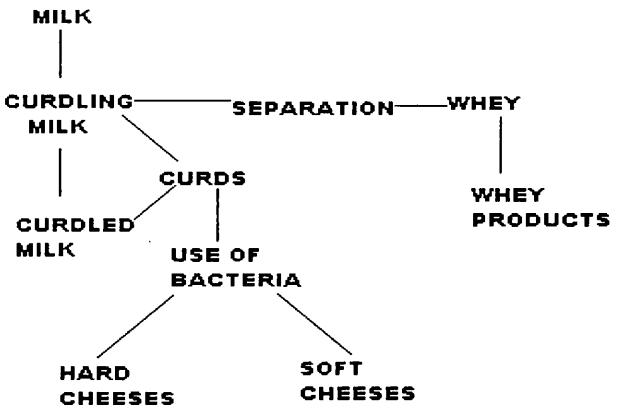


Figure 1.1. : Cheese manufacture: Separation Process (Fields, 1979)

1.3. Cottage Cheese

Cottage cheese is an unripened skimmed milk curd to which NaCl and sweet or cultured cream is added prior to consummation (Frazier, 1978).

For the manufacture of cottage cheese a mixed strain starter culture is used. It is consisted by strains of lactic acid producers, *L.lactis* and *L.cremoris*. In order to prepare the bulk starter culture, the starter milk is inoculated with commercially prepared, frozen concentrated culture of these starters. The bulk culture is prepared under at least 3 different sets of combinations between time of incubation, temperature and percentage of the inoculum (Board, 1983).

1.3.1 Cheddar Cheese

Cheddar cheese is made by whole unripened pasteurized milk. The formation of curds begins with the addition of a starter culture and rennin in the milk. Starters may be mixed strains of *L.lactis* or a mixture of *L.lactis* and *L.cremoris* .In cheddar cheese, the use of starters is vital, as the acidic reaction favors the function of rennin in order to curdle the κ-casein into pH 4.7, helps at the separation (cutting) of whey and favours the proper diffusion of the curd particles (Cutting, Carr, & Whiting, 1975).

The *Lactococci* remain active in the curd after pressing and reach their maximum population within 48 hours, and then their numbers starts declining. The flavour of cheddar cheese can also be affected by the use of *L.lactis var maltigenes* in the starter culture which offers a malty flavour. This flavour occurs by the formation of methyl isobutanol and methyl butanol from leucine and isoleucine. Another microorganism which may also influence the taste of cheddar cheese is *L.lactis var diacetylactis* by producing carbon dioxide from citric acid in the milk and contributes to the openness and gas pockets in the cheese (Fields, 1979).

1.3.2 Blue Cheese

There are several types of blue cheese according to the country of manufacture. In these varieties of cheese the milk is inoculated by a starter culture of several strains of *L.lactis* and *L.cremoris*. The LAB primarily produces cheese curd and a clear acid flavour, whether *Penicillium roqueforti* produces the typical flavour, taste, aroma and colour. The presence of LAB and their acid production provide the optimum conditions (acidic pH 4.5- 4.7, high salt content and oxygen conditions) for the proper growth of the mould (Rose, 1982).

1.4 Use of L.lactis in other dairy products

1.4.1. Fermented Milks

Fermented milks include cultured buttermilk, yogurt, Bulgarian buttermilk, acidophilus milk, kefir, kumis, skyr, taette and sour cream.

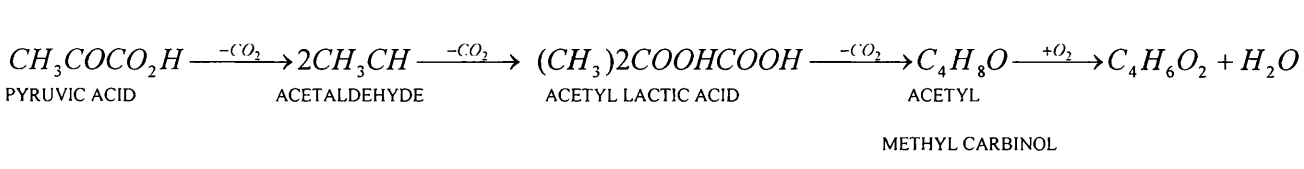
Cultured buttermilk, kefir and sour cream employ the action of mixed strain cultures with one strain being mainly responsible for the production of lactic acid while another one provides the aroma and the flavour .In the majority of cases, *L.lactis* and *L.cremonis* are used to produce acidity and *Leuconostoc spp.* and *L.lactis var diacetylactis* are the flavour and aroma forming strains which produce diacetyl which is the characteristic flavour compound of buttermilk (Miller & Litsky, 1976).

1.4.2. Butter

Butter is mostly developed from cream which has been pasteurised, inoculated with known species of bacteria and then incubated. In some cases the cream is left to develop an amount of lactic acid from the growth of bacteria already present.

In order to have the desirable flavour development in the cream , the production of lactic acid should be enough as to provide the proper pH ,on which acetylmethylcarbinol and diacetyl (<2-4 ppm),the basic flavour compounds of the butter will be formed (May, 1978).

L.lactis and *L.cremoris* are the starters used to produce lactic acid in order to low the pH to 4.7.Their presence in butter manufacture reassures the proper acidification of cream for the growth of secondary starters. Then *Str.citrovirus* and *Str.paracitrovirus*, the bacteria used for flavour production can act. They catabolise pyruvic acid according to the following chemical reaction:



(Nickerson & Sinskey, 1972).

1.5 Lactococcus lactis Microbiological and Genetical features and Biochemical abilities

L.lactis belongs to the family of *Streptococcaceae* .*Streptococci* are Gram positive (+) ovoid or spherically shaped coccoi. Their cells are arranged in chains, of varying lengths (Dawes& Sutherland, 1976).

In this family the following genera belong: (Holt, 1974).

- *Streptococci*
- *Leuconostoc*
- *Pediococci*
- *Aerococci*
- *Gemella*

The main characteristic of *Streptococcaceae* genera is their ability to fermentate hexoses and mainly glucose. The fermentation occurs in two ways, either by a homogenous way (homofermentation) with major end product, being lactic acid or by a heterogeneous way (heterofermentation) where lactic acid with, other carbon compounds and carbon dioxide are produced. The glycolysis occurs via the hexose diphosphate pathway (Casida, 1968).

L.lactis was until recently, considered to be a member of *Streptococci* genus but due to its pleomorphic nature was reclassified. According to the recent nomenclature *L.lactis* belongs to the genus *Lactococci* which also includes other coccoi used in the dairy industry. (Ayres, Mundt & Saudrine, 1980). Despite the change in its binomial nomenclature *L.lactis* possesses many if not all the basic characteristics of the *Streptococci* (Skinner &Carr, 1974).

1.5.1 Main Microbiological Characteristics of L.lactis

The genus *Lactococcus* involves the following variants:

- *L.lactis var lactis*
- *L.lactis var tardi*
- *L.lactis var hordni*
- *L.lactis var pisciu*
- *L.lactis plantarum*
- *L.lactis var garvieae*
- *L.lactis holandicus*
- *L.lactis anonxophilus*
- *L.lactis diacetylactis*
- *L. lactis maltigenes*

L.lactis natural habitants are plants, soil, the oral cavity and the intestinal tract of animals. Its cells are ovoid mostly structured in the direction of short chains .Their diameter in vivo is about 0.5 μm to 1 μm occurring predominantly in pairs or short chains. *L.lactis* is a thermoduric coccus, being able to grow into temperatures between 10°C to 40°C with its optimum temperature in growing conditions is 30°C, and its reproduction occurs by binary fusion. (division of cell resulting into two bacterial daughter cells of equal size and structure)(Doelle,1969). It possesses a capsule, which serves as a barrier between the cell and the external environment (Alcamo, 1997; Wannamaker & Matsen, 1972).

L.lactis is a Gram-positive (+) non motile, non spore formulating coccus which has a single layer cell wall structured mainly by peptidoglycan and teichoic acids. The C substance in *L.lactis* is synthesised by glycerol and teichoic acid combined with α-alanine and galactose phosphate. (N group streptococci according to Lancefield taxonomy).The polysaccharide is cited intracellulary between the cell wall and the cell membrane. (Skinner& Carr, 1974). Its growth is inhibited in high temperatures (>45°C) and in the presence of NaCl in a higher quantity than 5% w/v.

During its, in vitro, development *L.lactis* has complex nutritious needs. It is an auxotrophic coccus for a number of amino acids and vitamins, especially vitamins of the B complex which are involved on the biosynthesis of purines and pyrimidines. As *L.lactis* has a very limited biosynthetic capacity, for optimal growth conditions to be achieved, the exogenous supply of sugars, phosphorus, potassium and magnesium is necessary (Skinner& Quensel, 1978).

L.lactis lacks the components of the cytochrome system used by bacteria for aerobic respiration. As a result it cannot use oxygen as an electron acceptor source and very little use of amino acids is done in order to achieve energy. As an electron acceptor *L.lactis* uses NADH, hydrogen and pyruvic acid. By its homofermentative fermentation of glucose *L.lactis* mainly produces L-isomer form of lactic acid. During its growth on red blood cell agar *L.lactis* may produce a weak α-haemolysis or a γ-haemolysis (Holt, 1974).

The differentiating characteristics are demonstrated on the following table:

Metabolism and Growth Characteristics	***Response***
Growth at 50°C	-
Growth at 39.5°C	+
Growth at pH 9.5	_
Growth at pH 4.5	_
Growth at 5-6.5% NaCl	_
Formation of ammonia (NH_3) from arginine	+
Formation of acid from maltose	+
Formation of carbon dioxide and diacetyl from citrate	-
Presence of N antigen	+

Table 1.2: Differentiating Characteristics of L.lactis (Holt, 1974).

1.6 Biochemical abilities of L.lactis

1.6.1. Carbohydrate Metabolism by L.lactis

The purpose of *L.lactis* fermenting carbohydrate is primarily to achieve energy in the form of ATP capturing the primary sugar existing in the milk which is lactose. Fermentation can be described as a genre of anaerobic respiration as oxygen is not used as a final electron acceptor. In fermentation an organic molecule, in most cases a chemical intermediary accepts the electrons. In the case of *L.lactis* pyruvate is used as an electron receptor and nitrogen as an electron acceptor, being able to accept the electrons and the proton from NADH. This process is done via NAD which exists in a very small rate within the cell cytoplasm and has to be constantly regenerated so that glycolysis can continue (Alcamo, 1997).Many cycles of oxidation are required to give rapid metabolism of sugars.

L.lactis performs homolactic fermentation in which carbons 1 and 6 of glucose become the methyl carbon, carbons 2 and 5 form carbinol carbon and 3 and 4 the carboxyl carbon of lactic acid. (Sokatch, 1969).

Fermentation process can be inhibited by the Pasteur Effect which means the inhibition of glycolysis by the presence of oxygen (Atlas & Bartha, 1993).

Lactose in order to be catabolised has to be transferred into the internal of the cell. *L.lactis* uses the Phosphoenolpyruvate: carbohydrate phosphotransferace system (PTS system) which is located in the cellular membrane of the coccus (Stanier &Gunsulus, 1961; Gerhard, 1979).

The primary step is the conversion of lactose into galactose and glucose. This done by the enzyme β-galactosidase which belongs to the family of oxidases and cleaves off the β-oxygen bonded attachments to galactose. (Fytou-Pallikari, 1997). In *L.lactis* β-galactosidase is not strongly binded with the cell wall but it floats freely within the cell.

L.lactis catabolises glucose to pyruvate acid by the Embden-Meyerhof (EMP) glycolytic pathway and galactose by the Leloir pathway. The pathways are connected via phosphate-6-glucose which is the final end product in the Leloir pathway and through this form can enter the glycolysis pathway and be further converted to pyruvate acid (Davidson &Sittman, 1999).

1.6.2 Transport Systems of Sugars in L.lactis :Phosphoenolpyruvate:carbohydrate phosphotransferace system (PTS system)

The PTS system is a transport system responsible of catalysing the translocation of sugar molecules through cellular membranes. PTS system is performing an additional phosphorylation in a variety of carbohydrates concluding in the intracellular accumulation of the carbohydrate phosphates. (group translocation) (Postma & Lengeller1985). Each carbohydrate phosphate compound acts as a starter intermediate for the further catabolism of sugars, via several chemical pathways performed by enzymes in the intracellular space.

PTS provides a strong and steady bond between the uptake of sugars and their further degradation. This is highlighted by the fact that no unbound carbohydrate is found in the intracellular space. It can be said, that PTS acts as a symporter allowing for each sugar molecule entering the intracellular space, another phosphorylated sugar molecule is released (Postma & Lengeller, 1985).

$$\text{CARBOHYDRATE OUT} + \text{PHOSPHOCARBOHYDRATE IN} \underset{\leftarrow}{\overset{E2}{\rightarrow}} \text{PHOSPHOCARBOHYDRATE IN} + \text{CARBOHYDRATE OUT}$$

PTS system is consisted by numerous membrane bound and cytoplasmic proteins each of them being able of existing both in a phosphorylated and in a non phosphorylated form. By its biological role can also be characterised as a chemoreceptor system over carbohydrates and as a pacemaker regulatory system for catabolic pathways. (Postma & Lengeller, 1985).

PTS basic compounds are four groups of protein molecules which are the following Enzyme I, HPr-protein, Enzymes II group and Enzymes III group. The uptake of all PTS carbohydrates is primarily dependent on the function of Enzyme I and HPr-protein. The general reaction diagram which describes the uptake of all Phosphoenolpyruvate (PEP)-dependent phosphotransferase is the following:

STEP ONE PEP+ ENZYME I →PHOSPHOENZYME I +PYRUVATE
←

STEP ONE PHOSPHOENZYME I + HPR-PROTEIN → ENZYME I+PHOSPHO-HPR
←

STEP TWO PHOSPHO-HPR +ENZYME II →HPR +PHOSPHOENZYMEII
←

STEP THREE PHOSPHO-HPR + ENZYME III → HPR+PHOSPHOENZYME III
←

STEP THREE PHOSPHOENZYME III +ENZYME II → ENZYME II + CARBOHYDRATE PHOSPHATE

←

PTS uses as a phosphoryl donor, phosphoenolpyruvate acid (PEP) in order to phosphorylate the sugar molecules. The primary step of the phosphotransfer procedure requires the presence of Enzyme I and HPr-protein. The phosphoryl group in the phosphoenzyme created is linked to the N3 position of the imidazole ring of a histidine residue. Enzyme I is not highly specific over the sugar substrate but it can be linked to almost all carbohydrates dependent of the PTS system. Also phosphoenzyme I and the soluble phosphoproteins of the PTS system. Also, phosphoenzyme I and the soluble phosphoproteins of the PTS, have the highest phosphoryl transfer potential of all known natural phosphoryl compounds such as ATP. It can also act reversibly by transferring its phosphoryl group back to pyruvate in order to form PEP, and this can be also done to other keto-structure acids and their analogs. After transferring its phosphoryl group to the histidine –containing ,heat stable, phosphocarrier protein HPr, the enzyme can be restored in its original position and be reused (Postma & Lengeller, 1985).

HPr-protein, the second protein involved in the phosphoryl transfer reaction sequence, is a small protein molecule consisted by 84 amino acids and histidine fragments at positions 15 and 75. HPr-protein lacks cysteine, tyrosine and tryptophane. (Postma & Lengeller, 1985).The phosphoryl group deriving from phosphoenzyme I is linked on the N1 position of the imidazole ring of His-15 which is a part of the active side of the protein. All HPr-protein activity is located in the intracellular space .This protein in *L.lactis* can also be phosphorylated by an ATP- dependent reaction over a seryl fragment. It is important to highlight that Enzyme I and HPr-protein are not involved directly to the translocation of sugars, they simply offer the phosphoryl group and

they may participate indirectly to the regulation of other enzymical transport system (Peppler & Periman, 1979).

As the reaction develops, the phosphorylation of sugars is finally achieved by two groups of enzymes, Enzymes II and III groups. Each enzyme of these families is highly specific in order to react with its respective carbohydrate compound .These enzymes may not be a single membrane bound polypeptide but they can be composed by multiple protein chains (Doelle, 1969).

In order to differentiate the specific substrate enzymes of these groups abbreviated superscripts are used, as for example II lac, III gluc, II man each of them phosphorylating lactose, glucose and mannitol. Moreover, due to their high specification they react with each other only in specific combinations in contrast with HPr-protein which interacts with all enzymical compounds of group II enzymes (Gerhard, 1979).

In the case of lactose by *L.lactis* the PTS system functions under the following reaction scheme:

$$\text{PEP} + \text{HPr} \xrightarrow[\leftarrow]{\text{ENZYME I}} \text{P HPr} + \text{Pyruvate}$$

$$\text{Enzyme}^{3}\text{lac} + 2\,\text{Pi-HPr} \xrightarrow[Mg^{2+}]{} \text{Enzyme}^{3}\text{lac} + 2\text{Pi} + 2\text{HPr}$$

$$\text{Enzyme}^{3}\text{lac} + 2\text{Pi} + 2\,\text{Lactose} \xrightarrow[\leftarrow]{\text{Enzyme}^{2}\text{lac}} 2\,\text{Lactose-Pi} + \text{Enzyme}^{3}\text{lac}$$

The in vivo metabolic control of the system in *L.lactis* is achieved by the allosteric glycolitic enzyme pyruvate kinase (PK).PK's function is regulated by the presence of ATP and acetyl-coA both of which can act as inhibitors when necessary. PK regulates the flow of the intermediates from glucose-6-phosphate to pyruvate. Finally phosphorylated at the carbon 6 of galactose, lactose is cleaved by an enzyme of galactosidases family, β-D-phosphogalactosidase galactohydrase. Lactose is cleaved to glucose and galactose (Mandolstan & Macquillen, 1973).

1.6.3 Catabolism of Galactose

Galactose is broken down according to a series of chemical reactions called Leloir pathway, a series of four chemical reactions catalysed by several enzymes and cofactors. In a separate final reaction glucose-1-phosphate will be converted to glucose-6-phosphate in order to follow the glycolysis pathway (Mathwes et al., 1999).

Galactose is a crystalic substance with melting point 146°C.It is not a water soluble molecule and it belongs to the hexose family of monosacharides having the same chemical formula with glucose but differing in the stereochemistry in the fourth carbon of its molecule. (Roberts &Juinner ,1983). Galactose is firstly phosphorylated to galactose-1-phosphate by the enzyme galactokinase under the presence of ATP and $Mg^{2}+$.

$$\text{Galactose + galactokinase (ATP: D-glucose-1-phosphotransferase) +ATP} \xrightarrow{Mg^{2+}} \text{Galactose 1-Phosphate +ADP}$$

Then galactose -1-phosphate is converted to UDP galactose by the enzyme UDP-glucose-galactose-1-phosphate uridyl transeferase.

$$\text{Galactose-1-phosphate} \xrightarrow[\text{UDP-glucose- galactose-1-phosphate uridyl transeferase.}]{} \text{UDP-galactose}$$

Next, UDP-galactose is epimerised in its C4 carbon by the enzyme UDP-glucose-4-epimerase and the presence of NAD to UDP-glucose.

$$\text{UDP-galactose} \xrightarrow[NAD+]{\text{UDP-glucose-4-epimerase}} \text{UDP-glucose + NADH}$$

The reaction follows an analogy of 1:1 molecule. Then UDP-glucose-1-phosphate by the enzyme UDPG-pyrophosphorylase transferase.

$$\text{UDP-glucose} \xrightarrow{\text{UDPG-pyrophosphorylase transferase}} \text{Glucose -1-phosphate}$$

After the end of Leloir pathway, the end product glucose-1-phosphate is converted to glucose-6-phosphate in order to enter glycolysis (Stanier et al, 1977).

Galactose-1-phosphate can be further metabolised by the use of D-tagatose-6-phosphate pathway and the end product of this process can re enter the glycolysis circle.

The whole pathway can be summarised according to the following diagram:

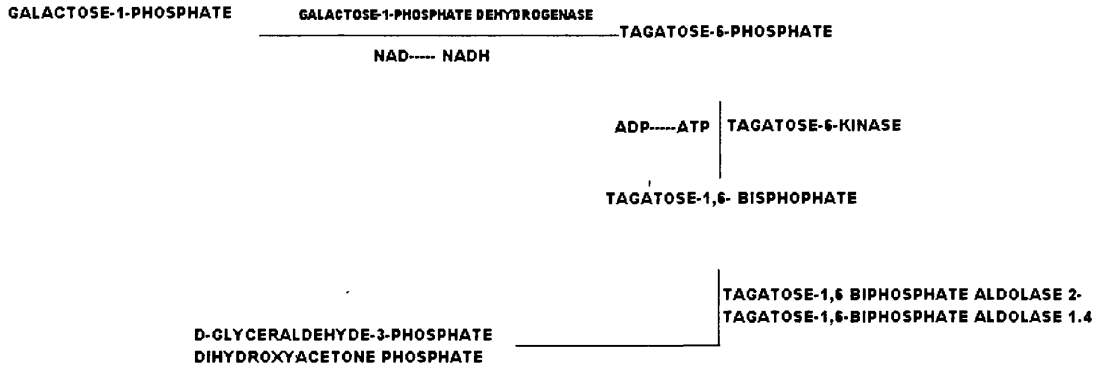


Diagram1.3: Catabolism of Galactose-1-phosphate (Mandolstan & Micquillen, 1973)

1.6.4 Catabolism of Glucose, Description of the Embden-Meyerhof glycolytic pathway (EMP)

Glycolysis is a ten reaction .cyclic procedure and can be divided into two major phases, each phase represented by a set of reactions. Phase one is considered to be the energy investment phase (reactions 1 to 5) and phase two which is the energy generation phase (reactions 6 to 10).The end product of glycolysis is pyruvate acid and two molecules of ATP. (Alcamo, 1997). The glycolysis begins with the investment phase .In reaction 1; glucose reacts with ATP which is the basic carrier of phosphate under the presence of $Mg^{2}+$ to form glucose-6-phosphate. Nextly (reaction 2) glucose-6-phosphate (G6P) is transformed to fructose-6-phosphate (F6P) by the enzyme phosphoglycoisomerase. Then (reaction 3), fructose- 6- phosphate is converted to D-fructose-1, 6 biphosphate by the enzyme phosphofructokinase. Then D-fructose-1, 6 biphosphate is broken down into two three-carbon intermediates (reaction 4), glyceraldehyde-3-phosphate and dihydroxy acetone phosphate. The enzymic catalyst of the reaction is fructose-1, 6 biphosphate aldolase. Then dihydroxy acetone phosphate (DHAP) is isomerised by the enzyme triose phosphate isomerise to glyceraldehyde-3-phosphate (G3P) (reaction 5). Then glyceraldehyde-3-phosphate is oxidised on its carbonyl carbon (loss of two electrons) by the enzyme glyceraldehyde-3-phosphate oxidase under the presence of NAD and inorganic phosphorus. The

product is 1, 3-bisphosphoroglycerate acid with NADH and H ions (reaction 6).Nextly, 1; 3-bisphosphoroglycerate is phosphorylated to 3-phosphoglycerate acid. (reaction 7).The enzyme participating is phosphoglycerate kinase with ADP and Mg (reaction 8). The following step, is the formation of phosphoenolpyruvate by a dehydration step of two-phosphoglycerate acid. The dehydration is achieved by an enolase under the presence of Mg^{2}+(reaction 9). Finally, with the presence of pyruvate kinase phosphoenolpyruvate is transformed to pyruvate .The reaction is done with ATP, and if pyruvate is further degraded ATP will be regenerated.(Fytou-Pallikari,1997;Alcamo,1997;Doelle,1969).EMP pathway can be schematically represented as follows:

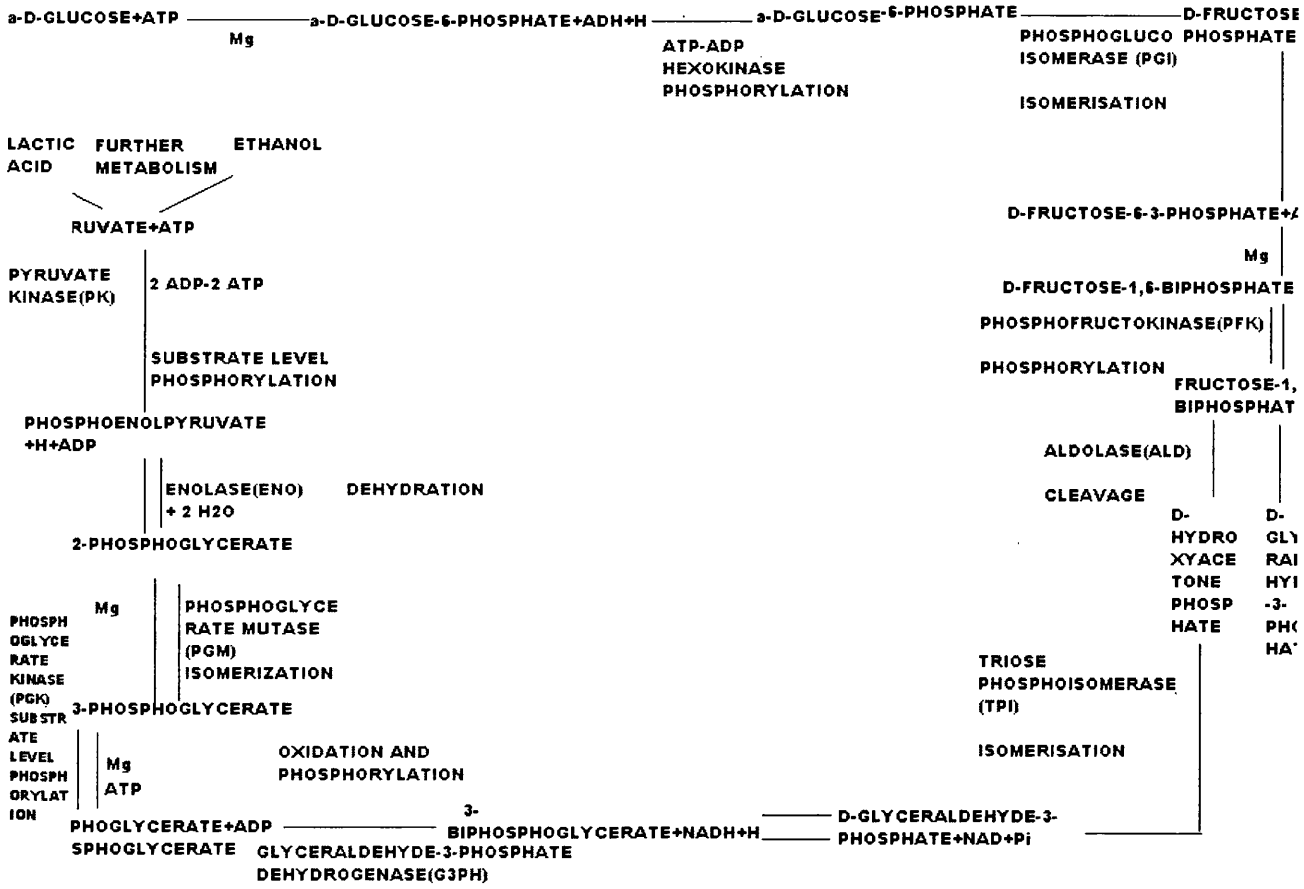
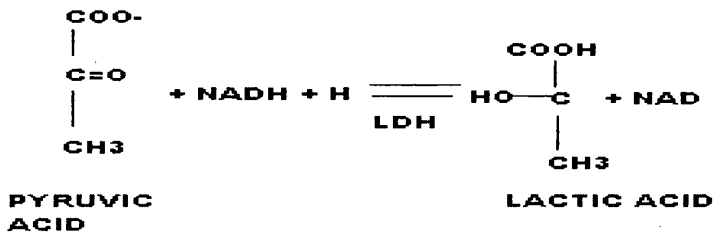


Diagram 1.4:Embden-Meyerhof glycolytic pathway (Backberry& Teesdale, 2001)

1.6.5 Conversion of Pyruvate to Lactic Acid

Pyruvate is the anion form of pyruvic acid (CH_3COCOO^-).Is an alpha-keto acid of the keto-acid group and under the presence of the enzyme lactate dehydrogenase (LDH) and the coenzyme NAD. Pyruvate is the major end product of glycolysis. In the case of *L.lactis* pyruvate is converted to lactic acid via lactate dehydrogenase isoform (L-lactate: NAD oxidoreductase). Lactate dehydrogenase oxidises the C=O and the CH-OH (carbinol) part of pyruvic acid. The lactic acid produced is of L-stereo isomeric form (Board, 1983).

The reaction can be described as follows:



1.6.6 Industrial Importance of Lactic Acid

Lactic acid or 2-hydroxypropanoic acid ($C_3H_6O_3, CH_3CHOHCOOH$) is an important chemical substance widely used in food industry and in pharmaceutical and cosmetics industry. (Wasewar et al, 2003).

Lactic acid is a carboxylic acid with a hydroxyl group and it is considered to be an Alpha hydroxyl acid. (AHA). Lactic acid has two optically isomer forms L-(+) - lactic acid and D-(-) – lactic acid and is a chiral acid (Fytou-Pallikari, 1997).The reaction of production of L- lactic acid which is the most important form biologically, is catalysed by the enzyme lactate dehydrogenase (LDH) and its isoenzymes. Lactic acid has a melting point of 53 °C, though the racemic form (D/L) has a boiling point of 122°C at 12 mm Hg.

Lactic acid can be produced into large amounts, biotechnologically, through fermentation process performed by bacteria or fungi. Usually, the product of fermentation is a racemic mixture conglomerate mixture of D (-) - and L (+) – isomers but there are also strains which produce optically pure forms of one of the stereoisomer's. (Martau et al, 2003).

Another modern application is the use of lactic acid as a monomer participating in the synthesis of biodegradable homopolymers and co-polymers, such as polylactide (Choi & Hong, 1999). For the synthesis though of such fine polymers highly purified forms of lactic acid are demanded. Most of these polymers are used in the pharmaceutics industry especially for artificial prosthesis and controlled drug delivery.

Traditional recovery methods for fermentation products (crystallization, extraction with solvent, filtration, carbon treatment evaporation) have high operational cost, so other methods such as distillation and distillation simultaneously with reaction are proposed due to low cost (Choi & Hong, 1999).

1.7 Other metabolic pathways used by L.lactis

Lactococcus lactis as being a homofermentative microorganism converts mainly pyruvate acid to lactic acid to a percentage almost of 90%. A small percentage of pyruvate though, can be converted to ethanol, acetate and 2, 3 butanediol. This does not influence the quality of the fermented milk products in which *L.lactis* is used as starter culture. The previously referred chemical compounds are formed by the following reactions:

- Ethanol

Pyruvate, by the enzyme pyruvate dehydrogenase (PDH) and the simultaneous presence of NAD is converted to acetyl-coA and NADH. During the reaction CO_2 is produced.

$$\text{Pyruvate} + \text{NAD} \rightarrow \text{acetyl-coA} + \text{NADH} + \text{CO2}$$

Acetyl-coA can be converted by the enzyme acetaldehyde dehydrogenase (ADHE) to acetaldehyde with the participation of NADH.

$$\text{Acetyl-coA} + \text{NADH} \xrightarrow{\text{ADHE}} \text{Acetaldehyde} + \text{NAD}$$

Finally, acetaldehyde by the presence of the alcohol dehydrogenase (ADHA) enzyme and NADH is transformed to ethanol.

$$\text{Acetaldehyde} + \text{NADH} \xrightarrow{\text{ADHA}} \text{Ethanol} + \text{NAD}$$

(Cutting, Carr, & Whiting, 1975).

- Acetate

Pyruvate can also be converted to acetate with the enzyme pyruvate formate lyase (PFLS) and the simultaneous production of formate acid.

$$\text{Pyruvate} \xrightarrow{\text{PFL}} \text{acetyl –coA} + \text{Formate}$$

acetyl-coA with the use of phosphotranferase lyase (PTA) can be converted to acetyl phosphate.

$$\text{acetyl-coA} \xrightarrow{\text{PTA}} \text{acetyl-P}$$

Then acetyl-P by the enzyme acetate kinase (ACKA) and the presence of ADP is converted to acetate.

$$\text{acetyl-P} + \text{ADP} \xrightarrow{\text{ACKA}} \text{acetate} + \text{ATP}$$

(Glazier & Nikaido, 1995).

- 2,3 Butanediol

In order to form 2, 3- Butanediol pyruvate is firstly converted to acetolactate by the catabolic and anabolic system of 2-acetolactate synthase (ALS/ ILVB)

$$\text{Pyruvate} \xrightarrow{\text{ALS/ ILVB}} \text{Acetolactate} + CO_2$$

Acetolactate is oxidised and then is converted to diacetyl.

$$\text{Acetolactate} + \text{O2} \rightarrow \text{Diacetyl} + CO_2$$

Diacetyl is converted to acetoin by the enzyme diacetyl reductase (BUTA) under the presence of NADH.

$$\text{Diacetyl} + \text{NADH} \xrightarrow{\text{BUTA}} \text{Acetoin}$$

Acetoin can also be formed by acetolactate with the use of the enzyme acetolactate decarboxylase (ALDB)

$$\text{Acetolactate} \xrightarrow{\text{ALDB}} \text{Acetoin} + \text{CO2}$$

Then acetoin with the enzyme acetoin reductase (BUTB) and NADH is converted to 2, 3-Butanediol.

$$\text{Acetoin} \xrightarrow{\text{BUTB}} \text{2, 3 Butanediol} + \text{NAD} + \text{NADH}$$

These three end products do not suffer any further metabolism by *L.lactis.*

(Board, 1983, Glazier, & Nikaido, 1995).

1.8 Proteolytic activity of L.lactis

L.lactis has a small proteolytic activity using a system consisted by a cell envelope proteinase, an oligopeptide system of transport and several intracellular peptidases. This complex system provides by the degradation of κ-, β- and α-casein, and the essential amino acids for growth, especially isoleukine, leukine and methionine. The primary catabolism is achieved by a cell wall bound serine protease (PtP).The casein peptides produced by the above reaction are degraded by different peptidases. These peptidases are located intracellularly. The secondary catabolism is achieved by the aminopeptidase PepN and the x-prolyl amino peptidase Pep Xp (Meijer, Marragg, Hegenholtz, 1996).

The possession of cell bound extra cellular proteinases ensures the production of peptides close to the cell and in proximity to their oligopeptide and dipeptide uptake systems. The loss of proteolytic activity of *L.lactis* is combined with the loss of plasmid DNA (Efstathiou and McKay,1976 as cited by Rose, 1978).

1.9 Genetics of L.lactis

1.9.1 Introduction

L.lactis possesses numerous genetic systems controlling the regulation of its growth, productivity and metabolism. The most commonly utilised system for both in vitro research and industrial production scale is the NICE (Nisin Controlled Gene Expression System) system. This system is responsible for the regulatory production and transcription of an important lactococcal bacteriocin named nisin. Nisin fuctions as a natural preservative and antibiotic of the products which are inoculated with LAB strains, due to its function as a natural inhibitor of growth of numerous harmful bacteria (Mierau, Lei et al, 2005).

1.9.2 Components of the NICE system

NICE system is part of the deeply studied pheromone-dependent quorum sensing systems which are widely distributed on the Gram positive (+) bacteria. (De Vos, 1999).

It is an auto regulatory two component system which can be activated fully by nisin in very low sub toxic amounts (ng/ml) (Mierau & Lei, 2005).

The system operates as following: The nisin A operon is activated by the gene product nisin .Nis A and Nis F are functioning as promoters. They are bonded to the response regulator, receptor and signal transducer NisK. NisK the basic sensor of nisin is an histidine protein kinase. It activates the NisR under the presence of phosphorus. The activated phosphorylated Nis R induces the Nisin operon at the Nisin A promoter. (Mierau, Olieman et al, 2005). for the production of nisin. The response regulator NisR responds to extra cellular nisin, so addition of nisin to the extra cellular media above the tolerant amount results in the induction of gene expression from nisin A operon. (De Vos, 1999).

The NICE system was firstly identified through the molecular characterization of the production of nisin. (De Vos, 1999).As previously referred NICE has various applications such as the use foe expression of homologous and heterologous proteins of Gram positive (+) and Gram negative (-) bacteria, protein secretion and membrane proteins of prokaryotic and eukaryotic cells, cloning of toxic genes, bacterial and viral antigens and cytokines. The majority of these applications have been performed on acidifying cultures (medium acidification due to the production of lactic acid) which have as major disadvantage the low final cell density. On the contrary, pH controlled fermentations end up in higher biomass yields and cell densities. (Mierau&Lei et al 2005; Pavan,2000; Hols.et al, 2000).

The system can be schematically represented as following:

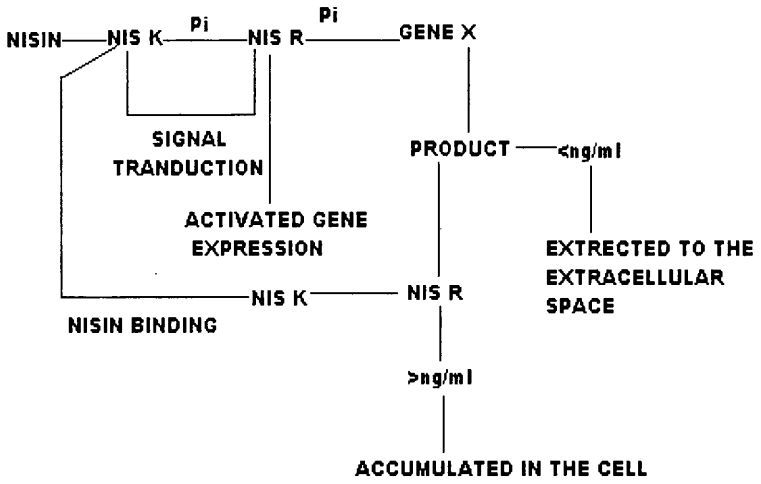


Diagram 1.5 : NICE system (Mierau &Olieman, 2005)

1.9.3 Nisin

Nisin is a 34 amino acid lactococcal bacteriocin, a small polypeptide of 2, 9 kD (Todorov & Dicks, 2004). Nisin has two variants A and Z with a difference of only one amino acid on their molecular structure (Beasly & Saris ,2004)The use of nisin as a food additive is permitted in the United States(Food and Drug Administration) in the E.U. and the U.K.(Guillet,2003).

Nisin can inhibit the growth of many pathogen bacteria and bacteria causing food spoilage such as *Enterococcus faecalis, Escherichia coli, Lactobacillus plantarum, Lactobacillus casei, Pseudomonas aureginosa,* and *Staphylococcus aureus.* In the dairy industry, this lactabiotic is used to prevent spore germination of *Clostiridium botulinum* and *Bacillus cereus.* Through several studies has been identified that nisin is also found in human milk and it may protect mothers from breast infections during feeding and infants from toxication due to pathogenic skin flora such as *Staphylococcus aureus* (Beasly & Saris, 2004).

Nisin can interact with various different microbiomolecules. Nisin binds on the membrane localised cell wall precursor lipid II, enabling efficient membrane binding and also enhancing pore formation. This means that nisin by forming short-lived pores in biological membranes thereby killing the target bacteria (cell wall lysis).

Nisin production is strongly dependent on the pH of the nutrient medium, the nutrient sources (carbohydrates, K, vitamins, N, P) and incubation temperature. (temperature range between 20°C to 40°C optimum 30°C). The activity levels of nisin do not always correlate with cell mass or growth rate (μ) of the reduced strain. (Oliveira, Nielsen& Forster, 2005).

1.10 Fermentation Process and Fermentation Technology for Cell Production

Fermentation is a biotransformation process (also called zymosis) which can be described as the anaerobic metabolic breakdown of an organic nutrient molecule, a carbohydrate, such as fructose, galactose or glucose without net oxidation. Fermentation and product recovery are the basic aspects of biotechnology science.

The majority of fermentation processes are performed either by yeasts, fungi or bacteria. This procedure is achieved by the intra- of extra cellular enzymical compounds possessed by microorganisms. Zymosis procedures are applied mainly in industrial scale, in food industry (enrichment of products with flavours, aromas and textures, preservation) and in the pharmaceutics industry (production of antibiotics, proteins, enzymic molecules, acids).Fermentation process is used for the production of microbial biomass, microbial enzymes and metabolites and modification through biotransformation of compounds added in the medium. (Demain & Davies, 1999).

Every fermentation process is consisted by several preparatory steps such as: the formulation of medium to be used in culturing the chosen microorganism for inoculum developed and for the fermentation itself, the sterilisation of the nutrients and all the auxiliary mechanical equipment and the development of a suitable size pure inoculum for inoculation of the industrial fermenters and reactors (Miller & Litsky, 1976). Factors of mechanical structure of the reactors such as mass and heat transfer, agitation, aeration (if required), pressure, air and liquid flow have to be taken into consideration. Physical parameters such as pH, temperature, osmolality, growth and death rate of the microorganism, nutritional requirements and sensitivity to end products have also to be carefully measured. (Doran, 2004). Usually, for the successful application of industrial scale fermentation numerous small scale experimental procedures should be performed. (Fiechter, 1990).

1.10.1 Types of Fermentation

Fermentation procedure can be divided according to several factors which can be summarised as following

Fermentation procedure

NATURE OF THE SUBSTRATE	OXYGEN DEMAND	OPERATION MODE	VOLUME OF THE FERMENTED MATERIALS
Solid State Fermentation (SSF)	Anaerobic	Continuous Fermentation	Small scale (laboratory, experimental form)
Submerged Fermentation (SmF)	Aerobic	Batch Fermentation	
		Fed-Batch Fermentation	Large scale (industrial fermentation)

On analysing further the above types further subdivisions can be made.For example, according to the dependence of product formation on energy metabolism:

- *Type One:* Product is directly derived from primary metabolism used for energy production. Growth, carbohydrate catabolism and product formation run simultaneously.

  Substrate A→Product

  Substrate A→B→C→Product

- *Type Two:* Product is derived from the substrate used for primary energy metabolism, but product takes place in a secondary pathway which is separate from primary metabolism

  Substrate→ A→ B→ C→ D→ Primary Metabolism

  ↓

  E→ F→Product

Products such as antibiotics, small molecular weight compounds, organic acids (citric acid, tartaric acid) are produced by this type of fermentation. Growth occurs by high substrate consumption without any product formation. The product formation starts coupled with high substrate high consumption rate as growth reaches its maxima.

- *Type Three:* Product formation and primary metabolism occur at completely separate times. Primary metabolism occurs first simultaneously with substrate consumption and growth. The product is formed through amphibolic pathways during intermediate metabolism.

Substrate → A + B + C → Primary Metabolism→D→E

↓

Intermediate Metabolism

↓

F→ G

↓

Final Metabolism

(Bu'lock & Kristiansen, 1978; Doran, 2004).

1.10.2 Analysis of Types of Fermentation

1.10.2.1 Solid State Fermentation (SSF)

Solid state fermentation can be defined as the process of fermentation occurring on a solid or a semisolid substrate or in a nutrionally inert support (Pandey ,2003, Demain & Davies, 1999). SSF process has unique characteristics and limitations which are strongly coupled with the fact that it takes place under low water conditions.

Several parameters have to be taken into consideration when setting up an SSF process. These include: selection of suitable microorganism and substrate key parameters, such as particle size, initial moisture, pH pre-treatment of the substrate, relative humidity, temperature of incubation, agitation and aeration, age and size of the inoculums, supplementation of additional carbon source, and extraction and purification of product should be carefully measured.

As in SSF solid material acts both as physical support and source of nutrients has to be carefully selected in order not to pose difficulties in the isolation of the product .Inert material such as polyurethane foam is preferable as it does not causes difficulties in product recovery.(Van De Lagemaat & Pyle, 2001).

1.10.2.2 Submerged Fermentation (SmF)

Submerged fermentations (SmF) also called liquid fermentations; represent the plurality of fermentations carried out both in experimental and industrial scale .In liquid fermentations both the substrate and the microbial culture are in a liquid form.

Many SmF processes can be run simultaneously, as they represent an economic process for the products of fine chemicals, antibiotics and primary and secondary metabolites of commercial importance. Product recovery is also easier in SmF through the use of filters for separation. (Stanbury &Whitaker, 1993).

According to the need of oxygen supply SmF can be further divided in aerobic and aerobic process. Aerobic processes represent a large number of fermentations and they are more difficult to control due to the continuous need for oxygen supply .Oxygen as being a difficulty soluble molecule creates many limitations concerning mass transfer rates between gas and liquid areas within the reactors. It is also very important to constantly monitor the oxygen flow within the reactor in relation to the oxygen accumulation by the growing microorganisms. (Aiba, Humphrey & Millis, 1973).

On the other hand, anaerobic processes have numerous advantages and disadvantages but in general they can be controlled and operated easier when comparing with aerobic processes. Advantages such as higher product yield due to characteristic metabolism of anaerobes, smaller demand of mass and energy sources as input, the production of unique products from the catabolism of complex substrates and the resistance of anaerobes to extreme in-vitro environmental conditions(e.g. low pH) make anaerobic fermentation an easily manipulated process. However, disadvantages such as the high rate of contamination, the production of toxic end products, the instability of microbial inoculum and the difficulties in genetic manipulation may endanger the process. (Malek et al, 1969).

In order to develop industrial scale fermentation several in vitro simulation fermentations should be firstly performed. When transferring small scale fermentation to a large scale process (scale up) several differences between the output data may occur. For example differences concerning biomass, metabolic by products and limiting substrate concentrations occur. So, for studying these effects and minimising

them mathematical models concerning fluid mixing and microbial kinetics have too be developed and integrated. (Casida, 1968).

1.10.2.3 Batch Fermentation

A batch fermentation can be defined as a closed system fermentation process .At the beginning of the fermentation process (when time equals to zero) the sterilised substrate (liquid nutrient solution) is inoculated with a proper quantity of the selected microorganism. Incubation process starts and proceeds under optimum physicochemical conditions (Gruger& Gruger, 1989). During the progress of the batch SmF no technical additives are put in the system except gaseous oxygen, an antifoam agent and an acid or base solution in order to control the pH. Advantages of batch fermentation include an easily operated process which can be performed in a simple batch fermenter with avoidance of contamination. On the other hand, disadvantages include an inefficient growth circle and the demand of a large quantity of inoculum for the process. (Patnaik, 2002).

1.10.2.4 Fed- Batch Fermentation

An adhension of the batch process is the fed-batch fermentation. During this process substrate is fed intermittently, with specific time intervals between each dose .In a modelled fed-batch SmF a small amount of biomass and substrate are added in the fermenter. As most of the initially added substrate has been accumulated by the microorganism, the substrate feed starts. This process is designed according to the catabolite repressor effect possessed by the bacteria for the production of valuable secondary metabolites. Fed-batch fermentation processes are applied especially when the substrate is inhibitory for high growth rates (vinegar and amylase production) and the end products can be achieved into low substrate concentrations (production of antibiotics).(Lancini & Lorenzetti,1993).

A major difficulty in operating a fed batch process the nonlinear behaviour occurring. All the key variables (volume, product concentration, and biomass) are constantly changing in this time limited procedure.(Gregensen & Jorgensen ,1999).These difficulties result into the absence of dynamic mathematical models for such kind of systems. In order to set up fed-batch fermentation numerous optimization experiments should be performed. Another obstacle when applying fed-batch process into large scale is the absence of highly accurate and precise on line sensors for the

measurement of all key variables. So indirect control methods are applied. To analyze the data obtained through these techniques, multivariate statistical control methods have been applied. (Zhang & Lennox, 2004).These methods can detect the fault conditions in the operating system with Partial Least Squares (PLS) method being the most preferable for analysis. (Patnaik ,2002).

Biomass concentration in a fed-batch system at any time can be given by the following equation:

$$X_t = Xo + Y(S_r - S)$$

where Xt stands for biomass concentration at time t (hours), Xo is the original inoculum concentration, Y is the yield coefficient S, is the substrate concentration and Sr is the residual substrate concentration. In such a system the input of substrate is equal with the consumption of the substrate by the cells) so the cell concentration remains constant and the total biomass concentration augments with time. In this system the growth rate (μ) equals with the dilution rate (D) and this situation is termed a quasi-state operating system. (Stanbury &Whitaker, 1984).

1.10.2.5 Continuous Fermentations

Continuous fermentation represents an open system set up where sterile liquid nutrient medium is constantly fed up by a peristaltic pump or a solenoid gate system to the reaction vessel. Simultaneously an equal amount of converted nutrient including microorganisms is extracted. This system can be run either in a turbidostat or a chemostat fermenter in a set-up of homogenously-mixed reactor such as plug and flow reactor. For the successful operation of a continuous culture system sufficient mixing should be ensured, so all the material is equally mixed and dispersed (Malek et al, 1969).

At a constant flow rate, steady state conditions occur and all the parameters (substrate content, rate of product formation, biochemical reactions) can be maintained in a steady state. As it can be easily understood in this type of fermentation all the parameters of the system are correlated and completely dependent of flow rate (Malek et al, 1969).

Advantages of operating fermentation in a continuous mode include the use of smaller size of bioreactors, production of uniform end products due to the maintenance of

cells into synchrony (exponential growth phase) during the process. But in continuous cultures the preservation of aseptic conditions is difficult and several problems concerning downstream processing may occur (Powell, Evans & Strange, 1967).

1.11 Microbial Selection for Fermentation Process

1.11.1 Microbial Growth and Inoculum Development

In order to achieve a successful in vitro propagation of microorganism several requirements concerning growth should be fulfilled.

It has been generally accepted, that it is necessary to simulate technically the natural environment, in which the microorganism physically grows. Usually, the optimum conditions of each parameter have to be used in order to achieve the maxima in growth (biomass), productivity (end products) and yield (primary and secondary metabolites)(Glazier & Nikaido, 1995).

In general, the growth development of any microorganism (yeasts, fungi, bacteria) can be represented by a curve which contains the basic four stages of bacterial growing. These stages are lag phase, exponential phase, stationary phase and death phase. Each phase corresponds to a specific stage of microbial growth.

Lag Phase: Lag phase is considered to be the phase of adaptation of the microorganism. The cells have been injected to a new environment and they are starting to adjust. During this phase, the cells increase in size but not in number. They are metabolically active and they either produce enzymes which can accumulate the rich in nutrients new medium or if the medium is poor in nutrients enzymes are produced from the organism so to maintain its viability. The length of the lag phase depends on the current environment as well as the previous physiological state of the cells (aged, dormant, active) and it comes to an end as soon as the cells have adapted to their new environment and begin division.(Gerhard,1979).

Exponential or Logarithmic Growth Phase: In this phase, the cells are actively divided at their maximum rate. Cell growth is dependent upon the current environment (nutrient composition, pH, temperature of incubation) but it is not dependent upon the previous physiological state. In this phase, μ is defined as the cell number per time interval. At some point the cell growth rate will level off and become constant .The most probable cause for this change is the substrate limited inhibition

(exhaustion of the supply of essential nutrients).So a smaller number of cells divide and the transition from logarithmic to stationary phase begins.(Hewitt, 1977).

Exponential Growth Equation: $N_2 = N_1 2^a$ (Equation 1.1)

where N2: the number of bacterial cells at time t2, N1: the number of bacterial cells at time t1 and a is the number of doublings. Using natural logarithms the equation becomes:

$$\log N_2 = \log N_1 + n \log 2 \quad \text{(Equation 1.2)}$$

another form of the above equation is the following:

$$\mu\ (h^{-1}) = \frac{\ln N_2 - \ln N_1}{t_2 - t_1} \quad \text{(Equation 1.3)}$$

Another parameter which can be encounted is doubling time (td, h) which is the time between one cell division and the next meaning the time needed for the population of cells to double in size.

Doubling Time Equation: $\text{td} = \frac{(t_2 - t_1)}{n}$ (Equation 1.4)

Substituting equation s, the equation becomes

$$\log N_2 = \frac{\log N_1 + \log 2(t_2 - t_1)}{td} \quad \text{(Equation 1.5)}$$

and the relation between growth rate and doubling time is given. Another form of the above equation is the following:

$$\mu = \frac{1}{x}\frac{dx}{dt} = \frac{d(\ln x)}{dt} = \frac{\ln 2}{td} \quad \text{(Equation 1.6)}$$

where x cell is the cell concentration and t : time from which $\mu = 0.69/\text{td}$

(Bu'lock & Kristiansen, 1987).

Stationary Phase: The stationary phase occurs at the maximum population density, the point at which the maximum umber of cells can exist in an environment .This fact typically represents the carrying capacity of the environment .Factors such as pH, preservatives, antimicrobials, native micro flora, atmospheric composition and depletion of growth due to growth limiting factors can affect the maximum population density. During this phase there is no increase in the population size as the number of

cells being produced equals to the number of cells dying. This means that the microbial population stays in a suspended state (no longer dividing but still able to return to state of active growth if conditions become unfavourable). During this phase many bacteria produce secondary metabolites (byproducts of metabolism of low molecular weight) (Gerhard, 1979).

Death Phase: The death phase occurs when cells are being inactivated or killed because conditions no longer support growth or survival. Bacterial cell death can be defined as the state where the microbe is unable to reproduce when added to a suitable medium with the necessary nutritional requirements under suitable medium with the necessary nutrional requirements under suitable environmental conditions .In result, death occurs logarithmically like growth. The microbes under these declining conditions undergo alterations which affect their structural identity. The majority of microorganisms suffer non lethal changes on their cell structure resulting to a non typical appearance and a changing morphology. If these cells are inoculated in a new medium a new growth circle can begin.

Industrial microbiology is mainly focused in the exponential growth phase as estimating the growth rate of yeasts and bacteria is an important parameter which can be correlated with the productivity of the chosen microorganism. However, important information over the microbial growth behaviour can be also extracted from each phase of growth. In order to model microbial growth the lag and the growth phase are more useful as the production of many metabolites occurs before the stationary and the death phase are reached. Death and stationary phases can give important information over the tolerance of inactivation factors of the microorganisms (Orginsky & Wayne, 1959).

1.12 Evaluation and Prediction of Fermentation Performance

The evaluation of productivity, prediction of behaviour and control of fermentation can be achieved through mathematical modelling.

A mathematical model can be defined as a set of correlations between the parameters of interest of a studied system. This set of relations can be represented by several forms such as graphical or table representations or through mathematical equations which evaluate the dependence between the variables and can be used also for improvement of fermentation process. Variables during fermentation, include state of

agitation and aeration (stirring and mixing), heat transfer and temperature maintenance, viability and preservation of microorganism, product recovery, feed rate of nutrients, energy and power requirements.

Each of the above parameters can be mathematically represented and be differentiated according to the mode of operation of the fermentation system. (Zhang & Lennox, 2004).

Mathematical models according to their complexity can be divided into primary, secondary and tertiary and can judged especially if used for performance prediction according to the bias and the accuracy factor. Bias factor can be used to judge the response of the model to the growth of microorganism and the accuracy factor measures the overall model error. (Kahya et al, 2001).

Mathematical models when referring on cell growth can also be divided in segregated and structured, and unsegregated and unstructured. The majority of fermentation processes are evaluated according to unstructured models especially in the case of microbial kinetics where the Monod equation pattern is used. (Bu'lock & Kristiansen, 1987). The unstructured models are based on the scenario of a fixed cell composition which is treated as one component solute and the growth is balanced .They are based on experimental observations (empirical models), they do not consider stoichiometry of reactions and they do not consider individual cellular structure. They are simple and applicable but they can in general be valid in a single state , for example steady state continuous culture or on fed-batch culture or on the exponential growth phase when referring to batch culture .On the other hand segregated structured models , use a multicomponent description of cellular population based on the population heterogeneity. These models include internal kinetics and cellular regulation considering also stoichiometry of reactions within the cell population. They are not easily applicable due to the extended demand of experimental data for their structure. To describe cell growth kinetics an unstructured unsegregated model can be used taking into consideration the following parameters:

INPUT		VOLUME OF INTEREST		OUTPUT
O2		Cells in		n X (biomass)
Σ substrate	→	a bioreactor	→	Σ P (sum of products)
Hydrogen				
Gaseous Ammonia				

Basic Equations: ΣS: $\frac{ds}{dt}$, Σ P: $\frac{dP}{dt}$, n X $\frac{dx}{dt}$

The overall reaction can be easily described by the following simplified equation:

Substrate + cells	→	Byproducts + biomass
Σ s + n X		Σ P + n X

After the construction of a model , the values of the parameters should be obtained . The parameters can be divided into stoichiometric which are referring to the variables concerning energy and material balances between the phases and the kinetic parameters which concern nutrient consumption, generation rate of species and the end product formulation. For further developing the model three methods can be used, associating additional constants into the original Monod model that promote corrections in product inhibition, yield of products, substrate accumulation, construction of a completely new kinetic concept based both on physicochemical and mechanistic parameters of the system or referring on the influence of physicochemical parameters on the Monod model (Kovarova &Egli, 1998). Experimental data are required for numerical values of the model. There are two methods of fitting the experimental data to the mathematical model. The most commonly used is the straight line fitting which involves transformation of variables and the curve fitting method where data are used in their normal form. The most common example of straight line fitting is the Linewear-Burke plot. This plot is used to determine the maximum rate and saturation constant of a Monod type equation:

$$rx = \frac{\mu \max * S}{K_s + S} Xv \qquad \text{(Equation 1.7)}$$

In this system Rx, S and Xv are the variables and μm and Ks are the parameters (Vrabel, et al, 2001).

1.13 Fermentation Kinetics of Biomass Production

Usually zymosis procedures operate under the restriction of a substrate limited growth model. The relation between substrate concentration and specific growth rate is the basic parameter for the formation of kinetic models. For the majority of cellular fermentation processes these kinetics can be described by the Monod equation:

$$\mu = \frac{\mu \max + S}{Ks + s} \qquad \text{(Equation 1.8)}$$

Where μ is the specific growth rate(h^{-1}), Ks is the constant coefficient for substrate saturation (g/l) and S is the substrate limiting factor (g/l). Monod equation can describe adequately the process when the growth is relatively slow and the population density is relatively low (Coulson & Richardson, 1971; Stanbury & Whitaker, 1993).

Several other rate expressions have been proposed to cover different conditions parameters. For rapidly growing dense cultures the equation can be modified as following:

$$\mu = \frac{\mu \max * S}{KoSo + S} \qquad \text{(Equation 1.9)}$$

where μmax is the maximum specific growth rate (h^{-1}), S is the substrate limiting concentration (g/l), Ko is the substrate saturation constant in the feed (g/l) and So is the concentratioin of substrate in the feed (g/l) (Bu'lock & Kristiansen, 1987; Panikov, 1995).

Other alternatives to the Monod equation are the following:

The Blackman equation:

$$\mu = \mu\max \text{ if } S \geq 2\ Ks$$

$$\mu = \frac{\mu \max * S}{2Ks} \quad \text{If } S < 2\ Ks \qquad \text{(Equation 1.10)}$$

where μmax is the maximum specific growth rate (h^{-1}),S is the substrate limiting concentration (g/l), Ks is the substrate affinity constant (g/l).

This equation may fit data better than the Monod model but there is a discontinuity of data.

The Tessier equation: $\mu = \mu\max^{*}(1-e^{-Ks})$ (Equation 1.11)

where μmax is the maximum specific growth rate (h^{-1}),S is the substrate limiting concentration (g/l), Ks is the substrate affinity constant (g/l)

The Moser equation: $\mu\max^{*}(1+KsS^{-s})^{-1} = \frac{\mu\max^{*} S^{n}}{Ks+S^{n}}$ (Equation 1.12)

where μmax is the maximum specific growth rate (h^{-1}),S^{n} is the substrate limiting concentration (g/l), Ks is the substrate affinity constant (g/l)

The Moser equation is most general form of an substrate limited equations, and when n=1 it is equivalent to the Monod equation.

The Contois equation: $\mu = \frac{\mu\max^{*} S}{KsxX + S}$ (Equation 1.13)

where μmax is the maximum specific growth rate (h^{-1}),S is the substrate limiting concentration (g/l), Ksx is the substrate saturation constant proportional to cell concentration that describes substrate –limited growth at high cell densities (g/l) and X is the biomass concentration (g/l)

According to this equation the specific growth rate decrease with decreasing substrate concentrations, and eventually becomes inversely proportional to the cell concentration in the medium (Vrabel, 2001).

As most anaerobic fermentations are inhibited by the end products of the microbial metabolism, models for growth inhibition by them have been developed. (DiSerio, Tesser & Sanctanerria, 2001). Models such as Jerusakimsky, Hinselwood, Taylor and Leudeking and Piret for growth inhibition due to end production inhibition have been widely used. (Roels, 1983, Kovarova&Egli, 1996).For inhibition due to toxicity of the organic compounds Jerusalimsky model is the following:

$$\mu = \frac{\mu nax * S * Kp}{(Ks+S)*(Kp+P)}$$ (Equation 1.14)

where μ is the specific growth rate (h^{-1}) μ max is the specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and Ks (g/l) is the substrate affinity constant (g/l) , P is the end product concentration (g/l) and Kp is a constant of end product production(g/l)

Though the Hinselwood model is proposing the following equation for μ

$$\mu = [S(Ks + S) * (1 - Kp * P)] \qquad \text{(Equation 1.15)}$$

where μ is the specific growth rate (h^{-1}) μ max is the specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and Ks (g/l) is the substrate affinity constant (g/l), P is the end product concentration (g/l) and Kp is a constant of end product production(g/l)

Taylor model is proposing the following 2 equations for end product inhibition

$$\mu = \mu \max * S * \left[(Ks + S) * (\frac{1 - P}{Kp}) \right] \qquad \text{(Equation 1.16)}$$

$$\mu = \frac{\mu \max * S}{(Ks + S) * (1 - Kp * P)} \qquad \text{(Equation 1.17)}$$

where μ is the specific growth rate (h^{-1}) μ max is the specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and Ks (g/l) is the substrate affinity constant (g/l), P is the end product concentration (g/l) and Kp is a constant of end product production (g/l) (Schuler &Kargi, 2002; Roels, 1983; Boomee et al, 2003).

The Leudeking and Piret model is the following:

$$\frac{dP}{dt} = a * \frac{dN}{dt} + b * N \qquad \text{(Equation 1.18)}$$

where, dp/dt is the end product formation rate (g/l), dN/dt is the biomass formation rate (g/l), N is the biomass concentration (g/l), α is a growth associated constant in Leudeking and Piret model (g/g^{-1}) and b is a non growth associated constant in Leudeking and Piret model. (g/g^{-1}).

Another parameter which has to be taken into consideration when referring to a microbial kinetic model is the yield coefficient which is a measure for the conversion efficiency of a growth substrate into cell material or product. (Kovarova & Egli,

1998). The yield coefficient is normally expected to be constant in quantity as all the physicochemical parameters are kept constant (Coulson & Richardson, 1971).

Mathematically yield coefficient is expressed as following:

$$\frac{dx}{ds} = Y \qquad \text{(Equation 1.19)}$$

where dx represents the change in the biomass concentration X which results from a change a change ds in the substrate concentration. An increase in biomass concentration though will result in a decrease in the substrate or in nutrient concentration. (Gruger &Gruger, 1989; Aiba, Humphrey & Millis, 1973).

When the product is the microbial biomass the equation becomes:

$$\frac{dx}{ds} = Y\, x/_{s} \qquad \text{(Equation 1.20)}$$

where Y x/s is defined as the cellular yield coefficient (g/l), X is the final biomass concentration (g/l) and S is the initial substrate concentration in the feed. (g/l)

and if it is a metabolite or an organic acid, the equation becomes:

$$\frac{dPi}{ds} = Y\, i/_{s} \qquad \text{(Equation 1.21)}$$

where Y x/s is defined as the cellular yield coefficient (g/l), i is the final biomass concentration (g/l) and S is the initial substrate concentration in the feed. (g/l)

A correlation between the substrate material balance for the substrate consumption and the specific growth rate can be given by the next mathematical expression:

$$\frac{ds}{dx} = \frac{1}{Y\,x/s} = \frac{1}{YG} + \frac{m}{\mu} \qquad \text{(Equation 1.22)}$$

where μ is the specific growth rate (h^{-1}) Y x/s is the cellular yield coefficient (g/g), m is the specific constant requirement for maintenance of growth (g/g^{-1}) and YG is the growth yield from the substrate.

When considering the Monod equation which introduces the idea of growth limited substrate product inhibition of growth should also be expressed mathematically .This is given by:

$$\mu = \frac{S}{Ks + S + (\frac{S^2}{Ki})} \mu \max \qquad \text{(Equation 1.23)}$$

where μ is the specific growth rate (h^{-1}) μ max is the specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and Ks (g/l) is the substrate affinity constant (g/l), Ki is the end product inhibition constant (g/l).

Also substrate inhibition due to slow addition can be mathematically expressed. For non competitive substrate inhibition growth rate can be defined as:

$$\mu = \frac{\mu \max}{(1 + \frac{Ks}{S})(1 + \frac{S}{K1})} \qquad \text{(Equation 1.24)}$$

where μ is the specific growth rate (h^{-1}) μ max is the specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and Ks (g/l) is the substrate affinity constant (g/l), and K1 is a constant of end product production. (g/l)

If K>>K2 then the equation is transformed to:

$$\mu = \frac{\mu \max * S}{Ks + S + S^2/k1} \qquad \text{(Equation 1.25)}$$

where μ is the specific growth rate (h^{-1}) μ max is the specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and Ks (g/l) is the substrate affinity constant (g/l), X is the cell concentration (g/l) and K1 is a constant of end product production. (g/l)

Through for competitive substrate inhibition:

$$\mu = \frac{\mu \max * S}{Ks(1 + S/K1) + S} \qquad \text{(Equation 1.26)}$$

where μ is the specific growth rate (h^{-1}) μ max is the specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and Ks (g/l) is the substrate affinity constant (g/l), and K1 is a constant of end product production. (g/l) (DiSerio, Tessier& Sanctanerria, 2001).

1.13.1 Batch Cultures and Kinetics

As previously referred , a batch system can be defined as a closed system, commonly a batch fermenter, with heating and cooling systems, where all the materials (substrates, nutrient medium, microorganism) are placed in time zero(t=0) and left to interact. The biotransformation process will continue until the growth becomes limited due to substrate exhaustion. In a batch system no input or output of materials occurs. Concerning the control of a batch process many complications including non linearities and constraints occur (Bu'lock & Kristiansen, 1987).

For a simple batch process the growth rate is examined in the exponential growth phase where the microorganism grows at his maxima, and can be calculated by the following equation:

$$\frac{dx}{dt} = \mu * \mathrm{X} \qquad \text{(Equation 1.27)}$$

where X is the concentration of cells (g/l), μ is the specific growth rate (h^{-1}) of the selected microorganism and t is the time in hours (h). This equation though is valid only for balanced growth of a single strain culture with a constant cell composition. On integrating the above equation:

$$Xt = Xoe^{\mu t} \qquad \text{(Equation 1.28)}$$

where Xo is the original biomass concentration at time zero (0), Xt is the biomass concentration after a time interval of hours (t) and e is the base of natural logarithm.

Taking natural logarithms the equation becomes:

$$\ln Xt = \ln Xo + \mu t \qquad \text{(Equation 1.29)}$$

Calculating a natural log of biomass concentration against the time course, a straight line occurs which slope equals to μ (h^{-1})

Biomass can be directly correlated with the substrate concentration .An increase in the substrate concentration will result into a proportional increase of the biomass concentration. The relation can be described by the equation:

$$X = Y(Sr - S) \qquad \text{(Equation 1.30)}$$

where X stands for the concentration of biomass produced (g/l), Y is the yield factor (g/g) Sr is the residual substrate concentration (g/l) and S is the original substrate concentration. (g/l) (Bu'lock & Kristiansen, 1987; Stanbury & Whitaker, 1993).

Productivity in a fermentation process follows the general equation of:

Flow of material in + Formation of material by biochemical reaction – Flow of material out = Accumulation

Since in a batch process there is no inflow or outflow of substrate material the equation becomes:

Formation of material by biochemical reaction = Accumulation

In these formulas productivity is growth limited and can be described by the equation:

$$\frac{dp}{dt} = qpx \qquad \text{(Equation 1.31)}$$

where P is the concentration of product (g/l), qp is the specific rate of product formation (g/l) .The equation can be further developed as:

$$\frac{dp}{dx} = Y_{p/x} \qquad \text{(Equation 1.32)}$$

where dp is the concentration of product (g/l), dx is the biomass produced (g/l) and Y p/x is the yield of product in terms of substrate consumed (g/l). On integration, equation becomes:

$$qp = Y_{p/x} * \mu \qquad \text{(Equation 1.33)}$$

where qp is the specific rate of product formation (g/l), μ is the specific growth rate and Y p/x is the yield of product in terms of substrate consumed. (Webb, 1964)

The Yield coefficient of the previously referred equations can be calculated by:

$$\frac{dx}{dt} = -Y\, ds/dt \text{ or by } Ys = \frac{biomass(g)}{substrateconsumption(g)}$$

(Equation 1.34)

Yield coefficient stands for the ratio of cells produced to substrate consumed.

Batch fermentations are usually used to produce biomass, primary metabolites and secondary metabolites.

Another equation of evaluating the productivity in a batch system is the following:

$$Rbatch = \frac{X\max - Xo}{t_i - t_{ii}} \qquad \text{(Equation 1.35)}$$

where Rbatch is the output of culture in terms of cell concentration per hour, Xmax is the maximum cell concentration at the beginning of stationary phase (g/l), Xo is the initial cell concentration at inoculation (inoculum size) (g/l), ti is the time during which the organism is not growing at μmax (h) and tii is the time during which the organism is not growing at μmax (lag phase, death phase, sterilization time) (h)

As all the microbial growth kinetic models follow the Monod equation which introduces the idea of growth limited substrate ideas such as substrate inhibition or inhibition of growth due to other factors have been developed.

1.13.2 Continuous Cultures and Kinetics

A continuous culture, as previously referred, is an open system mode of operation where the inflow of nutrients equals and occurs simultaneously with the outflow of mediums. Continuous culture system although their superiority has been recognised towards batch and fed-batch system of operations is not widely applied into industrial scale because of the difficulties they present in their mechanical construction. Several kinetic equations following mostly the Monod pattern have been introduced for the evaluation of the continuous culture. An important parameter for the optimal operation of a continuous system is the dilution rate given by the equation:

$$D = \frac{F}{V}(h^{-1}) \qquad \text{(Equation 1.36)}$$

where D is the dilution rate (h^{-1}), V is the volume of the bulk liquid (L) and F is flow rate (L/h) in and outside the bioreactor. (Malek et al, 1969).

The total biomass concentration in such a system follows the pattern of:

Biomass in incoming medium+ Growth-Output-Death

Mathematically this formula is written as:

$$\frac{dx}{dt} = \frac{F}{V} Xo + \mu X - \frac{F}{V} x - Ax \qquad \text{(Equation 1.36)}$$

where dx/dt is the rate of accumulation of biomass per unit time and per volume (g/l), Xo is the biomass concentration in the incoming medium(g/l), X is the concentration in the bioreactor(g/l), and A is the specific death rate of cells(g/g^{-1}) , F is the flow of nutrients (L/h), V is the volume of nutrients (L) and μ is the growth rate (h^{-1}).As the microbial population remains in a steady state the equation is simplified into:

$$\mu * X = D * X \qquad \text{(Equation 1.37)}$$

where μ is the growth rate (h^{-1}), X is the biomass concentration (g/l) and D is the dilution rate. (h^{-1})

and further simplifying $\mu = D$ (Malek et al, 1969).

On applying the Monod equation for growth rate on a continuous system, the equation for a steady state becomes:

where μ is the growth rate (h^{-1}), X is the biomass concentration (g/l) and D is the dilution rate. (h^{-1})

$$S = Ks \frac{D}{\mu \max - D} \qquad \text{(Equation 1.38)}$$

where μmax is the specific growth rate (h^{-1}), X is the biomass concentration (g/l) and D is the dilution rate. (h^{-1})

For a steady state biomass concentration can be represented as:

$$X = Y_{x/s}(So - S) = Y_{x/s}(So - Ks \frac{D}{\mu \max - D}) \qquad \text{(Equation 1.39)}$$

where Yx/s is the cellular yield coefficient (g/l), So is the initial substrate concentration in the feed (g/l), S is the final substrate concentration (g/l), D is the dilution rate (h^{-1}), Ks is the substrate affinity constant (g/l) and D is the dilution rate (h^{-1}) where μmax equals μ when μmax=0.05

The productivity of a continuous culture can be judged by:

$$Rcont = Dx(1 - \frac{t_{iii}}{T}) \qquad \text{(Equation 1.40)}$$

where Rcont.is the output of cultures in terms of cell concentration per hour, Dx is the steady state cell concentration, T is the time period during which steady state conditions prevail and tiii is the time period prior to the establishment of a steady state and includes the vessel preparation, sterilisation and operation in batch culture prior to continuous operation. Dx increases with increasing dilution rate until the maximum value is reached and after that any further increase in D results in a decrease in Dx. As a result maximum productivity of microbial population can be achieved by the use of the dilution rate giving the highest value of Dx. The principal advantage of continuous culture is that the rate of microbial growth via a substrate limited medium is controlled by the rate of dilution .As long as the dilution rate is lower than the maximum growth rate attainable; the cell division rate exactly balances the cell washout rate. The critical dilution rate is affected by μmax, Ks and Sr and can be expressed mathematically:

$$D = \frac{\mu \max + Sr}{Ks + Sr} \qquad \text{(Equation 1.41)}$$

where D is the dilution rate (h^{-1}), μmax is the maximum specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l), Ks is the substrate affinity constant (g/l) and Sr is the residual substrate concentration (g/l).

On integrating the equation is simplified to:

$$D(Ks + Sr) = \mu \max Sr \qquad \text{(Equation 1.42)}$$

where D is the dilution rate (h^{-1}), μmax is the maximum specific growth rate (h^{-1}), Sr is the residual substrate concentration (g/l), Ks is the substrate affinity constant (g/l)

Dividing by Sr the relationship becomes:

$$\frac{DKs}{Sr} + D = \mu \max \text{ and finally } Sr = \frac{DKs}{\mu \max - D} \qquad \text{(Equation 1.43)}$$

where D is the dilution rate (h^{-1}), μmax is the maximum specific growth rate (h^{-1}), Sr is the residual substrate concentration (g/l), Ks is the substrate affinity constant (g/l)

All the above equation refers to a single vessel system. According to the number of vessels incorporated the model construction can be further developed. (Lombardi et al., 1999).

1.14 Membrane Reactors and Kinetics

1.14.1 Introduction

Membranes are thin, porous barriers which perform several degrees of separation (microfiltration, ultrafiltration, and nanofiltration) using differences in concentration, electrical potential and charge and pressure between two compartments they perform separation to. (Chang & Forsake, 1991). The driving forces used in membrane separation which are diffusion, electrical conductivity, hydraulic pressure.

These forces are interacting with the membrane creating phenomena such as osmosis, reverse osmosis, electrodialysis and electroosmosis. Also other physical parameters which influence the permeability of the membrane are the transport number, the membrane potential (surface charge), the streaming current and the streaming potential. As successful bioseparation process can be considered only the one where the membrane solvents and molecules of interest interact in equilibrium. Phenomena of ultrafiltration, microfiltration and nanofiltration are forced by differential pressure, dialysis and membrane extractions are performed through concentration differences and ionized chemicals are separated due to electrical potential differences (Oh& Kini, 1996).

In numerous cases there is only one separation mechanism which drives the separation mechanism. It is possible though to combine two or more separation mechanisms for membrane separation, although this procedure will be much more complicated and difficult but the separation factor will be large. If there is a combination of primary factors for separation properties of the membranes (porosity, surface charge, chemical structure, diffusivity) and kind of molecules to be separated, many membrane separation processes can occur.

According to the major factors which influence the bioseparation processes, membranes can be applied into various operational units. For example the pore size provides ultrafiltration, nanofiltration, microfiltration and gel filtration, diffusivity provides reverse osmosis and dialysis. Ionic exchange can provide electrodialysis, ion exchange absorbent, volatility results into distillation, vacuum distillation, evaporation, solubility of molecules provides solvent extraction, precipitation and flocculation (Wang, Seki, & Furusaki, 1995).

The majority of membranes are made of polymers but also other materials such as ceramics and stainless steel have been used. Polymeric membranes can be easily produced but are structurally weak and need solid support in order to function properly. Polymeric membranes can be also made very thin and can be applied in a variety for processes like gas permeation and electrodialysis.

Although new materials have been used for the manufacture of membranes, the membranes are not strong enough to support a membrane device alone nor can they obtain enough area for mass transfer. So, they are packaged in several forms like flat, spiral, tubular and hollow fibber types. The major advantage of this procedure is that high pressure can be applied. (Fiechter, 1990).

1.14.2 Classification of Membrane reactors

Membrane bioreactors are composed of two basic parts, a biologic unit and a membrane module used for physical separation of the mixed fluids. They are divided into two general groups according to their function.

In the first category, the membrane is used for retaining cells and separating the biocatalysts which were suspended in the reactor from the solution, resulting in the difference of hydraulic and solid reaction times (Chang & Furusaki, 1991).

In the second category the biocatalysts are immobilised, usually by microencapsulation on the surface or in the matrix of the membrane or they are immobilized within two membranes. All membrane devices though employ tangential flow filtration and the products can be removed either by dialysis extraction with solvent or by pervaporation. Numerous module designs of membranes have been used for MBR manufacture. These contain forms like tubular, spiral wound, flat sheet shape and frame, rotary disks and hollow fibres. According to the above criterion

types of MBRs include hollow fibber bioreactors, dual hollow fibber bioreactor and membrane recycle reactors (Fiechter, 1990).

The pore size of the membranes used ranges between 0.01 μm to 0.04 μm. The fluxes of the materials range between 0.05 $m^3m^{-1}d^{-1}$ to 10 $m^3m^{-1}d^{-1}$ being highly dependent on the systems configuration and on the membrane material. The biocatalysts can be immobilised within the membrane, segregated or flushed along. Methods such as entrapment, gelification, adsorption, ionic binding, covalent or cross-linking binding can be used. Choosing the suitable reactor configuration depends on the properties of the reaction system .For reaction example if the membrane is used to retain large components (enzyme and substrate) while permitting smaller molecules to pass through (product), concentration–polarization phenomena as well as membrane fouling will affect the performance of this type of the reactor (Cicek, 2003).

Another system of classification is according to the reactors configuration .The first group named integrated MBRs involves outer skin membranes that are internally placed in the reactor (Cicek, 2003).The driving force across the membrane is given by pressurizing positively or through negative pressure on the permeate side. Cleaning of this membrane is done by constant permeate back pulsing and occasional chemical backwashing. The second group named recirculated (external) MBRs involves the recycling of the mixed broth through a membrane device which is placed outside the bioreactor. The driving force for fluid circulation in inner and outer skin membranes is the pressure created by high cross flow velocity along the membrane surface. The applied pressure across the membrane surface varies from 20 to 500 kPa for inner skin membranes and from -10 to -80 kPa for outer skin membranes (Morao et al., 2006).

1.14.3 Membrane Bioreactors and Microbial Cell cultures

When MBRs are used for microbial cell cultures , the cells can either be immobilised in the membrane matrix or they can be retained in ultrafiltration unit membrane especially when using membrane recycle reactors and the end products can be removed either by dialysis(running water) or by extractive bio processing (solvent extraction) (Wang et al, 1995).

Unfortunately the majority of these applications have been limited to laboratory scale due to the technical difficulties occurring from the use of bioprocess systems in industrial scale.

The most popular type of reactor these type of process is the hollow fibre reactor and the dual hollow fibre reactor. In these types of reactors both anaerobic and aerobic cultures can be performed. The cells can be immobilised in the shell side of the hollow fibber reactor. They reproduce rapidly in the interstitial special space between the fibbers .Nutrients can be supplied through the membrane from the lumen side and products are removed in the opposite direction. The cells should be inoculated into large amounts for optimum performance (Fiechter, 1990).

Another type of reactor which can be used is the membrane recycle reactors which has been applicated in wastewater treatment for long time. The major advantage of this type of reactor at a higher dilution rate without considering washout, by separating hydraulic retention time (HRT) and solid retention time (SRT).

Membrane reactors using biological catalysts such as microbial cells or enzymes can be applied to production processing and treatment operations (Giorno & Drioli, 2000). Advantages over the use of MBRs include their ability of functioning into moderate temperatures and pressure, reduction of formation of by products and they do not demand additives in order to function.

Monod type kinetic equation can easily be applied on membrane reactors. The mathematical modelling concerning growth and productivity follows the common fermentation kinetics adjusting the parameters according the mode of operation (batch, fed-batch, continuous). An example of kinetics for a cell recycle bioreactor is the following:

Writing material balances for S (substrate), P (product) and X (biomass)

X (biomass): $\frac{dx}{dt} = (\mu - BD)X$ (Equation 1.44)

where dx/dt is the biomass formation rate (g/l), μ is the growth rate (h^{-1}), BD is the bleeding ratio (L/h) and X is the biomass concentration (g/l).

S (substrate): $\frac{S}{S+Ks}\mu\max = \mu$ and $\frac{dS}{dt} = D(So - S) - {\mu x}/{Ys/S}$ (Equation 1.45)

where ds/dt is the substrate consumption rate (g/l), μmax is the maximum specific growth rate (h^{-1}), μ is the growth rate (h^{-1}), D is the dilution rate (L/h) and X is the biomass concentration (g/l) and Ys/S is the substrate yield (g/g).

P (product): $\frac{dP}{dt} = -DP + q_p X$ (Equation 1.46)

At steady state where $dx/dt = 0$ and the condition for no washout is Dwashout $< \mu/B$ where B stands for bleed ratio. If there is no bleed Dwashout becomes infinite. (Fiechter, 1990)

The productivity of the system P consists of a two component equation:

$$P = (1 - B)DPa + BDP \quad \text{(Equation 1.60)}$$

The first term refers to the productivity obtained through membrane filtration and Pa stands for product concentration through the membrane. The second term refers to a product that that goes out with a bleed stream containing the cell mass. According to the above equation productivity (P) becomes lower with an increase of dilution rate (D). The same situation applies to an inhibitor, an inhibitory growth toxin and product formation if present (Lin & Hwang, 2005).

1.15 Aims and Objectives

The aim of this research project was to investigate the growth kinetics of *L.lactis* on 3 different culturing systems (batch, continuous, MBR) and provide a growth strategy for development of high cell density of the coccus.

This principal aim of the project was achieved through the use of several techniques. Firstly batch cultures of the coccus without pH control will be performed. The physicochemical properties and nutritional requirements of the coccus were investigated and an optimised culture medium was developed which was used for growth of *L.lactis* into large scale.

Secondly the growth of the coccus will be investigated in a 2L CSTR with working volume of 1.8L with continuous pH control system. The reactor will be operated batch wise and the optimum substrate concentrations will be confirmed. The optimum pH value of operation will be also selected based on growth parameters such as product and cellular yields.

The use of the optimised growth medium incorporating the optimum pH value will be aimed at shortening the lag phase and support the production of higher biomass yields.

The wider investigation of growth kinetics and the comparison of the different culturing conditions will be achieved through mathematical modelling with the use of numerical methods. Three mathematical algorithms will be developed, each one representing a different system of culturing conditions, will be used to predict the volumetric cell productivity (g/l/h) under different growth conditions. The validity of the formulas composing the models will be certified through comparison of the theoretical predictions with the available experimental data. Through mathematical models the relative performance of the reaction systems will be discussed.

Chapter Two

Materials and Methods

Phase One: Optimisation of Growth Media for Lactococcus lactis

2.1 Introduction

In order to achieve higher yields of lactococcal biomass and consequently higher productivity of metabolic end products such as lactic acid, an investigation for an optimised growth medium was performed. The coccus was primarily propagated into basal medium .In addition a study over the inhibition effects on growth by the metabolic end products was performed.

2.2 Maintenance and Preservation of the Inoculum

2.2.1 Inoculum source

The microbial strain of *Lactococcus lactis* was provided in a liquid form by Dr.R.W. Lovitt, Department of Chemical Engineering Science, University of Wales, Swansea.

2.2.2 Inoculum Preservation

The coccus was revived by inoculating the strain into 50 ml serum vials containing basal medium and was statically incubated at 30°C for 48 hours. Stock culture solution of the strain was made through cryopreservation method. The coccus was firstly inoculated into 50 ml serum vials containing basal medium and was incubated for 12 hours until the exponential growth phase was reached. The cells were separated from the liquid medium via centrifugation method, (4000 rpm for 10 min.)(Biofuge Stratos Sorall, Kendro Products, Germany). After rejecting the centrifuged liquid; the remaining solution was again centrifuged in order to further concentrate the cells. The final cell paste created was mixed with an equal amount of glycerol solution (BDH Chemicals, UK) (50% v/v) which was used as a cryoprotective agent. The final solution was dispersed under aseptic conditions into criovials (200 μl) and preserved into deep freezing (-70°C) (Heraus Ultra-freezer, UK). The coccus was revived by inoculating after thawing 2 criovials (400 μl) into 10 ml basal culture medium contained into pressure tubes. The culture tubes were statically incubated into 30°C for 24 hours.

For constant use, the coccus was regularly reinoculated (on a weekly basis) into 25 ml serum vials containing basal medium and were preserved at 4 °C.

2.2.3 Purity of Cultures

The purity of cultures was controlled via optical microscopy. (Olympus CX21, UK) Two samples were taken from each culture and colourless liquid preparations were made. The samples were checked for morphology, cell damage and mutation using phase contrast microscopy.

2.3 Growth Media, Carbon Sources and Chemical Solutions

2.3.1. Basal Medium Composition (Liquid Form)

1.	Glucose ($C_6H_{12}O_6$)	1% w/v
2.	Yeast Extract (Y.E.)	0.5% w/v
3.	Bacteriological Peptone	0.5% w/v
4.	Sodium Chloride (NaCl)	0.5% w/v
5.	Potassium Hydrogen Phosphate (KH_2PO_4)	0.4% w/v
6.	Di-Potassium Hydrogen Orthophosphate Anhydrous (K_2HPO_4)	0.15% w/v
7.	Resazurin Dye ($C_{12}H_6No_4Na$)	0.005% v/v

All the previously referred chemical compounds were provided by three companies, Oxoid Chemicals, UK, BDH Chemicals UK, and Sigma-Aldrich Chemicals, USA.

Methodology

The above amounts of powdered materials were weighted into an electronic balance (Sartorius, CP4202S, JENCONS-PLS, Germany) and they are added into an Erlenmeyer flask containing 1L. of distilled water. The flask **(1)** contained a magnetic stir bar and it was placed on a magnetic stirring plate (Rotary Mixer, HAT, UK) where it was continuously stirred (700 rpm) until all materials were completely dissolved. In order to remove the existing dissolved oxygen, the medium was boiled using a Bunsen burner. Resazurin dye functioned as an anaerobiosis indicator (negative redox potential) changing its colour from deep purple to colourless. (Willis, 1977). The material was then dispersed via sterile syringe connected with rubber tube

(6) into serum vials **(2&3)** (50ml and 100ml) and pressure tubes **(4)** (10ml) under the presence of gaseous nitrogen flow **(5)** in order to achieve complete anaerobic conditions. The serum vials are then sealed with butyl rubber stoppers and alumina seals.

So to avoid a caramelization reaction, glucose solution was autoclaved (120°C for 20 min.) (Priorclave: Tactrol 2, RSC/E, UK) and prepared separately under aseptic conditions. The medium's pH was set at 6.8. with NaOH 1M alkali solution. The basal medium solution was then autoclaved (120°C for 20 min.) (Priorclave: Tactrol 2, RSC/E, UK) glucose was introduced into the basal medium after autoclaving using a sterile syringe.

Initial doubling time and maximum specific growth rate of the microbial strain were determined into basal medium under a 10 hour circle of static incubation into 30°C. In addition basal medium was used for inoculum preservation.

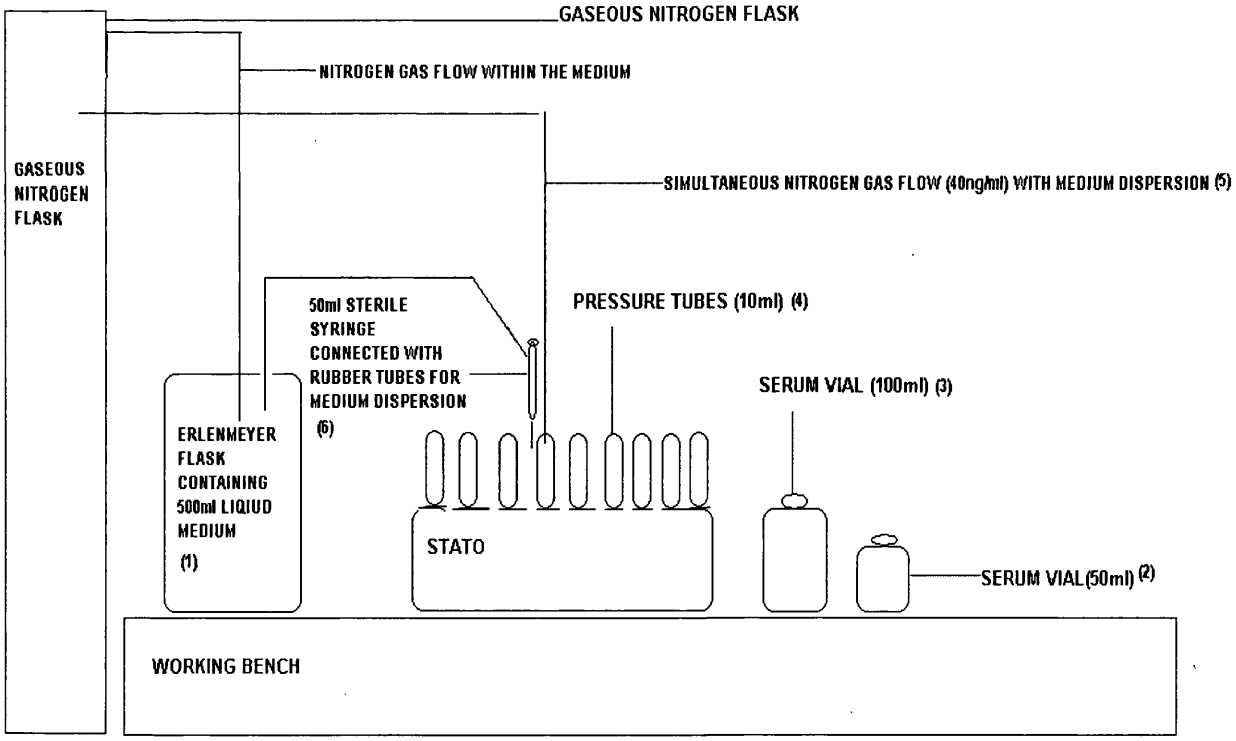


Diagram 2.1: Medium dispersion within the tubes and the serum vials. The numbers in the parenthesis are referring on the section 2.3.1

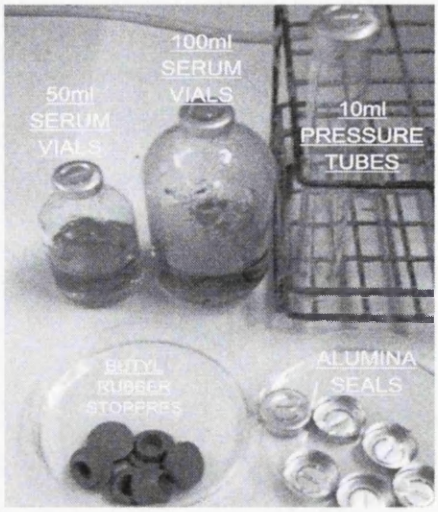


Figure 2.1: Liquid culture equipment used in medium preparation showing 50 &100ml serum vials and 10ml pressure tubes.

2.3.2 General method for amelioration of Growth medium composition

Introduction

Glucose, peptone and yeast extract were present in the culturing basal medium. Each major component effect over the growth of the coccus was separately tested using the same experimental methodology in the series of trials aiming to the development of an optimised growth medium. The specific changes in the medium and the effect of them are presented and discussed separately in the Results section. No glucose was added prior to autoclave in the medium as to avoid caramelization effect, but added to the required concentrations to test the effect on the growth.

Methodology

Each major component (glucose, peptone, yeast extract) was separately prepared and autoclaved in a concentrated form solution. The effect of each component was tested within a concentration range between 0% to 5% and introduced to the medium via a sterile syringe in the desired amounts. Each differential concentration was duplicated to obtain the average data. (statistical data variation <5%) giving an overall of 24 pressure tubes.

The total volume of the basal medium prepared was 500 ml. (The medium was prepared according to the analytical procedure presented in section 2.3.1) The medium was dispensed into the pressure tubes under the presence of gaseous nitrogen flow so to achieve complete anaerobic conditions. The pH of the medium was set at

6.8 with NaOH 1M alkali solution. After the introduction in the tubes of the desired amounts of each component, the tubes were gently mixed into a vortex mixer and then inoculated with 10% inoculum size. The effect of each component was evaluated through a 10 hour static incubation at 30°C achieving specific growth rate and doubling time parameters. After the determination of the optimum concentration for growth of every component, all the components resulting into the manufacture of an optimised medium.

2.3.3. Optimisation of the Growth Medium

Optimum Medium Concentration (Liquid form)

1.	Glucose($C_6H_{12}O_6$)	2% w/v
2.	Yeast Extract(Y.E.)	1% w/v
3.	Bacteriological Peptone	0.4% w/v
4.	Sodium Chloride(NaCl)	0.5% w/v
5.	Potassium Hydrogen Phosphate(KH_2PO_4)	0.4% w/v
6.	Di-Potassium Hydrogen Ortho phosphate Anhydrous(K_2HPO_4)	0.15% w/v
7.	Resazurin Dye($C_{12}H_6No_4Na$)	0.005% v/v

Methodology

In order to evaluate the influence of the optimised medium on growth of *L.lactis*, 10 pressure tubes (10 ml overall concentration) were fabricated. The total volume of medium fabricated 500 ml. (The medium was prepared according to the analytical procedure presented in section 2.3.1.) The medium was dispersed into the pressure tubes under gaseous nitrogen flow in order to achieve complete anaerobic conditions. The medium's pH was set at 6.8. with NaOH 1M alkali solution. The pressure tubes were sealed with butyl rubber stoppers and alumina seals and autoclaved (20 min at 120°C) (Priorclave: Tactrol 2, RSC/E, UK). As to avoid a caramelization reaction, glucose solution was separately prepared, autoclaved and introduced within the pressure tubes after autoclaving via a sterile syringe. The tubes were gently mixed in a vortex and inoculated with 10% inoculum size. The effect of optimised medium was evaluated under a ten hour growth circle static incubation at 30°C, achieving doubling time and specific growth rate parameters. (experimental data variation <5%).

2.4 Inhibition Effects on Growth of Lactoccocus Lactis of Metabolic End Products

As being a homofermentative coccus *L.lactis* major metabolic end product is lactic acid. *L.lactis* also produces a small amount of acetate. (Holt, 1974) Similarly to all the anaerobic fermentations the growth of the coccus is inhibited by the constantly augmenting concentrations of its end products. Sodium lactate and sodium acetate solutions were used to evaluate the inhibitory effects on growth.

2.4.1. Sodium Lactate Effect

Basal Medium and other reagents Composition (Liquid Form)

1.	Glucose($C_6H_{12}O_6$)	1% w/v
2.	Yeast Extract (Y.E.)	0.5% w/v
3.	Bacteriological Peptone	0.5% w/v
4.	Sodium Chloride(NaCl)	0.5% w/v
5.	Potassium Hydrogen Phosphate(KH_2PO_4)	0.4% w/v
6.	Di-Potassium Hydrogen Ortho phosphate Anhydrous(K_2HPO_4)	0.15% w/v
7.	Resazurin Dye($C_{12}H_6No_4Na$)	0.005% v/v
1.Sodium Lactate solution ($C_3H_5NaO_3$)		10% w/v

Methodology

Sodium lactate solution (10% w/v) was separately prepared and autoclaved (120°C for 20 min) (Priorclave: Tactrol 2, RSC/E, UK).500 ml of basal medium were prepared. (The medium was prepared according to the analytical procedure presented in section 2.3.1) The medium was dispersed into 24 pressure tubes under the presence of gaseous nitrogen flow so to achieve complete anaerobic conditions. The medium's pH was not preset due to the introduction of sodium lactate. Sodium lactate was introduced into the medium after autoclaving via a sterile syringe. Each tube contained a different sodium lactate concentration, from 0% to 5%. Each different concentration was duplicated in order to avoid statistical errors of the obtained data. (experimental data variation <5%). The tubes were sealed with butyl rubber stoppers

and alumina seals and autoclaved. (120 °C for 20 min.) (Priorclave: Tactrol 2, RSC/E, UK) The tubes were gently mixed in a vortex and inoculated with 10% inoculum size. The effect of sodium lactate was evaluated under a ten hour growth circle static incubation at 30°C. The doubling time and specific growth rate parameters were determined.

2.4.2 Sodium Acetate Effect

Basal Medium and other reagents Composition (Liquid Form)

1.	Glucose($C_6H_{12}O_6$)	1% w/v
2.	Yeast Extract(Y.E.)	0.5% w/v
3.	Bacteriological Peptone	0.5% w/v
4.	Sodium Chloride(NaCl)	0.5% w/v
5.	Potassium Hydrogen Phosphate(KH_2PO_4)	0.4% w/v
6.	Di-Potassium Hydrogen Ortho phosphate Anhydrous(K_2HPO_4)	0.15% w/v
7.	Resazurin Dye($C_{12}H_6No_4Na$)	0.005% v/v
1. Sodium acetate solution (CH_3COONa)		10% w/v

Sodium acetate solution (10% w/v) was separately prepared and autoclaved. (120 °C for 20 min) (Priorclave: Tactrol 2, RSC/E, UK).500 ml of basal medium were prepared. (The medium was prepared according to the analytical procedure presented in section 2.3.1.) The medium was dispersed into 24 pressure tubes under the presence of gaseous nitrogen flow so to achieve complete anaerobic conditions. The medium's pH was not preset due to the introduction of sodium acetate. (neutral pH) Sodium acetate was introduced into the medium after autoclaving via a sterile syringe. Each tube contained a different final sodium acetate concentration, from 0% to 5%. Each different concentration was duplicated in order to avoid statistical errors of the obtained data (experimental data variation <5%).The tubes were sealed with butyl rubber stoppers and alumina seals and autoclaved. (120°C for 20 min) (Priorclave: Tactrol 2, RSC/E, UK) The tubes are gently mixed in a vortex and inoculated with 10% inoculum size. The effect of sodium acetate was evaluated under a ten hour

growth circle static incubation at 30°C, to determine doubling time and specific growth rate parameters.

2.4.3 Measurement of Biomass Concentration

Biomass concentration (X), growth rate (μ) and doubling time (td) were evaluated by means of optical density measurements. The optical density of each sample was measured on 660nm wavelength on a Spectrophotometer (Philips, PO8625 UV/VIS, France).The optical density measurements were converted into dry weight units (g/l).

2.4.4 Dry Weight Determination of L.lactis

In order to convert the optical density measurements into dry weight units (g/l) of the coccus a dry weight determination experiment was performed.

The coccus was initially inoculated and propagated (10% inoculum size) into a 5L pyrex glass reaction vessel containing 4.5L of optimum medium. The vessel was statically incubated at 30°C for 36 hours until the coccus reached the late exponential growth phase.

The cells were harvested from the nutrient broth via microfiltration method. A microfiltration device (CFP-2-F-8A, Needhaw, UK) equipped with a heat exchanger was used giving a final concentration of cells of 500 ml. The cells were further concentrated via centrifugation method (4000 rpm for 10 min.)(Biofuge Stratos Sorall, Kendro Products, Germany) using 50 ml plastic conical sterile tubes. (Fisherbrand, UK). The optical density of the cell paste was measured (660 nm, 1cm) (Philips Spectrophotometer PO8625 UV/VIS, France) and the sample was further diluted with A.D. using an automatic pipette (BCL, ANT pipette 5000F, UK) (10 fold dilutions). The diluted samples were filtered with filter papers (Whatman papers 70mm, qualitative, UK) and the cell paste was left on the filter. The samples were then weighted using a high precision electronic scale (0.1 mg Ohaus, V12140 Voyager, Switzerland).Then; the samples were collected and dried for 24 hours into an furnace (Heraus Furnace, UK) at 105°C. After drying the filters were weighted again. The difference between the original and the final weight measurement was determined as the dry weight of the coccus. A correlation between the optical density measurements and the weight of each sample was made resulting into the equation for the dry weight determination. The equation resulting have a linear form of $y = a\chi + c$.

Dry Weight Determination Equation $Y = 0.21405 \mathbf{X} + 0.00347$.

where X stands for the O.D. measurements.

Phase Two: Investigation of pH Controlled Fermentations in a CSTR Fermenter

2.5 Introduction

The majority of trials concerning the productivity of LAB have been done on acidifying cultures due to the production of acids as metabolic end products. It has been suggested though that pH controlled fermentations may result into higher yields of end products. (Mierau,Lei et al.2005;Pavan and Hols, 2000).*L.lactis* growth on pH controlled fermentations was evaluated on a pH range of 4.5 to 7 in order to determine the optimum pH value for growth and it was performed on an Stirring Tank Reactor (STR)

2.4.1. Design of the CSTR Fermenter

A 2L Pyrex glass fermenter has been selected for the procedure. The fermenter was equipped with an hydrargiric thermometer for temperature control, **(1)** a pH probe(Fischer Scientific, UK) for pH control **(2)** , a magnetic stir bar for agitation,**(3)** an glass aeration port,**(4)** a sampling and inoculation port,**(5)** a gas flow stainless steel port connected with a filter (Polyvent filter, 0.2μm, Whatman Filters, UK) **(6),** a port for alkali/acid feed**(7)** and stainless steel coils for heat emission**(8).**All the ports were made of stainless steel and were connected with plastic tubes of several lengths. The working volume of the reactor was set at 1800ml.

The pH probe was connected with a pH controller apparatus (Electrolab FerMac 260, UK) **(9)** and it was calibrated with suitable acidic and alkalic solutions (pH 4 and pH 7) to adjust the pH range. The gas flow port was equipped with a filter (Polyvent filter, 0.2 μm, Whatman Filters, UK).The filter was connected with a gaseous nitrogen flask**(10)** via rubber tubes and the flow was set up at 40 ng/ml. The alkali feed port was connected with a plastic bottle containing an alkali solution of 100 ml of KOH 5M **(11)** which was placed on an electronic scale (Ohaus portable advanced, Switzerland**) (12)** so to measure the volume of alkali used for pH maintenance. The coils were connected with a water bath (Grant Water bath, UK) **(13)** and a pump (Watson Marlow Digital, 505S, UK) **(14)** for continuous preservation of steady temperature (30°C).The fermenter was placed on a magnetic stirring plate (SM1,

Stuart Scientific, UK) **(15)** and was constantly stirred at 350 rpm as being an anaerobic bacterium. The sampling was performed on an hourly basis on a 10 hours circle via the sampling port with a 10 ml sterile syringe and all the samples were measured for biomass concentration via optical density measurements (660 nm, 1cm) (Philips Spectrophotometer PO8625 UV/VIS, France).Also the pH of the samples was re-evaluated into an electronic pH meter. (Metler Toledo, Seven Gasy, Switzerland).

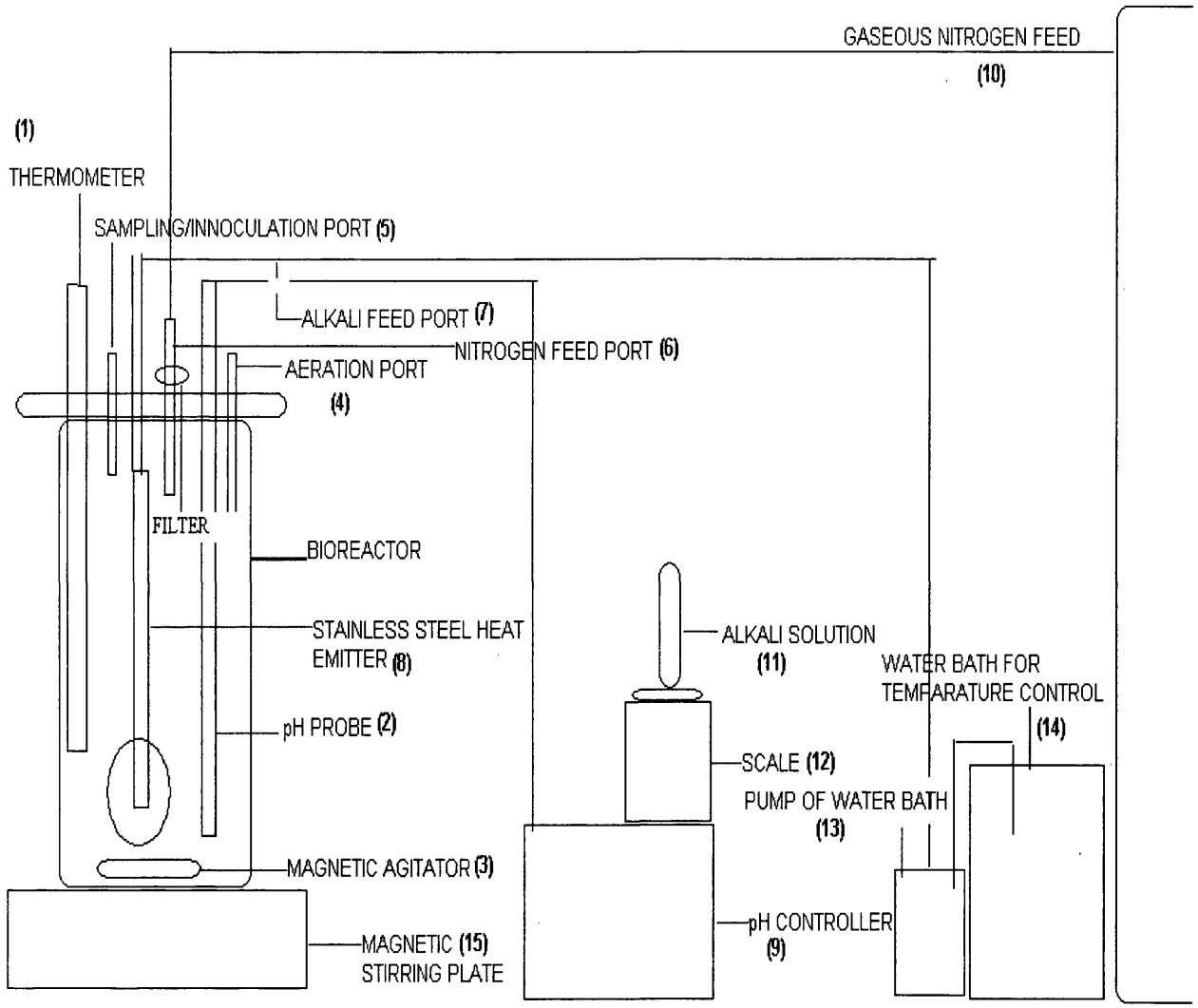


Figure 2.2: Experimental Design of pH control fermentation. The diagram is described in section 2.4.1

Figure 2.3: Photographs of the equipment for the pH controlled STR

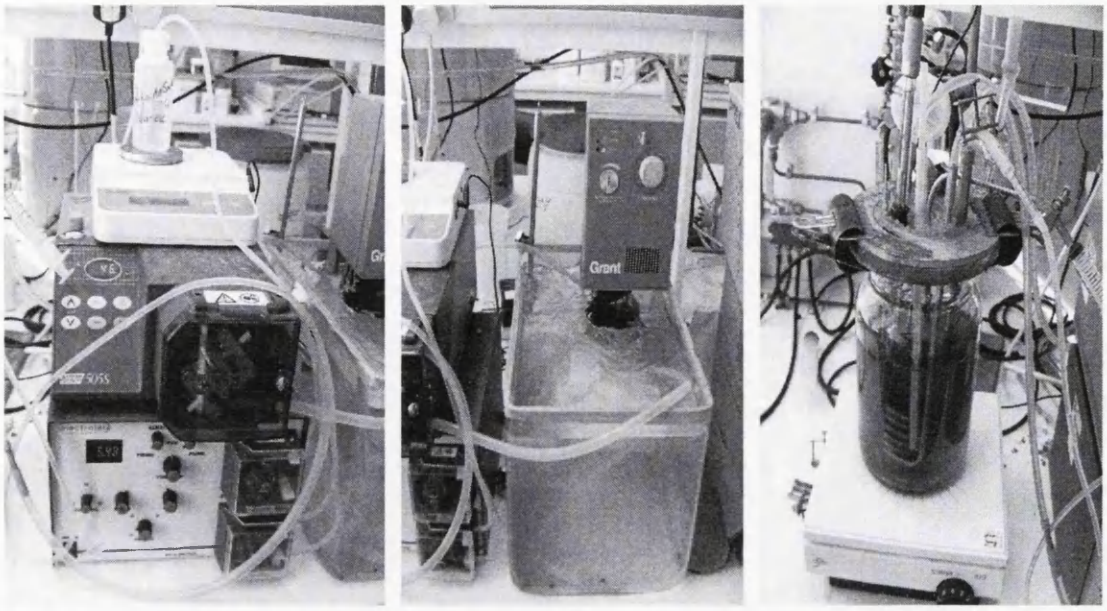


Image 2 pH controller, pump, scale, Alkali solution feed

Image 3 pH Waterbath for temperature control

Image 4 STR Fermenter on a stirring plate

For detailed explanation see Figure 2.2

2.5.2. Medium Preparation and Sterilisation

The fermentation process was performed with optimum medium for growth of *L.lactis*. 1.8 L of optimum medium were prepared and introduced in the fermenter. (The medium was prepared according to the analytical procedure presented at the experiment 2.3.1.).The glucose solution was not prepared separately and in order to avoid caramelization the medium's initial pH was set before autoclaving at 5.5 and reset again before every trial at the desirable stage. The fermenter was sealed with two metallic fasteners and all the ports were sealed with metallic clips and alumina foil, before autoclaving. The pH probe was autoclavable. One aeration port was left open during autoclaving for gas exchange. Then it was sealed with a metallic lock (water seal) covered with cotton wool and alumina foil. The STR was autoclaved at 120°C for 15 min. (Priorclave: Tactrol 2, RSC/E, UK)

2.5.3 Inoculum Preparation

A 10% inoculum size was prepared for the experimental process. 4 serum vials containing 50 ml of optimum medium were inoculated with 10% inoculum size concentration and were incubated at 30°C for 20 hours until the coccus reached late

exponential growth phase. The serum vials were inoculated via the inoculation port in the fermenter using a 50 ml sterile syringe under aseptic conditions.

2.5.4 Alkali and Acidic Solutions

For the pH controlled fermentations and for the pH adjustment of the medium, alkali solution of 5M KOH (BDH Chemicals, UK) and acidic solution of 1M of HCl (BDH Chemicals, UK) were prepared. The solutions were freshly prepared for every fermentation process. (It was assumed that these extreme chemical conditions destroy all contamination sources)

2.6 Determination of Fermentation Pathway used by L.lactis

2.6.1 Determination of Organic Acids Production

In order to determine whether *L.lactis* strain used is a homofermentative or heterofermentative organic acids concentration measurements where performed. All the analysis of organic acids was done using High Performance Liquid Chromatography (HPLC) method.

2.6.2 Sample Preparation

All the samples were taken by the fermented liquid nutrient medium resulted after the 10 hour pH controlled fermentations. As growth of the coccus occurred in a pH range between 5 and 7, samples of 50ml were taken by the broths used in this pH range. The samples were centrifuged (4000 rpm for 5min) (Biofuge Stratos Sorall, Kendro Products, Germany) using 50ml sterile plastic conical tubes (Fisherbrand, UK) in order to remove the cellular biomass. The clarified liquid was further centrifuged twice (4000 rpm for 5min) for complete removal of biomass. One ml of each sample was collected into pressure tubes and diluted 100 times with distilled water. After the completation of the measurements all the samples were collected and preserved at 4°C until analysed.

2.6.3 HPLC Unit Operation

The HPLC system was connected with a conductivity detector (ED 40 Electrochemical Detector, Dionex, UK) and fitted with an ion exchange column. (Varian Co. Canada.)The solvent (mobile phase) delivery system was constructed by 2 pumps (pumps A and B) (Varian Co.Canada.) with a pressure operation range

between 1500 and 1900 mbar. Temperature control of the solvents was maintained with a hotplate (Millipore Co., UK) at 27°C.

The mobile phase was represented by two solution of NaOH of 0.05mM and 100mM concentration. The solutions were delivered to the pumps via rubber tubes and valves. The mobile phase was organised as following: NaOH 0.05 mM was poured for 5min., then the concentration of the solution increased to 5.5mM for 10min. and then to 100mM for 5min.At the final phase the NaOH concentration was kept at 50.5 mM for 15min and then to 0.5 mM for 3 min. The flow rate of the samples and of the mobile phase was set at 1ml/min.

The operation of the system was controlled automatically using Prostar Workstation Data analysis software package (Varian Co., Canada). Every cycle of measurements lasted for 38 min.

All the samples were injected in the system via a sterile HPLC plastic syringe at a 20 μl injection loop connected with the HPLC system.

2.6.4 Calibration Curves

L.lactis was reported to produce mainly lactic acid and a very small amount of acetic acid during the exponential growth phase of its metabolism (May, 1978).In order to determine this fact reference curves of different concentrations of and lactic acid and acetic acid were made. The concentration of the solutions varied between 5mM to 30mM. The solutions were fabricated from pure liquid lactic acid and acetic acid (Sigma Aldrich Chemicals, UK).The retention time of lactic acid was 12min though acetic acid had 15min. 20 μl of each solution was injected in the system via the injection loop and the concentration of the solutions was expressed in terms of conductivity (mVolts) of the column width occurring. The equations resulting have a linear form of $y = a\chi + c$.

Organic Acids	*Resulting Equations*
Lactic Acid	79277 **X**+ 237815
Acetic Acid	42457 **X**+ 241361

where X was the organic acids concentration (g/l) and Y for the main peak area.

2.6.5 Sample Measurements

All the diluted samples were injected in the injection loop in a quantity of 20 μl. Each measurement lasted for 38min. After the end of the measurements the equipment was cleaned overnight using the mobile phase solutions. The concentration of lactic acid in the samples was obtained by the following formula: Concentration (mM) = Column Area (mVolts) – Constant (79277)/ Slope (237815)

2.7 Carbohydrate Consumption rate Determination

In order to investigate the amount of carbohydrate accumulated and define the carbohydrate consumption rate during the fermentation performed by the coccus glucose concentration was measured using an enzymical method using GOD and POD enzymes. Moreover through this method the productivity of lactic acid by the coccus can be investigated. The measurements were done on samples deriving from the broth of pH controlled fermentation on 6.5 pH, as this pH has been selected as the optimum.

2.7.1 Sample Preparation

All the samples for the determination of the final glucose concentration were prepared according to the section 2.5.2.In order to define the rate of carbohydrate consumption all the preserved samples taken on hourly basis during the pH control fermentation were defrosted. The samples were taken from the fermentation conducted at pH 6.5 which was selected as the optimum pH value. The samples were transferred into 10ml conical plastic tubes (Fisherbrand, UK) and centrifuged twice (5000rpm for 10 min.)(Biofuge Stratos Sorall, Kendro Products, Germany) in order to remove completely the biomass. The clarified liquid was diluted into pressure tubes with A.D. (10 fold dilutions).

2.7.2 Essay Preparation

The GOPOD glucose determination assay (Megazyme Int., Ireland) is an enzymical method for the determination of glucose. Glucose is oxidised to gluconic acid with the production of hydrogen peroxide under the presence of glucose oxidase. The

hydrogen peroxide is then used with peroxidise to oxidise a reagent containing p-Hydroxybenzoic acid and aminoantipyrine to produce a coloured solution. The integration of the colour of the solution is proportional to the concentration of glucose. Three solutions were prepared, the glucose reagent buffer, the glucose determination reagent and glucose standard concentration solution (1% w/v) (Sigma Aldrich Chemicals, UK). The measurements were performed in quartz cuvettes in a spectrophotometer (Unicam, UK) at 510nm wavelength. The cuvettes, after the measurements, were cleaned with 50% ethanol solution (Aldrich Chemicals, UK) and A.D.

2.7.3 Sample Measurements

100 µl of each sample was gently mixed with 3ml of glucose determination reagent in pressure tubes, and then incubated in water bath of 40°C for 20min.Then all the samples were transferred into quartz cuvettes and the absorbance was measured against reagent blank. The concentration of the glucose in the sample was calculated by the following equation:

Glucose µg/0.1ml= O.D. Sample / O.D. of Glucose Standard *10

Phase Three: Mathematical modelling of L.lactis intensive culture on Batch Culture, Continuous culture and on Membrane Reactor

2.8 Mathematical Modelling of L.lactis

2.7.1 Introduction

In the previous experimental phases the cultural characteristics of *L.lactis* were studied .The optimal conditions for biomass formation and lactic acid production were investigated. It was demonstrated that the strain used is purely homolactic. In order to develop further the understanding of fermentation patterns and develop an optimal process, mathematical modelling technique was used.

2.8.2 Mathematical Model Formation for Batch Mode Operation

An unstructured kinetic mathematical model was proposed to describe the growth of *L.lactis* on a STR reactor operating in batch mode with continuous pH control.

The mathematical algorithm was based on the Monod model for microbial kinetics and was represented by three differential equations each one of them representing product formation (dp/dt), substrate consumption (ds/dt) and biomass formation (dx/dt).

As it was confirmed experimentally *L.lactis* is a homolactic strain. Its growth is inhibited by the metabolic end product's constantly augmenting concentration. A mathematical term was incorporated in the model so as to describe inhibition effects. Maintenance of the coccus into stationary phase was represented by a maintenance coefficient ($k_d X$) resulting from the slope of the line of growth on optimum medium (Doran, 2004, Pirt, 1985).

In order to develop the model several assumptions had to be used.

- The main model is constructed on a volume of 1L.
- The concentration units of biomass, substrate and end products are in g/l
- All the mechanical and physical parameters (temperature, agitation, aeration, media sterilisation) within the system were kept steady.

All the equations were developed in Excel 2003 Microsoft office software written on VBA language with the use of numerical method, using the primary estimates of the

coefficients in parallel with the initial values of the parameters to create a series of time course curves that were compared to the experimental data.

All the equations and the model development were performed on a spreadsheet. The time step was set at $0.01\ h^{-1}$ on a 10 hour circle. The model was primarily developed without any product inhibition term. The product inhibition term was incorporated within the model after unsuccessful fitting with the experimental data available. 7 columns of data were used. The 1st column was representing the time scale, the 2nd the substrate diminish during time, the 3rd represented the biomass development during time, the 4th represented the growth rate, the 5th the biomass formation rate (dx/dt), the 6th the substrate consumption rate (ds/dt) and the 7th the product formation rate per time (dp/dt).

The 1st column (time scale,h) was made by multiplying the column number with the time scale. The 2nd (substrate consumption during time,S) was made by adding to the initial substrate concentration the substrate consumption rate per time. To the final result, the next substrate consumption rate per time was again added and so on, in order to achieve the substrate diminish in every time step. The 3rd column (biomass development during time, X) resulted from the addition to the initial concentration of biomass, of the biomass formation rate per time. To the final result, the next biomass formation rate per time was again added and so on, in order to achieve the biomass development in every time step. The 4th (growth rate, μ), 5th (biomass formation rate, dx/dt), 6th (substrate consumption rate, ds/dt) and 7th (product formation rate per time, dp/dt) column resulted from the equations used in the mathematical model development for the growth of *L.lactis* on a STR. (section 3.4&3.7).

For the construction of the model the following coefficients and parameters were used: S(substrate, g/l), X (biomass g/l), μmax (maximum growth rate, h^{-1}), Y x/s (cellular yield coefficient, g/g), Y p/s (product yield coefficient, g/g), Ks (constant for substrate consumption, g/l), Kp (constant for lactic acid production, g/l).All the above parameters were defined experimentally (section 3.4).

The parameters representing the constants for product formation (Kp) and substrate consumption rate (Ks) are considered to be pH depended constants were defined according to the following equations, which resulted from the slopes of the lines for substrate consumption rate and end product rate:

Constants	*Resulting Equations*
Ks (g/l)	71.1363* pH+ 252.2041
Kp (g/l)	7.2498* pH + 0.06050

Prior to correlate the model formula simultaneously and validate the goodness of fit of the model initial estimates of all constants were performed. Each model formula was taken in term and the respective experimental data were used to achieve the best estimates of all the coefficients used. The basic estimates and the standardisation of all the model constants were performed on every set of experimental data for the analysis of each set of batch of cultures. Several trials were also done for the calibration of the model using different values for time base, constants and coefficients and parameters. The best fitting was used in order to avoid any confusion in the results. (Appendix 7)

2.8.3 Mathematical Model Formation for Continuous Mode Operation

Based on the experimental results obtained during the experimental procedures of growth of *L.lactis* on a STR on a batch mode with constant pH control and on the theoretical predictions obtained by the model constructed for batch growth, an unstructured unsegregated kinetic mathematical model was constructed in order to predict theoretically the growth of *L.lactis* on a continuous substrate feed operation system, including end product inhibition term and a maintenance coefficient ($k_d X$) term as the growth of the coccus was kept on stationary phase. The time step was set at $0.01\,h^{-1}$.

The model was developed according the same assumptions referred in section 2.7.2.

The mathematical algorithm used in this model is based on the same equations developed for biomass formation rate (dx/dt), substrate consumption rate (ds/dt) and product formation rate (dp/dt) used for the model representing batch growth. (sections, 2.7.2, 3.7).As the growth is strongly related by the dilution rate (D, h^{-1}) several flux rates were used in order to calibrate the model. The model was also tested against different substrate concentrations and different pH values.

Additionally, equations representing the substrate inflow and outflow, the biomass outflow, the biomass maintained in the system, the end product outflow and the product formation in the system were incorporated.

All the previously referred equations were developed in Excel 2003 Microsoft office commercial software using numerical method and graphical representations of the numerical predictions.

The primary estimates of the coefficients (Ks, Kp, Yx/s, Yp/s, $k_d X$) were used simultaneously with the initial values of the parameters in order to create series of time course curves.

All the numerical equations and the model formation were performed on a spreadsheet on 14 hours circle.11 columns of data were used. The 1st column was representing the time scale (time step 0.01 h^{-1}), the 2nd column was representing the substrate maintained in the system (Sins), the 3rd column was representing the substrate outflow during time(Sout), the 4th column was representing the substrate inflow(Sin), the 5th was representing the biomass maintained in the system(Xin) ,the 6th column was representing the biomass outflow (Xout), the 7th column was representing the growth rate(μ) , the 8th column was representing the biomass formation rate(dx/dt), the 9th column was representing the substrate consumption rate (ds/dt), the 10th column was representing the end product formation rate in the system(dp/dt) and the 11th column was representing the product outflow.(Pout)

The 1st column (time scale) was made by multiplying the column number with the time step. The 2nd (substrate in the system, Sins) was made by adding to the initial substrate concentration the substrate consumption rate per time. To the final result, the next substrate consumption rate per time was again added and so on, in order to find the substrate diminish in every time step. All the other columns resulted from the equations used for the mathematical model development. (Appendix 8)

For the formation of the model the following parameters and coefficients were used:

S (substrate, g/l), X (biomass, g/l), μmax (maximum growth rate, h^{-1}), Yx/s (cellular yield coefficient, g/g) Kp (constant for lactic acid production, g/l), $k_d X$ (maintenance coefficient during stationary phase, g/g/h), F (flux of nutrient, L/h) and

dilution rate (D, h^{-1}).All the above parameters excluding the fluxes and the dilution rate were defined experimentally.

The model was calibrated against different values for time step, parameters and coefficients. The best fitting was used in order to avoid any confusion in the results.

2.8.4 Mathematical Model Formation for Intensive culture on MBR

Based on the experimental results obtained during the experimental procedures of growth of *L.lactis* on a STR on a batch mode with constant pH control, and on the model proposed for growth on a continuous mode of operation an unstructured unsegregated kinetic mathematical model was constructed in order to predict theoretically the growth of *L.lactis* on a MBR operated with continuous substrate feed and with fed-batch substrate feed. An end product inhibition term and a maintenance coefficient ($k_d X$) term as the growth of the coccus was kept on stationary phase were included. The time step was set at 0.01 h^{-1} for continuous mode of operation and 0.004 h^{-1} for fed-batch feed.

The model was developed according the same assumptions referred in section 2.7.2.

The mathematical algorithm used in this model is based on the same equations developed for biomass formation rate (dx/dt), substrate consumption rate (ds/dt) and product formation rate (dp/dt) used for the model representing batch and continuous growth. (sections, 2.7.2, 2.7.3). As the growth is strongly related by the dilution rate (D, h^{-1}) several fluxes and flux rates were used in order to calibrate the model. The model was also tested against different substrate concentrations and different pH values. (Appendix 9, 10)

Additionally, equations representing the substrate inflow and outflow, the biomass maintained in the system, the end product outflow and the product formation in the system were incorporated.

All the previously referred equations were developed in Excel 2003 Microsoft office commercial software using numerical method and graphical representations of the numerical predictions. For continuous feed of substrate the equations were developed continuously in 1 working sheet though for fed-batch substrate feed the equations were developed in separate worksheets each one representing 2 hours of growth. For

every next worksheet the final concentrations of product, biomass and substrate of the previous one were used as initial concentrations.

The primary estimates of the coefficients (Ks, Kp, Yx/s, Yp/s, $k_d X$) were used simultaneously with the initial values of the parameters in order to create series of time course curves.

All the numerical equations and the model formation were performed on a spreadsheet on 14 hours circle.11 columns of data were used. The 1st column was representing the time scale (time step 0.01 h^{-1}), the 2nd column was representing the flux of substrate(F), the 3rd column was representing the flow rate(dF/dt), the 4th column was representing the substrate in the reactor (Sins), the 5th was representing the substrate outflow(Sout) ,the 6th column was representing the biomass inflow(Xin), the 7th column was representing the biomass maintained in the system , the 8th column was representing the growth rate (μ) the 9th column was representing the biomass formation rate(dx/dt), the 10th column was representing the substrate consumption rate(ds/dt), the 11th columns the end product formation rate in the system(dp/dt) and the 12th column was representing the product outflow (Pout).

The 1st column (time scale) was made by multiplying the column number with the time step. The 2nd (substrate in the system, Sins) was made by adding to the initial substrate concentration the substrate consumption rate per time. To the final result, the next substrate consumption rate per time was again added and so on, in order to find the substrate diminish in every time step. All the other columns resulted from the equations used for the mathematical model development (Appendix 9, 10).

For the formation of the model the following parameters and coefficients were used: S (substrate, g/l), X (biomass, g/l), μmax (maximum growth rate, h^{-1}), Yx/s (cellular yield coefficient, g/g) Kp (constant for lactic acid production, g/l), $k_d X$ (maintenance coefficient during stationary phase, g/g/h), F (flux of nutrient, L/h) and dilution rate (D, h^{-1}). All the above parameters excluding the fluxes and the dilution rate were defined experimentally.

The model was calibrated against different values for time step, parameters and coefficients. The best fitting was used in order to avoid any confusion in the results

3.1.1 Evaluation of the Effect of t Glucose Concentrations on Growth

As the yield of biomass in all anaerobic bacteria is strongly dependent on carbohydrate feed (Mandolstan & MacQuillen, 1973) the effect of glucose concentration was studied. Glucose was supplied in a range of concentrations between 0% to 5% (277 mM) according to the experimental process described in section 2.3.2.1As it can be seen by the Figure 3.3 the maximum growth rate rises simultaneously with the rise of glucose concentration, at low concentration. At the point of 4%w/v (222 mM) concentration glucose becomes highly inhibitory for growth. Furthermore, as it can be seen by the table 3.1 growth into glucose concentrations between 0.05% to 0.3% is poor.

According to the Figure 3.2 doubling time is very slow on low glucose concentrations and increases when glucose concentration rise. Biomass concentration also rises steadily into higher glucose concentration. At the point of 2% w/v (111mM) glucose concentration the highest maximum growth rate $0.18\,h^{-1}$ and td 3.8 h. were achieved. The biomass in this concentration after a 10 h cycle of static incubation is 3.2 g/l.

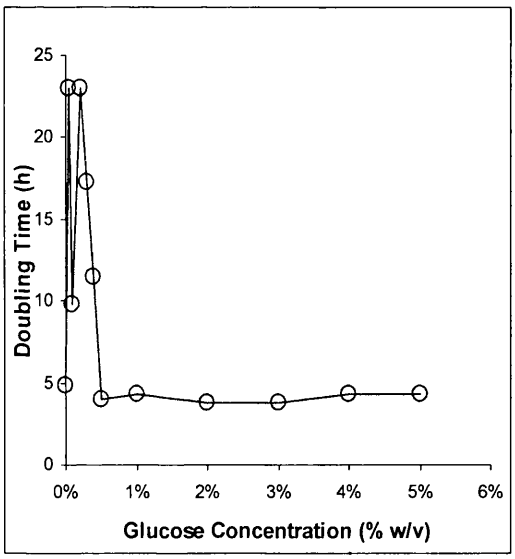


Figure 3.2
Doubling Time (td, h) (○) of L.lactis on Different Glucose Concentrations (%)

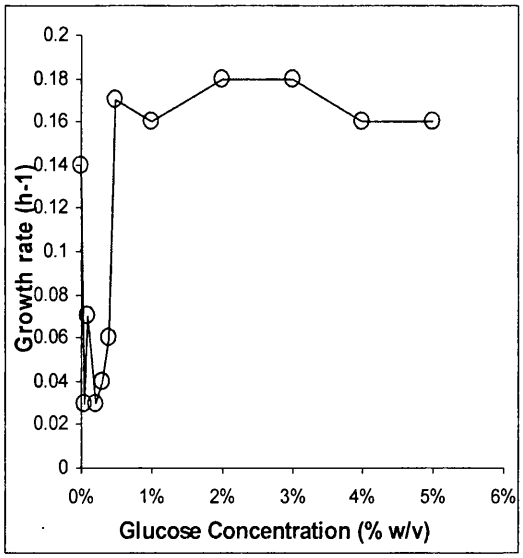


Figure 3.3
Maximum growth rate (μmax, h-1) (○) of L.lactis on Different Glucose Concentrations (%)

Glucose Concentration (w/v, %)	*Doubling Time (td, h)*	*Maximum Growth rate (μmax, h^{-1})*	*Final Biomass Concentration (g/l)*
0	23	0.03	0.44
0.05	23	0.03	0.6
0.1	17.25	0.04	0.7
0.2	11.5	0.06	0.8
0.3	9.85	0.07	1
0.4	4.3	0.16	2.5
5	4.05	0.17	2.9
1	4.05	0.17	3.2
2	3.8	0.18	3.2
3	3.8	0.18	3.0
4	4.3	0.16	2.6
5	4.3	0.16	2.8

Table 3.1:The effect of glucose concentrations

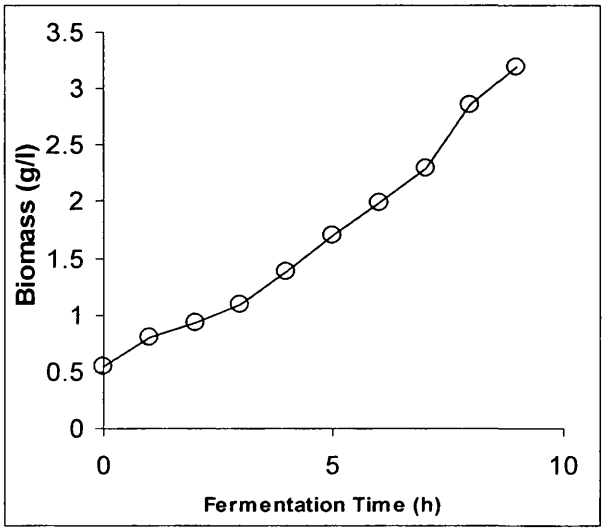


Figure 3.4 Growth of L.lactis on optimum concentration of Glucose (2%)

In conclusion the growth of *L.lactis* is strongly dependent on carbohydrate feed. Higher concentration of glucose above 4% w/v result into inhibition of rapid growth. 2% w/v was set as optimum glucose concentration point.

3.1.2 Evaluation of the Effect of Yeast Extract Concentrations on Growth

Several researchers (Liu, 2003) have introduced the idea of partial dependence of biomass and lactic acid productivity on the amount of yeast extract introduced within the growth media. The effect of yeast extract on growth was studied on a range of concentrations between 0% w/v to 2% w/v according to the experimental procedure described in section 2.3.

According to the Figure 3.5, maximum growth rate of the coccus rises simultaneously with yeast extract concentration and doubling time becomes faster. At a concentration of 1% w/v of yeast extract the highest maximum growth rate 0.20 h^{-1} and a doubling time of 3.45 h are achieved. The final biomass concentration is 3.0 g/l.

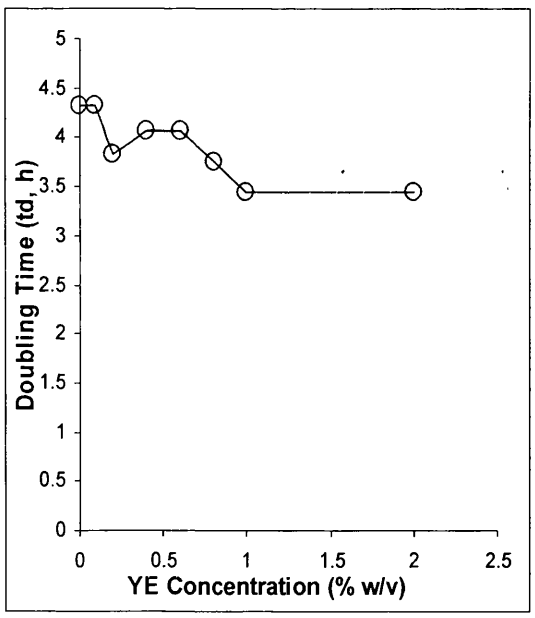


Figure 3.5
Doubling time (td) (○) of L.lactis On Different Y.E. Concentration

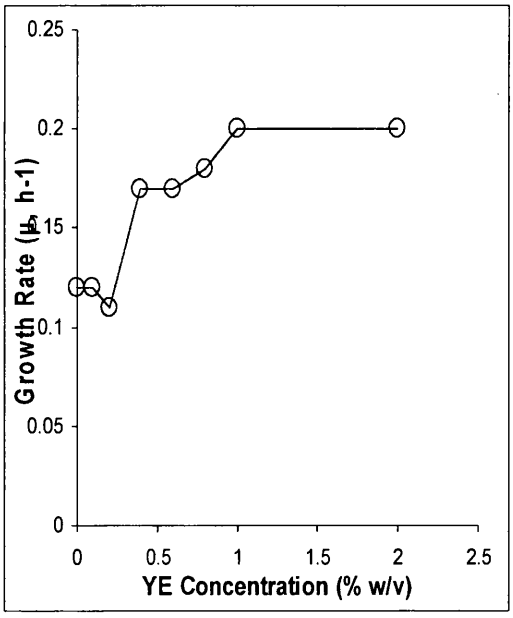


Figure 3.6
Maximum growth rate (μmax, h-1) (○) of *L.lactis* on Different Y.E. Concentration

Yeast Extract Concentration (w/v, %)	*Doubling Time (td, h)*	*Maximum growth Rate (μ max, h^{-1})*	*Final Biomass Concentration (g/L)*
0	4.31	0.12	1.8
0.1	4.31	0.12	2.0
0.2	3.83	0.11	2.6
0.4	4.07	0.17	2.7
0.6	4.07	0.17	3.0
0.8	3.75	0.18	2.9
1	3.45	0.20	3.2
2	3.45	0.20	3.2

Table 3.2 Y.E. Effect on Growth of *L.lactis*

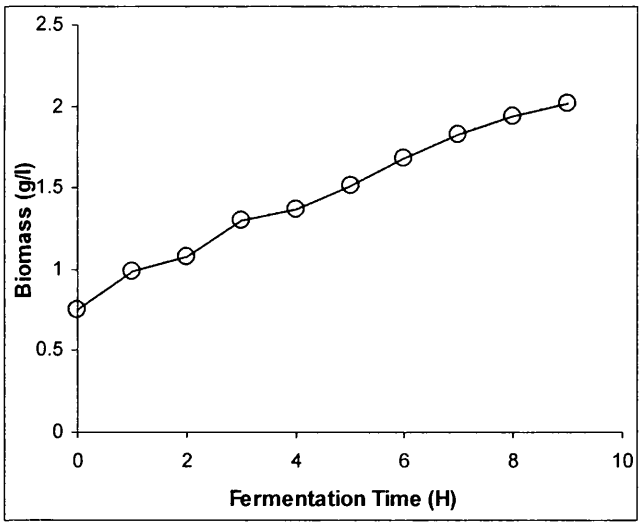


Figure 3.7 Growth (○) of *L.lactis* on Optimum Y.E. Concentration (1%)

Table 3.2 shows all of the data numerically and demonstrates the steady increase in maximum growth rate and all concentration with yeast extract concentrations up to 1% w/v. Further increase was not observed most probably due to the exhaustion of glucose. 1% w/v yeast extract was considered to be the optimum concentration value. Lower yeast extract concentrations result into slower doubling time and lower yields of biomass.

3.1.3 Evaluation of the Effect of Peptone Concentrations on Growth

As complex source of protein and nitrogen sources, peptone was selected. The effect of peptone on growth was studied on a range of concentrations between 0% w/v to 2% w/v according to the experimental procedure described in section 2.3.2.3

The results re shown in Figures 3.8 and 3.9. Maximum growth rate remains high even at low peptone concentrations. Highest maximum growth rate $0.22\,h^{-1}$ (td 3.13 h) were observed into 0.4% w/v peptone concentration. The final biomass concentration is 2.6 g/l.

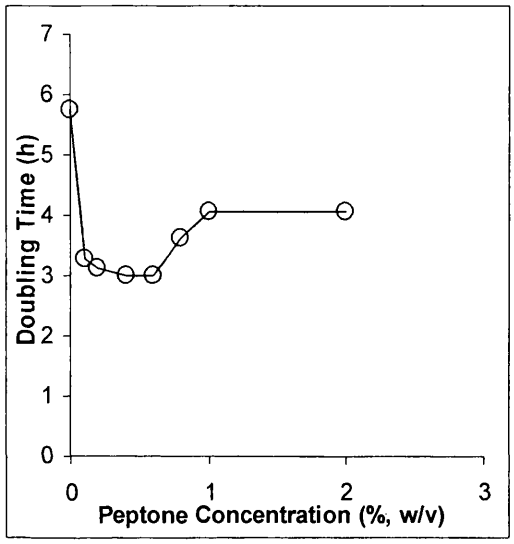


Figure 3.8
Doubling Time (td, h) (○) of L.lactis on Different Peptone Concentrations

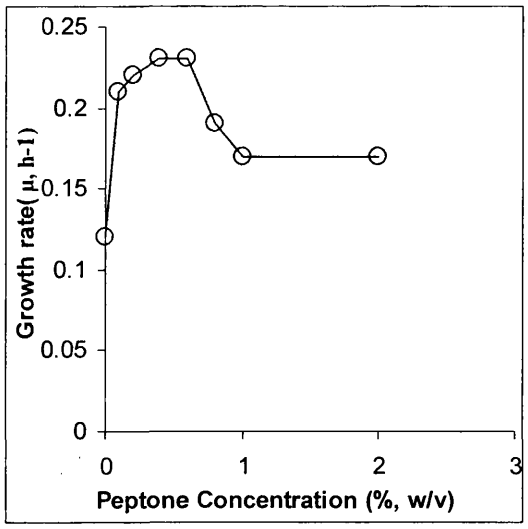


Figure 3.9
Maximum growth rate (μ, h-1) (○) on Different Peptone Concentrations

Peptone Concentration (w/v, %)	*Doubling Time (td, h)*	*Maximum growth rate (μ max, h^{-1})*	*Final Biomass Concentration (g/l)*
0	5.75	0.12	0.12
0.1	3.28	0.21	0.19
0.2	3.13	0.22	0.20
0.4	3	0.23	0.22
0.6	3	0.23	0.24
0.8	3.63	0.19	0.25
1	4.05	0.17	0.25
2	4.05	0.17	0.25

Table 3.3 Peptone Effect on Growth of *L.lactis*

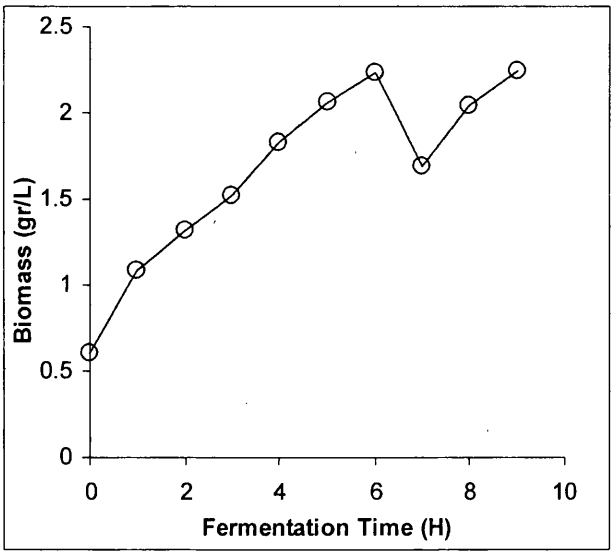


Figure 3.10 Growth (○) of L.lactis on Optimum Peptone Concentration (0.4%)

Table 3.3 shows the data numerically together with the final biomass concentrations. The final biomass concentrations do not increase after 0.6% w/v peptone concentrations. This indicates that peptone did not substantially support growth after this concentration.

With the results considered, a peptone concentration of 0.4% w/v was considered to be the optimum value for the growth of the coccus. Peptone strongly supports the growth of the coccus but becomes inhibitory into higher concentrations than 0.8%.

3.1.4 Growth on Optimised Medium

Introduction

Thus, to achieve the optimum maximum growth rate and of the bacterium and enhance its productivity, the coccus was inoculated in a medium of liquid form containing all the optimised parameters.(2% glucose, 1% yeast extract, 0.4% peptone). The experimental process was conducted according to the process described in section 2.4

The maximum growth rate of *L. lactis* on the optimised medium was $0.29 h^{-1}$ and the doubling time reduced to 2.4 h. The final biomass concentration was 2.3 g/l. When compared to the basal medium where maximum growth rate was 0.2 h^{-1} a significant increase in the maximum growth rate was achieved. Similarly the final cell concentration of the fermentation has been raised from 1.5 g/l to 2.3 g/l. The optimised medium will be used as a medium for further investigation.

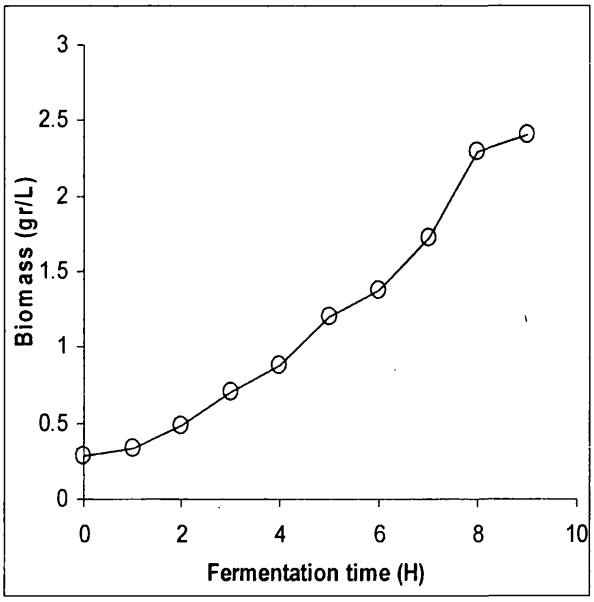


Figure 3.11 Growth (○) of L.lactis on optimised medium

3.2 Evaluation of the Inhibition Effect of Concentrations of Lactic acid on Growth

As a homofermentative LAB, *L.lactis* growth is strongly inhibited by the end products of its metabolism. (Board,1983).The effect of a wide range of different lactic acid concentrations between 0% w/v to 5% w/v (609 mM) on *L.lactis* growth was investigated to determine the effect of inhibition by adding known concentrations in the medium. Sodium lactate was used. The methods f study were as given according the experimental procedures described in section 2.4.1

Sodium Lactate Concentration (w/v, %)	*Sodium Lactate Concentration (mM)*	*Doubling Time (td, h)*	*Maximum growth rate (μmax , h^{-1})*	*Final Biomass Concentration (g/l)*
0	0	3.45	0.20	2.3
0.05	6	2.76	0.25	2.3
0.1	12	3.28	0.20	2.2
0.2	24	3.45	0.20	2.1
0.3	36	3.45	0.20	1.7
0.4	48	3.63	0.19	1.6
0.5	61	3.45	0.21	1.5
1	122	3.45	0.20	1
2	244	6.9	0.10	0.4
3	366	-	<0.01	0.2
4	488	-	<0.01	0.1
5	609	-	<0.01	0.1

Table 3.4 Effect of Sodium Lactate on Growth of *L.lactis*

The results of the experiment are tabulated above (Table 3.4). The results in Figures 3.12 show that *L.lactis* growth remains relatively unaffected up to 0.4% w/v (48 mM) sodium lactate, while higher concentrations inhibit the coccus growth, and complete inhibition occurs on 2% (243 mM) as shown in Table 3.4. The biomass yield is affected at 0.2% w/v although the maximum growth rate has not highly change; the cell yield is affected below 0.4 w/v sodium lactate suggesting that small concentrations of lactate may enhance growth.

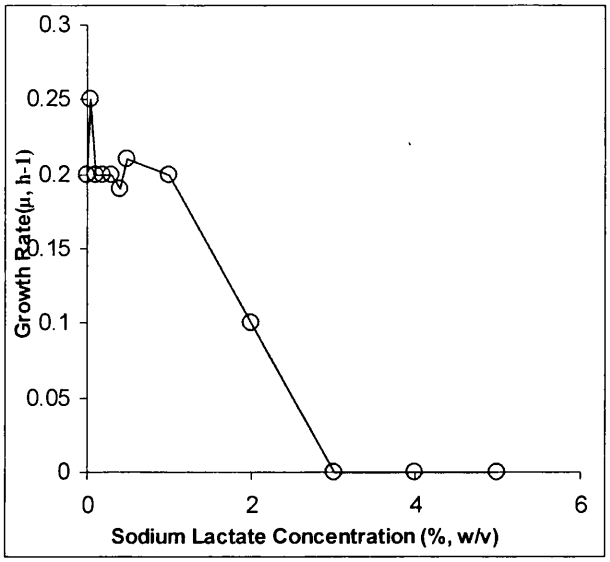


Figure 3.12 Maximum growth rate (μmax, h-1) of *L.lactis* on Different Sodium Lactate Concentration (%)

The coccus cannot stand concentration higher than 3% w/v (366 mM). Lower concentrations of lactate can inhibit the growth of the coccus down to 0.2% w/v (Table 3.4). Very low concentrations of sodium lactate may stimulate growth as the sodium lactate acts as a buffer to pH as lactate has a pKa of 4.7

3.3 Evaluation of the Inhibition Effect of Concentrations of Acetic acid on Growth

L.lactis produces also small amounts of acetic acid. The effect of different concentrations of acetic acid was studied. The sodium salt was used in concentrations between 0% w/v to 5% w/v (607mM). The effect was studied according the experimental procedure described in section 2.3.2. The experiments were carried out in pressure tubes which were periodically measured for the O.D. of the culture.

Figures 3.13 show the effect of acetic acid on the maximum growth rate of *L.lactis*. As it can be observed by the following Figures sodium acetate does not inhibit the growth of the coccus as much as equivalence of concentrations of lactate but significant inhibition of growth in terms of maximum growth rate can be observed.

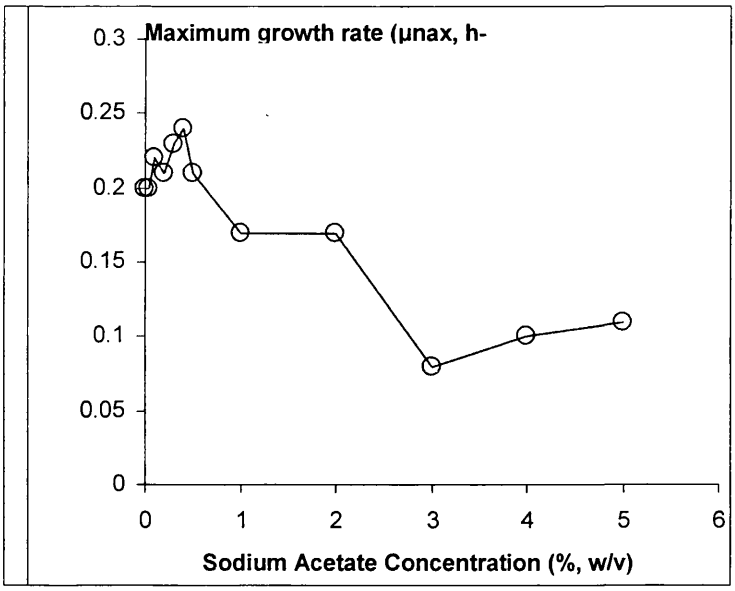


Figure 3.13 Maximum growth rates ($\mu max, h^{-1}$) of *L.lactis* on Different Sodium Acetate Concentrations

Table 3.5 tabulates the maximum growth rate results and also shows the overall yields. Again small amounts may stimulate maximum growth rate and growth yield. But above 0-2% w/v the rate of growth becomes inhibited over the range of studied concentrations. However at 1% w/v the maximum growth rate and the growth yield were reduced by 2 and 3 times respectively.

Sodium Acetate Concentration (w/v, %)	*Sodium Acetate Concentration (mM)*	*Doubling Time (td, h)*	*Maximum growth rate (μmax, h^{-1})*	*Final Biomass Concentration (g/l)*
0	0	3.43	0.20	2.2
0.05	4	3.45	0.20	2.2
0.1	9	3.13	0.22	2.6
0.2	18	3.28	0.21	2.3
0.3	27	3	0.23	1.9
0.4	36	3	0.24	2.1
0.5	45	3.28	0.21	1.9
1	89	4.05	0.17	1.2
2	178	4.05	0.17	1.0
3	268	9.3	0.08	0.70
4	357	7.4	0.10	0.70
5	446	7.5	0.11	0.70

Table 3.5 Effect of Sodium Acetate on Growth of *L.lactis*

Phase Two: Investigation of Growth of L.lactis on a pH control system

3.4 Growth of L.lactis on a STR with a pH control system

Introduction

In order to obtain a better maximum growth rate and higher growth yields and improved productivity a pH controlled STR system was developed. The system was designed and operated according to the experimental procedure described in section 2.4.1.As to investigate the influence of pH over growth in terms of μ, td and product and biomass yields the system was operated with a continuous pH control maintenance system. The influence of pH was tested in a range of highly acidic (4) to neutral (7) pH. All the process was performed in batch mode. The optimised medium was used (Table 3.6)

The results of the experiments are shown in Figures 3.14 and 3.15 and Table 3.6. There is a strong correlation between the pH and the growth of the coccus. The maximum growth rate was enhanced when the culture was controlled pH 6.5 and 7. Maintenance of pH on a steady state throughout the 10 h fermentation process was combined with the use of the optimised liquid medium gave highest biomass yields (Yx/s) and maximum growth rates as compare to the uncontrolled pH growth systems.. It can be also observed that on acidic pH values of 4 and 4.5, the growth of the coccus is completely inhibited. The amount of lactic acid produced by the coccus was identified as being equal to the amount of NaOH used for pH maintenance. Over the 10 h fermentation the pH 5, 5.5 and 6 the coccus was still growing as they had slower maximum growth rates and long lag periods prior to growth. Samples ere measured on an hourly basis and they were analysed for biomass, pH and in some occasions the glucose and the end product were also analysed. This technique is described in detail in section 2.5 of Materials and Methods.

The effect of reduced pH is strong where no growth was observed at pH 4 and pH 4.5.The optimum pH was 6.5 in the conditions studied here.

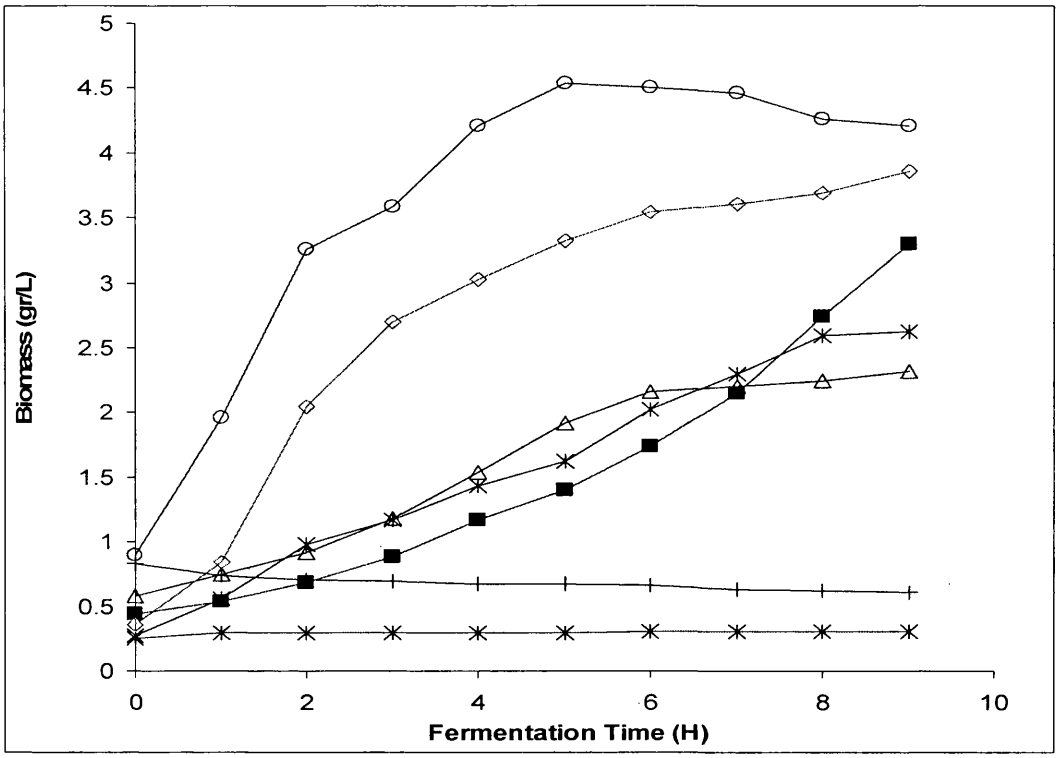


Figure 3.14 Growth of L.lactis on Different pH range in a 2L STR, Growth(+) on pH 4, Growth (*) on pH 4.5, Growth (Δ) on pH 5, Growth (☼) on pH 5.5, Growth (□) on pH 6, Growth (⌂)on pH 6.5, Growth on pH 7(◊)

pH	*Maximum growth rate (μmax, h^{-1})*	*Doubling Time (td, h)*	*Final Biomass Concentration (g/l)*	Cellular Yield Coefficient Yx/s (g/g)
4	0	0	0.6	0
4.5	0.02	34.5	0.6	0.003
5	0.19	3.63	2.3	0.1
5.5	0.22	3.13	2.6	0.13
6	0.24	2.87	3.3	0.16
6.5	0.66	1.04	4.2	0.2
7	0.72	0.95	3.8	0.19

Table 3.6 Effect of Different pH range on Growth of *L.lactis* on a STR

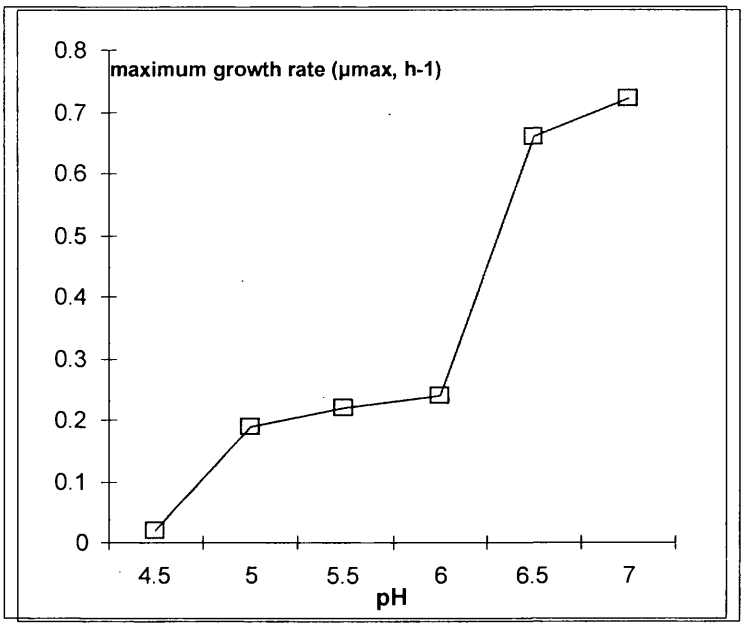


Figure 3.15 Maximum growth rate (μmax, h-1) (□) of *L.lactis* on pH control Fermentation on a 2L STR

Table 3.6 tabulates all the results from the analysis of the cultures. The highest μ are between 0.66 h^{-1} and 0.7 h^{-1} at pH 6.5 and 7 respectively. Compared to the uncontrolled pH system these are 3 times faster and the yield is almost doubled. (see Table 3.6). The performance of the uncontrolled system is closely to the one at pH 5.0 in this system.

3.5 Determination of Organic Acids Production during Growth of L.lactis

Samples for the pH controlled fermentation were analysed for glucose and organic acids concentration. This was done in order to determine the metabolism of the strain of *L.lactis* used in this research project the final organic acids concentration in the fermented liquid nutrient mediums used for the investigation of optimum pH for growth was measured. All the experimental procedure was performed according to the experimental procedure described in section 2.6 of Materials and Methods. Figure 3.16 shows the amount of lactic acid, higher yields of lactic acid were achieved in pH 6.5 (289 mM) and 7 (336 mM).

Lactic acid strongly correlates to the amount of growth. The pH 4.0 and 4.5 cultures show little or no lactate and the production that is observed is thought derived from the

inoculum .Figure 3.18 shows the amount of lactic acid production into correlation with the final biomass concentration on the pH controlled fermentations. Analytical data are tabulated in Table 3.7. At pH 5.0, 5.5 and 6.0, the growth was only partially complete so that relatively small quantity of lactic acid was produced. At 6.5 and 7.0 the quantity of lactic acid implies complete consumption of glucose. (i.e. 20 g/l, 110 mM give 220 mM for complete consumption of glucose). This suggests that the yeast extract and peptone components must contribute to the lactic acid production together with an addition of lactic acid due from the inoculum.

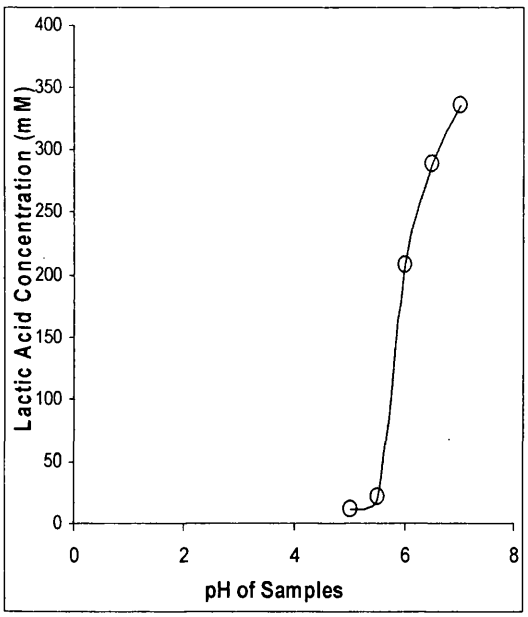


Figure 3.16 Lactic Acid Production (mM) (○) on Different pH Nutrient medium

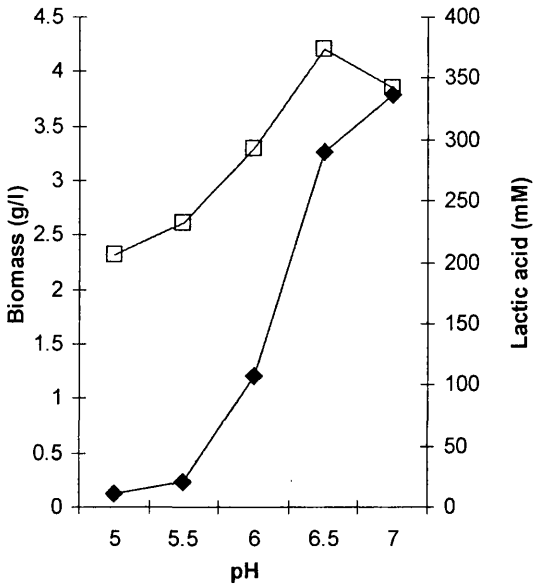


Figure 3.17 Lactic acid production (♦) and Final Biomass concentration (□) on different pH media

By the analysis of organic acids the strain of the coccus used is homofermentative. As in pH 6.5 higher yields of biomass were achieved also the productivity of lactic acid was higher.

pH of Fermented liquid nutrient medium	Concentration of Lactic acid (mM)	Final Biomass Concentration (g/l)	Initial glucose Concentration (g/l)
5	11	2.32	20
5.5	20.75	2.62	20
6	207.30	3.30	20
6.5	289.40	4.20	20
7	336.25	3.85	20

Table 3.7 Correlation between the pH and lactic acid production

3.6 Carbohydrate Accumulation Rate Determination

In order to correlate the amount of glucose accumulated with the productivity of lactic acid from the coccus the final concentration of glucose in the fermented samples had to be determined. Furthermore, the product yield coefficient (Yp/x) had to be determined and the maintenance term ($k_d X$) for the growth of the coccus could be established. High amounts of lactic acid result into growth inhibition though higher carbohydrate feed supports biomass productivity.

As it can be seen in Figure 3.20 the glucose consumption is inversely proportional with the amount of lactic acid produced during the growth. The higher amount of glucose accumulation was observed on pH 6.5 (16 g/l, 88 mM) and (17g/l, 94 mM). The rate of glucose accumulation per hour during the exponential growth phase was determined as 30.6 mmol/h. It can be assumed that the lactic acid production rate for the coccus per hour is 55 mmol/h during the exponential growth phase as the Y p/x was determined theoretically at 1.8 mole/mole in Section 1.6.1 of the Introduction during the exponential growth phase. The $k_d X$ value may be interpreted as the reciprocal of the mean of the lifespan of the organisms and was set at 0.0057 g cell/g cell/h.(section 2.8.2 of Materials

and Methods) (n figure 3.18 the carbohydrate consumption rate is demonstrated at the optimum pH 6.5.

Glucose Initial Concentration (g/L)	pH	Glucose Final Concentration (g/1.8L)	Glucose Accumulated (g/1.8L)
36	4.5	31.73	4.26
36	5	31.98	4.019
36	5.5	30.61	5.38
36	6	11.63	24.36
36	6.5	6.85	29.15
36	7	6.56	29.44

Table 3.8 Glucose consumption during growth on different pH range

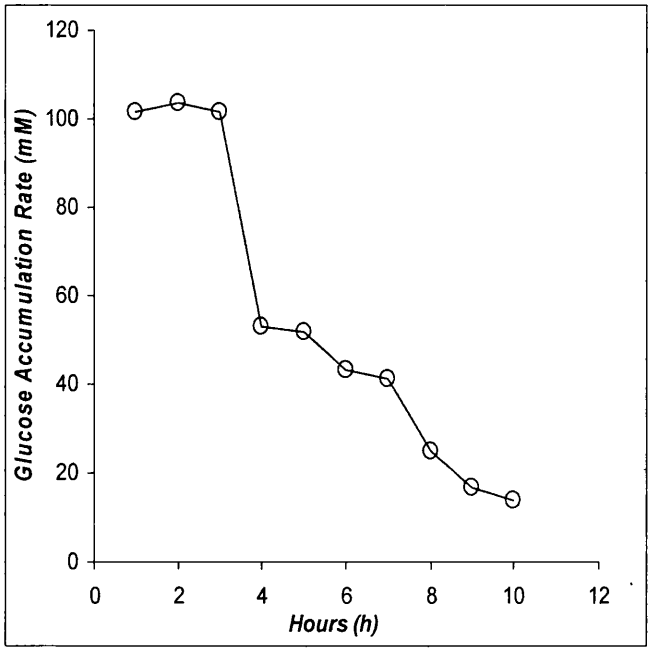


Figure 3.18 Glucose consumption rate per hour in pH 6.5 control fermentation

Chapter Four

Mathematical modelling of L.lactis intensive culture on Batch mode, Continuous mode and MBR on continuous and fed-batch substrate feed operation

4.1. Introduction

As previously described (section 2.7) mathematical modelling is a widely used technique for deeper understanding of microbial growth. 3 mathematical models were developed for the growth of *L.lactis* on different systems of operation (batch, continuous, MBR). All the equations were developed according to the mathematical equations referred on the Introduction part (sections 1.12-1.14). The model once developed was valuated with the data already gathered .Theoretical predictions over the volumetric cell productivity on every system were made. The various factors such as dilution rate , concentration of feed rate etc were then systematically investigated.

4.1.1. Mathematical modelling for Batch growth of L.lactis

4.1.2 Analysis

General structure of Model

Describing the Growth Rate

An unstructured kinetic mathematical model for non-competitive growth inhibition was formed to describe the growth of *L.lactis* strain on a STR. The equations used to describe the rate of formation of biomass ($\frac{dx}{dt}$, g/l) are the following:

$$\frac{dx}{dt} = \mu * \mathrm{X} \qquad \text{(Equation 3.1)}$$

A maintenance term ($k_d X$)can be incorporated into the equation:

$$\frac{dx}{dt} = \mu * X - k_d X \qquad \text{(Equation 3.2)}$$

where μ is the specific growth rate (h^{-1}) and X is the cell concentration (g/l).

In the above equation the specific growth rate is expressed by the following Monod equation (Roels, 1983; Kovarova&Egli, 1996)

$$\mu = \frac{\mu \max * S}{K_s + S} \qquad \text{(Equation 3.3)}$$

where μ is the specific growth rate (h^{-1}) μ max is the specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and K_s (g/l) is a constant of substrate consumption dependent on pH maintenance. (section 3.6)

Describing Substrate Consumption during Growth

The equation representing substrate accumulation ($\frac{ds}{dt}$, g/l) (section 1.16.1) is directly linked to cell growth. The Monod equation then is linked to a yield coefficient for cell growth ($Y^{x}/_{s}$, g/g) as follows:

$$\frac{ds}{dt} = \frac{-1}{Y^{x}/_{s}} * \frac{dx}{dt} \qquad \text{(Equation 3.4)}$$

On further analysing:

$$\frac{ds}{dt} = \frac{-1}{Y^{x}/_{s}} * \frac{\mu \max * S}{Ks + S} \qquad \text{(Equation 3.5)}$$

where $Y^{x}/_{s}$ is yield of biomass from substrate (g/g), μ max is the maximum specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and Ks is a constant of substrate consumption dependent on pH maintenance (g/l) and X is the cell concentration (g/l).

Product Formation

Product formation rate equation ($\frac{dp}{dt}$,g/l) proposed is based over the simplified assumption that the rate of product formation is directly related to the rate of substrate consumption, through a yield coefficient $Y\,{}^{pLacticacid}/_{s}$ (g/g)

For lactic acid formation the proposed equation is the following:

$$\frac{dP(Lacticacid)}{dt} = -Y\,{}^{p(lacticacid)}/_{s} * \frac{ds}{dt} \qquad \text{(Equation 3.6)}$$

where $\frac{dP}{dt}$ is the product formation rate (g/l) $Y\,{}^{pLacticacid}/_{s}$ is the yield coefficient of lactic acid production from substrate and $\frac{ds}{dt}$ is the substrate consumption rate (g/l)

4.1.3 Model Simulation

Primary Investigation

The model simulation was done with the experimental result of the pH control fermentations performed on a 2L STR reactor using optimised medium for growth of *L.lactis*. The estimation of parameters used in the model was allied to the experimental results available.

Table 4.1, lists the values of the model constants that gave the fit for the batch culture performed on a pH control fermentation on a STR.

Parameter	*Definition*	*Dimension*	*Values on different pH*	
μ max	Maximum Specific growth rate	h^{-1}	5.5 0.22 6 0.24 6.5 0.66 7 0.72	Sections 3.4
Ks	Constant	g/l	5.5 0.40 6 0.435 6.5 0.471 7 0.750	2.8.2
$Y_{x/s}$	Yield coefficient (cell growth)	g /g	5.5 0.11 6 0.13 6.5 0.20 7 0.19	3.4
$Y_{pLacticacid/s}$	Yield coefficient(lactic acid production)	g /g	1.8	3.5
$k_d X$	Maintenance coefficient	g /g/h	0.057	3.6

Table 4.1 : Coefficients and parameters used for mathematical modelling

The simulation was carried out as described in the Materials and Methods and involved the use of simple spreadsheet in which the calculations can be carried out. The method studies to confirm the validity of the model were based upon simple known constants and parameters and these to confirm the results. The time step of the integration proved an important parameter and was typically 0.01 h.

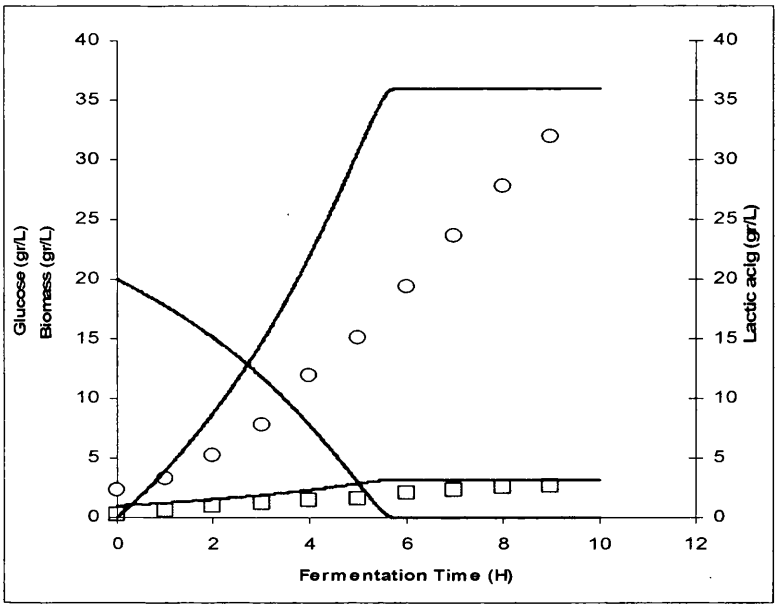


Figure 4.1: A comparison of the mathematical model predictions with the experimental data where (o) lactic acid production, (□) biomass formation and on pH 5.5 on a STR excluding end product inhibition

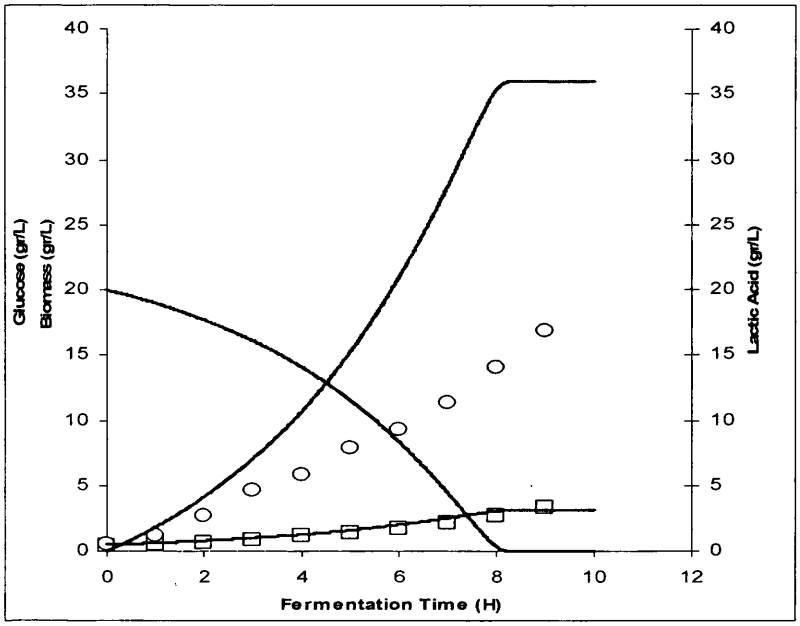


Figure 4.2: A comparison of the mathematical model predictions with the experimental data where (o) lactic acid production, (□) biomass formation and on pH 6 on a STR excluding end product inhibition

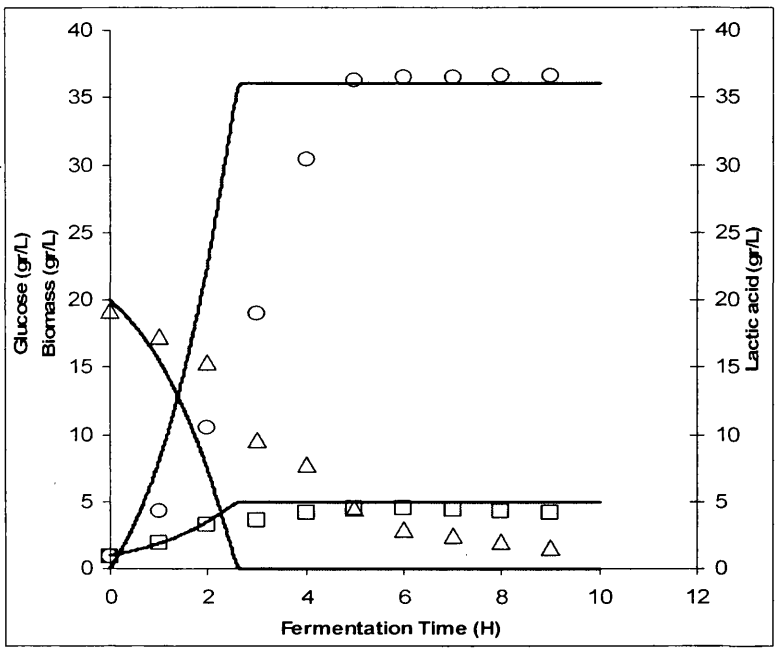


Figure 4.3: A comparison of the mathematical model predictions with the experimental data where (o) lactic acid production, (□) biomass formation and (Δ) substrate consumption on pH 6.5 on a STR excluding end product inhibition

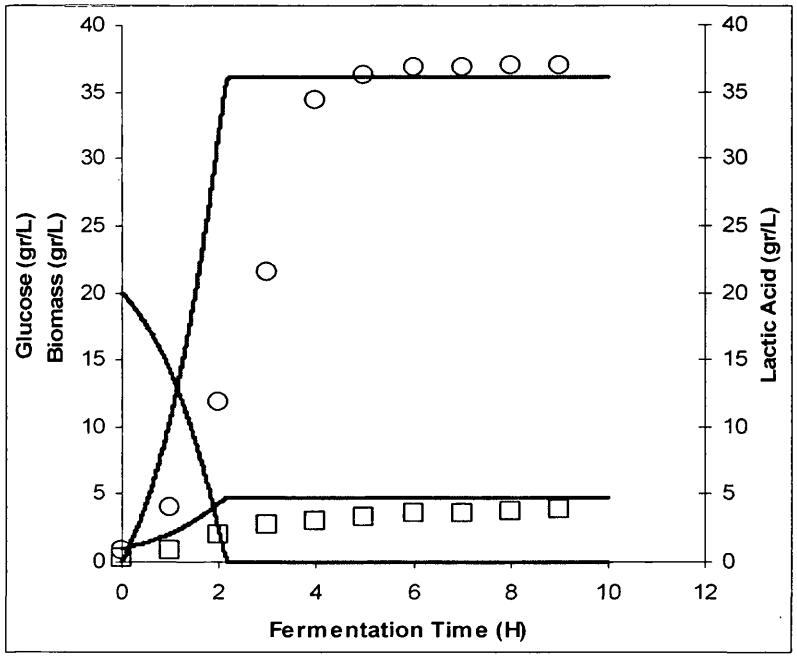


Figure 4.4 : A comparison of the mathematical model predictions with the experimental data where (o) lactic acid production, (□) biomass formation on pH 7 on a STR excluding end product inhibition

Figures 4.1, 4.2, 4.3 and 4.4 show the results of the investigation and compare the effect pH on experimental and simulated data without product inhibition. As it can be seen the simulated data do not fit to data well in term of predicting product formation and the growth. In these case the product and cell formation are too fast and finish prematurely in relation to the actual data. As it can be seen by the above plots , the end product inhibition term is required in the mathematical model as growth according to the experimental data available is limited most probably, due to end product inhibition. As a result the model was further developed to include a mathematical term representing end product inhibition.

4.1.4 Model Development

End Product Inhibition Kinetics

As previously referred (section 2.7.2) *L.lactis* is homofermentative coccus which growth is inhibited by the major end product of its metabolism, lactic acid. As it can be seen from section 3.2 the threshold value for complete growth inhibition due to lactic acid is 366 mM while growth kinetics are affected down to about 30 mM. During growth of *L.lactis* on pH control fermentation complete growth inhibition does not occur (section 3.4) but there is a strong effect of lactic acid on biomass formation. A product inhibition term was incorporated in the model following the Taylor and Hinselwood end product inhibition model. (section 1.16)

$$\frac{dx}{dt} = \mu * X - k_d X \qquad \text{(Equation 3.7)}$$

$$\text{where } \mu = \frac{\mu \max * S}{K_s + S} / \frac{P}{K_p + P} \qquad \text{(Equation 3.8)}$$

where, μ is the specific growth rate (h^{-1}), μ max is the maximum specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and Ks is a constant of substrate consumption dependent on pH maintenance (g/l), X is the cell concentration (g/l), P is the end product concentration (g/l) and Kp is a constant of end product production (g/l).

On further integrating the equation μ becomes:

$$\mu = \frac{(\mu \max * S) * (Kp + P)}{(Ks + S) * (P)} \qquad \text{(Equation 3.9)}$$

where, μ max is the maximum specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and Ks is a constant of substrate consumption dependent on pH maintenance (g/l) , X is the cell concentration (g/l),P is the end product concentration (g/l) and Kp is a constant of end product production (g/l)

So the biomass formation on stationary phase where the maintenance coefficient (k_d) is included the formula becomes:

$$\frac{dx}{dt} = \frac{(\mu \max * S) * (Kp + P)}{(Ks + S) * (P)} - k_d X \qquad \text{(Equation 3.10)}$$

where, μ max is the maximum specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and Ks is a constant of substrate consumption dependent on pH maintenance (g/l), X is the cell concentration (g/l), P is the end product concentration (g/l), Kp is a constant of end product production (g/l) and $k_d X$ is the maintenance coefficient. (g substrate/g cells/h)

As biomass formation kinetics are strongly related with substrate consumption the equation for substrate consumption rate becomes:

$$\frac{ds}{dt} = \frac{-1}{Y\,{}^{x}\!/_{s}} * \frac{dx}{dt} \qquad \text{(Equation 3.11)}$$

where $Y\,{}^{x}\!/_{s}$ is yield of biomass from substrate (g/g), μ max is the maximum specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and $\frac{dx}{dt}$ is the biomass formation rate.

On further integration the equation becomes

$$\frac{ds}{dt} = \frac{-1}{Y\,{}^{x}/_{s}} * \frac{(\mu \max * S) * (Kp + P)}{(Ks + S) * (P)} + k_d X \qquad \text{(Equation 3.12)}$$

where, $Y\,{}^{x}/_{s}$ is yield of biomass from substrate(g/g) μ max is the maximum specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and Ks is a constant of substrate consumption dependent on pH maintenance (g/l) , X is the cell concentration (g/l),P is the end product concentration (g/l) , Kp is a constant of end product production (g/l) and $k_d X$ is the maintenance coefficient.(g cell/g cells/h).

To express the product formation rate the formulas were kept the same.

All the parameters are kept the same,(see table 4.1) but a new parameter is incorporated, the Kp constant. The values of the constant on different pH are listed on the table 4.2.

Parameter	*Definition*	*Dimension*	*Values on different pH*	
Kp	Constant	g/l	5.5 0.643 6 0.679 6.5 0.714 7 0.750	Section 2.8.2

Table 4.2 : Kp constant on different pH rate

These new equations were incorporated into the model. The model was run again and compared to batch data collected previously in section 3.4 Figures 4.5 to 4.8 show the result of the simulation over a range of pH from 5.5b to 7.0. Much better overall fits were obtain when these graphs were composed to the previously(Figures 4.1 to 4.4).There was still some poor fitting the end of growth phase where some inhibition could not be described by the model. eg Figure 4.7 product/substrate were not modelled accurately.

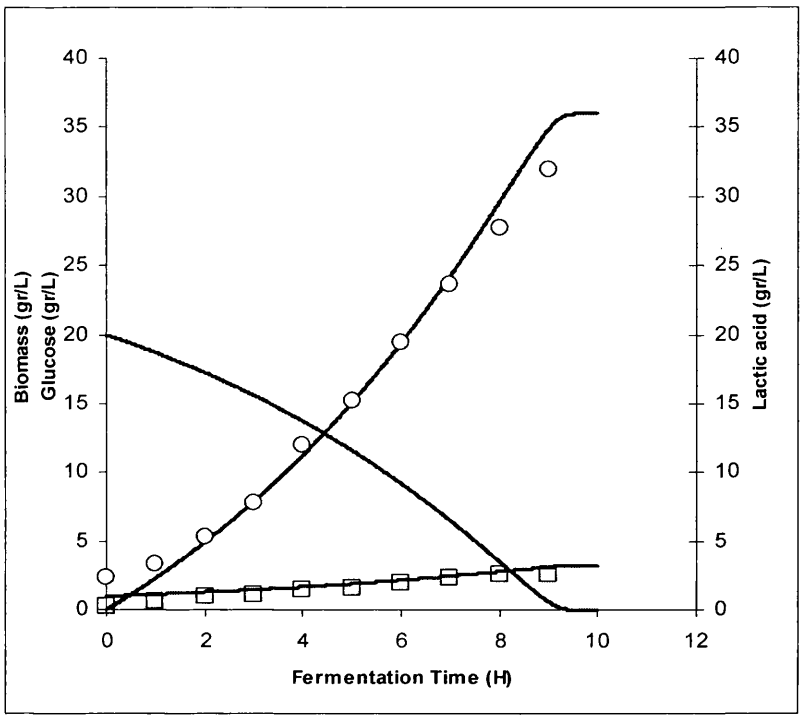


Figure 4.5: A comparison of the mathematical model predictions with the experimental data where (o) lactic acid production, (□) biomass formation on pH 5.5 on a STR including end product inhibition

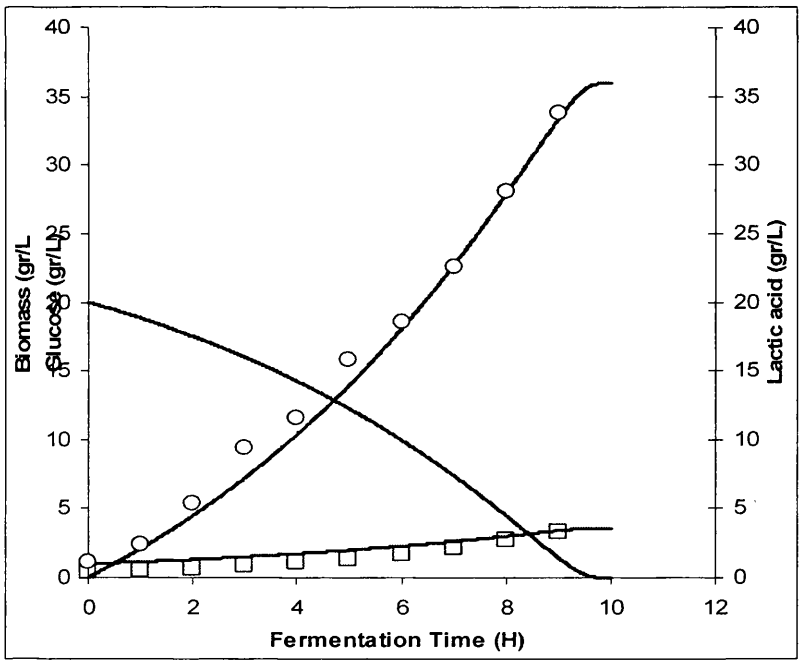


Figure 4.6: A comparison of the mathematical model predictions with the experimental data where (o) lactic acid production, (□) biomass formation on pH 6 on a STR including end product inhibition

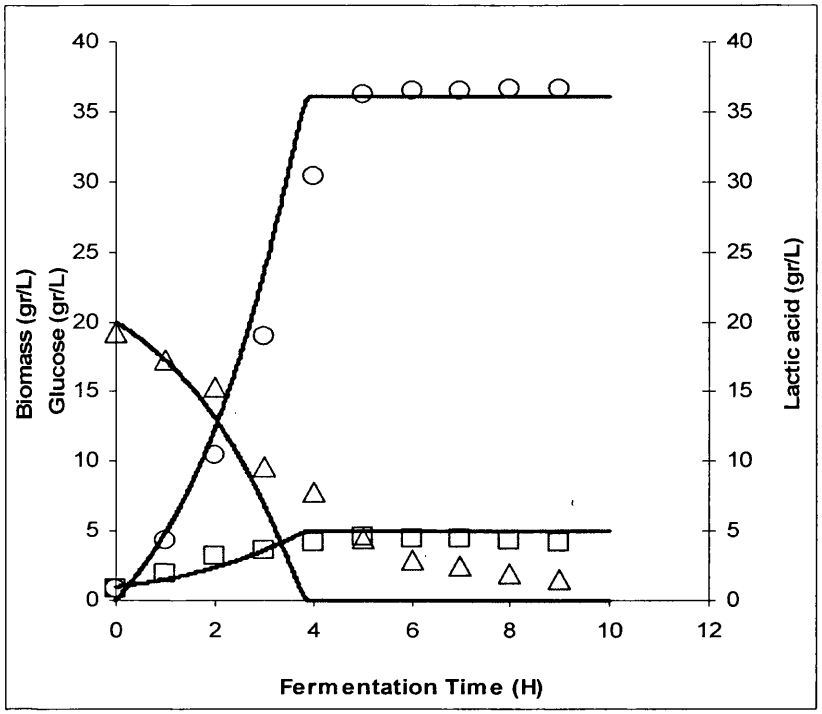


Figure 4.7: A comparison of the mathematical model predictions with the experimental data where (o) lactic acid production, (□) biomass formation and (Δ) substrate consumption on pH 6.5 on a STR including end product inhibition

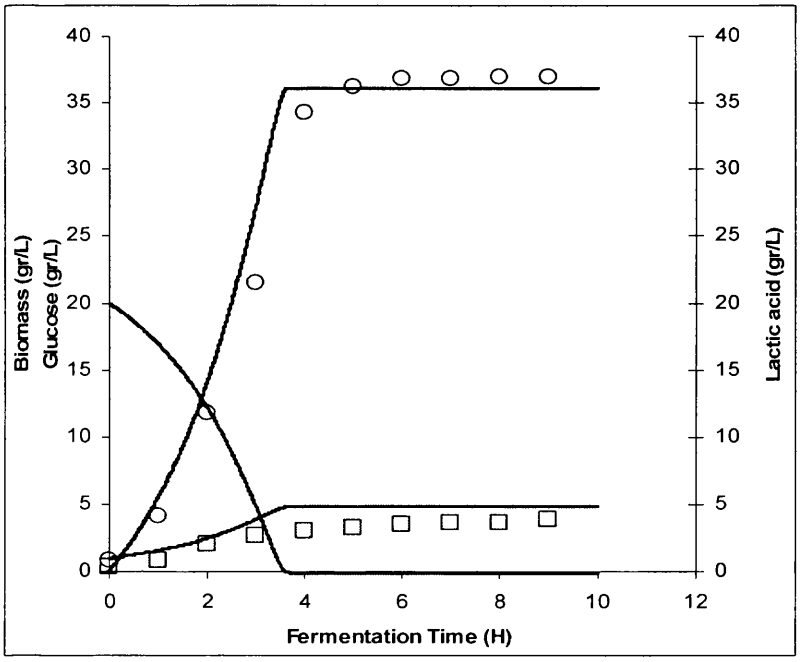


Figure 4.8: A comparison of the mathematical model predictions with the experimental data where (o) lactic acid production, (□) biomass formation on pH 7 on a STR including end product inhibition

However ,s it can be seen from the above graphs when end product inhibition term is incorporated in the mathematical model , the theoretical predictions are in much better accordance with the experimental values for biomass formation and lactic acid production.

4.1.5 Further Model Development

Introduction

In order to investigate the effect of substrate feed on different concentrations over growth and cell productivity of *L.lactis* on a continuous system, the model was tested against different substrate concentrations and theoretical predictions were made. The efficiency of the system was evaluated in terms of volumetric cell productivity which is given by the following equation:

Volumetric Cell Productivity (g/h) : Final biomass concentration(g)/ Total Fermentation time(h)

Using the model a series of simulation were made where the substrate concentrations varied between 2.5 g/l to 20 g/l All the conditions of the system were kept steady on a 10h fermentation circle. All the numerical values of the parameters and constants were kept the same. In figure 4.9 below the volumetric cell productivity (g/h) set as a function of substrate concentration are plotted.(i.e. Appendix)

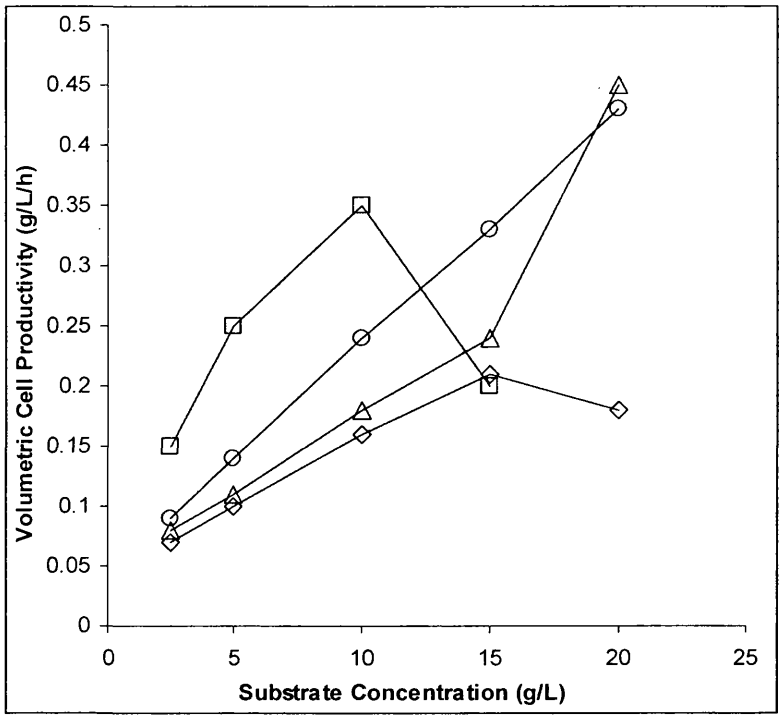


Figure 4.9 :Theoretical predictions of volumetric cell productivity on different substrate concentrations of batch growth of L.lactis on a STR on different pH 5.5, (◊) pH 6, (□) pH 6.5 (△), pH 7(☼)

The results show that the volumetric cell productivity (g/h) was higher in pH values of 6.5 and 7 and on substrate concentrations of 15 and 20 g/l. At lower substrate concentrations, the substrate becomes completely exhausted in a short period of time with a lower concentration of lactococcal biomass. At lower pH values the biomass formation rate is also lower with reduced productivity.

4.2 Mathematical Modelling of L.lactis on Continuous Culture

4.2.1 Introduction

Having investigated batch cultures An unstructured kinetic mathematical model was developed for theoretical predictions of growth of *L.lactis* on a continuous culture system operating on constant pH control. The mathematical algorithm was based on the previously developed equations over biomass formation, substrate consumption and lactic acid production for batch growth of *L.lactis*. A maintenance and an end product inhibition term was also incorporated. In order to evaluate the end product inhibition effects during growth the model was tested against different substrate concentrations, between 2.5 to 20 g/l. The efficiency and feasibility of the system was evaluated in terms of volumetric cell productivity (g/h).

4.2.2 Analysis

General Structure of the model

Describing the biomass formation rate, the biomass maintained in the system and the biomass outflow

The equations describing the biomass formation rate ($\frac{dx}{dt}, g/l$) is the following:

$$\frac{dx}{dt} = \mu * \mathrm{X} - k_d X \qquad \text{(Equation 3.13)}$$

where μ is the specific growth rate (h^{-1}) and X is the cell concentration (g/l).

A maintenance term ($k_d X$, g cells /g cells/h) is incorporated as the growth of the coccus is kept on stationary phase.

In the above equation μ is expressed by the following equation:

$$\mu = \frac{\mu \max * S}{Ks + S} / \frac{P}{Kp + P} \qquad \text{(Equation 3.14)}$$

where, μ is the specific growth rate (h^{-1}), μ max is the maximum specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and Ks is a constant of substrate

consumption dependent on pH maintenance (g/l), X is the cell concentration (g/l), P is the end product concentration (g/l) and Kp is a constant of end product production (g/l).

On further analysing the μ the equation becomes:

$$\mu = \frac{(\mu \max * S) * (Kp + P)}{(Ks + S) * (P)} \qquad \text{(Equation 3.15)}$$

where, μ is the specific growth rate (h^{-1}), μ max is the maximum specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and Ks is a constant of substrate consumption dependent on pH maintenance (g/l), X is the cell concentration (g/l), P is the end product concentration (g/l) and Kp is a constant of end product production (g/l).

The biomass (X, g/l) leaving the system is given by the following equation:

$$Xout = Xin * F * t \qquad \text{(Equation 3.16)}$$

where Xout (g/l) is the biomass outflow, Xin (g/l) is the biomass maintained in the system, F (L/h) is the flux of the nutrient per hour and t is the time step (h)

while biomass (X, g/l) maintained in the system is given by the formula:

$$Xin = Xo + \frac{dx}{dt} - Xout \qquad \text{(Equation 3.17)}$$

where Xout (g/l) is the biomass outflow, Xin (g/l) is the biomass maintained in the system and dx/dt (g/l) is the biomass formation rate during time

4.2.3 Describing substrate consumption rate during growth, the substrate feed rate, the substrate outflow rate and the substrate maintained within the system

The equation representing substrate accumulation rate ($\frac{ds}{dt}$, g/l) is directly linked to cell growth. A maintenance coefficient is also incorporated in the equation. The Monod equation then is linked to a yield coefficient for cell growth ($Y_{x/s}$, g/g) as follows:

$$\frac{ds}{dt} = \frac{-1}{Y_{x/s}} * \frac{dx}{dt} + k_d X \qquad \text{(Equation 3.18)}$$

On further analysing the equation becomes:

$$\frac{ds}{dt} = \frac{-1}{Y_{x/s}} * (\frac{\mu \max * S}{Ks + S}) / (\frac{P}{Kp + P}) + k_d X \qquad \text{(Equation 3.19)}$$

On further analysing the above equation:

$$\frac{ds}{dt} = \frac{-1}{Y_{x/s}} * (\frac{(\mu \max * S) * (Kp + P)}{(Ks + S) * (P)}) + k_d X \qquad \text{(Equation 3.20)}$$

where $Y_{x/s}$ is yield of biomass from substrate(g/g) μ max is the maximum specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and Ks is a constant of substrate consumption dependent on pH maintenance (g/l) , X is the cell concentration (g/l),P is the end product concentration (g/l) , Kp is a constant of end product production (g/l) and $k_d X$ is the maintenance coefficient.(g cells/g cells /h).

The substrate inflow rate (Sin, g/l) in the system is given by the following equation:

$$S_{in} = S_0 * F * t \qquad \text{(Equation 3.21)}$$

where Sin (g/l) is the substrate inflow rate per hour, So (g/l) is the initial substrate concentration within the nutrient, F (L/h) is the flux of the nutrient per hour and t is the time step (h).

The substrate outflow rate (Sout, g/l) in the system is given by the following equation:

$$Sout = Sin * F * t \qquad \text{(Equation 3.22)}$$

where Sout (g/l) is the substrate outflow rate per hour, Sin (g/l) is the substrate inflow in the system, F (L/h) is the flux of the nutrient per hour and t is the time step (h).

The substrate existing initially (Sins, g/l) in the system which is accumulated during growth is given by the following equation:

$$S_{ins} = So + \frac{ds}{dt} - Sout + Sin \qquad \text{(Equation 3.23)}$$

where Sins (g/l) is the substrate existing initially in the system, So (g/l) is the initial substrate concentration within the nutrient, ds/dt is the substrate accumulation rate per time (g/l), Sout (g/l) is the substrate outflow per time and Sin (g/l) is the substrate inflow is the system.

On further analysing the above equation the equation becomes:

$$S_{ins} = So + \frac{-1}{Y\,{}^{x}\!/_{s}} * \left(\frac{\mu \max * S}{Ks + S}\right) / \left(\frac{P}{Kp + P}\right) + k_d X \; - Sout + Sin$$

(Equation 3.24)

where $Y\,{}^{x}\!/_{s}$ is yield of biomass from substrate(g/g) μ max is the maximum specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and Ks is a constant of substrate consumption dependent on pH maintenance (g/l) , X is the cell concentration (g/l),P is the end product concentration (g/l) , Kp is a constant of end product production (g/l) and $k_d X$ is the maintenance coefficient.(g cells/g cells/h), Sout (g/l) is the substrate outflow per time and Sin(g/l) is the substrate inflow is the system and So (g/l) is the initial substrate concentration within the nutrient.

On further analysing the above equation becomes:

$$S_{ins} = So + \frac{-1}{Y\,x/_{s}} * (\frac{(\mu\,\max * S) * (Kp + P)}{(Ks + S) * (P)}) + k_d X - Sout + Sin$$

(Equation 3.25)

where $Y\,x/_{s}$ is yield of biomass from substrate(g/g) μ max is the maximum specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and Ks is a constant of substrate consumption dependent on pH maintenance (g/l) , X is the cell concentration (g/l),P is the end product concentration (g/l) , Kp is a constant of end product production (g/l) and $k_d X$ is the maintenance coefficient.(g cells/g cells/h), Sout (g/l) is the substrate outflow per time and Sin(g/l) is the substrate inflow is the system and So (g/l) is the initial substrate concentration within the nutrient.

4.2.4 Describing the rate of Product Formation, product maintained in the system and product outflow rate

Product formation equation proposed is based over the simplified assumption that the rate of product formation is directly related to the rate of substrate consumption, through a yield coefficient $Y\,pLacticacid/_{s}$ (g/g)

For lactic acid formation the proposed equation is the following:

$$\frac{dP(Lacticacid)}{dt} = -Y\,p(lacticacid)/_{s} * \frac{ds}{dt}$$ (Equation 3.26)

where $\frac{dP}{dt}$ is product formation rate (g/l) $Y\,pLacticacid/_{s}$ is the yield coefficient of lactic acid production from substrate and $\frac{ds}{dt}$ is the substrate consumption rate (g/l)

On further analysing the equation becomes:

$$\frac{dP(Lacticacid)}{dt} = -Y\,p(lacticacid)/_{s} * \{\frac{-1}{Y\,x/_{s}} * \{(\frac{\mu\,\max * S}{Ks * S})/(\frac{P}{Kp + P})\}\}$$

(Equation 3.27)

where $\frac{dP}{dt}$ is product formation rate(g/l), $Y^{pLacticacid}/_{s}$ is the yield coefficient of lactic acid production from substrate, $Y^{x}/_{s}$ is yield of biomass from substrate(g/g) μ max is the maximum specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and Ks is a constant of substrate consumption dependent on pH maintenance (g/l) , X is the cell concentration (g/l),P is the end product concentration (g/l) , Kp is a constant of end product production (g/l) and $k_d X$ is the maintenance coefficient.(g cells/g cells/h).

On further analysing the equation becomes:

$$\frac{dP(Lacticacid)}{dt} = -Y^{p(lacticacid)}/_{s} * \{\frac{-1}{Y^{x}/_{s}} * \{\frac{(\mu \max * S) * (Kp + P)}{(Ks * S) * (P)}\}\}$$

(Equation 3.28)

where $\frac{dP}{dt}$ is product formation rate(g/l), $Y^{pLacticacid}/_{s}$ (g/g) is the yield coefficient of lactic acid production from substrate, $Y^{x}/_{s}$ is yield of biomass from substrate(g/g) μ max is the maximum specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and Ks is a constant of substrate consumption dependent on pH maintenance (g/l) , X is the cell concentration (g/l),P is the end product concentration (g/l) , Kp is a constant of end product production (g/l

The rate on product maintained in the system is given by the formula:

$$P_{in} = \{Y^{p}/_{s} * (So - Sin) - Pout\} \qquad \text{(Equation 3.29)}$$

where Pin is the rate of product maintained in the system, $Y^{pLacticacid}/_{s}$ (g/g) is the yield coefficient of lactic acid production from substrate, So (g/l) is the initial substrate concentration, Sin (g/l) is the substrate inflow and Pout (g/l) is the product outflow.

The rate of product outflow (Pout, g/l) is given by the formula:

$$Pout = Pin * F * t \qquad \text{(Equation 3.30)}$$

where Pout (g/l) is the product outflow, Pin (g/l) is the product maintained in the system, F (L/h) is the flux of nutrient in the nutrient, and t (h) is the time step.

The volumetric cellular productivity (g/h) of the system was evaluated by the following equation:

$$VolumtricCell\ \Pr oductivity(g/L/h) = FinalXinTheSystem(g) * D(h^{-1})$$

(Equation 3.31)

where X out total is the biomass on steady state during the fermentation, t (h) is the time step and D (h^{-1}) is the dilution rate in the system during time.

The dilution rate is given by the equation (section1.16.2): $D = \frac{F}{V}(h^{-1})$ (Equation 3.32)

4.2.5 Model Simulation

Introduction

As previously referred (section 4.2), the model was tested against different substrate feed concentrations and on different dilution rates on every set of pH on which growth of *L.lactis* occurred.(section 3.4).Substrate feed concentrations varied between 2.5g/l to 20 g/l and dilution rate between 0.1 to 0.5 L/h. The efficiency of the system was evaluated according to the volumetric cell productivity (g/h) achieved on every test.

Theoretical predictions were made over the volumetric cell productivity on every pH set using several dilution rates and substrate concentrations. All the conditions of the system were kept steady and all the numerical values of the parameters and the constants were kept the same used in the mathematical model for batch growth. On the following tables the cell productivity is listed and the optimum values for dilution rates and substrate concentration are listed. Graphical representations of the optimum conditions are also included. The results of the simulation are shown in Figure 4.10.

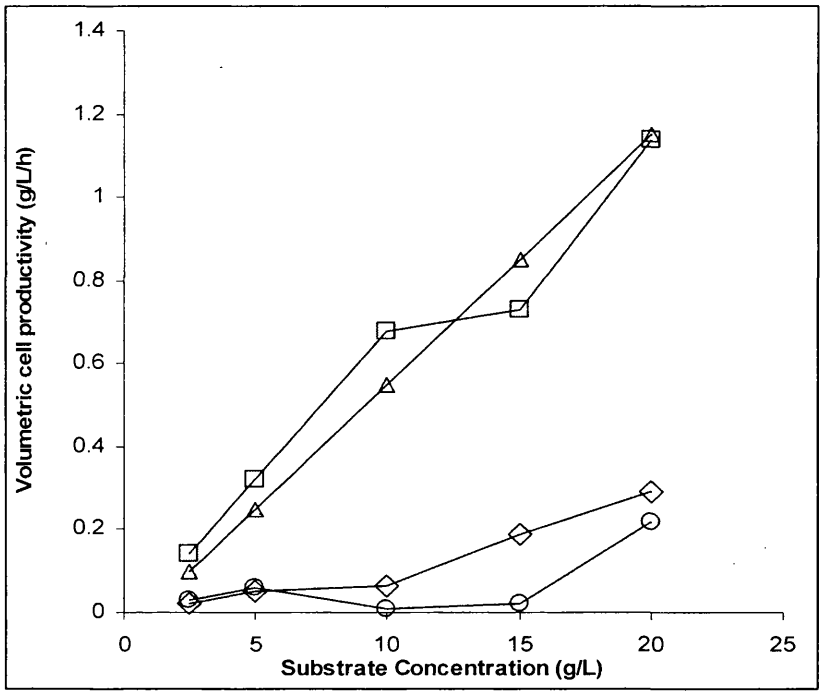


Figure 4.10: Theoretical predictions of volumetric cell productivity on different substrate concentrations of continuous growth of L.lactis on different pH 5.5 (☼) pH 6 (◊), pH 6.5, (⌂) pH 7(□)

As it can be seen by the figure 4.10 the volumetric cell productivity is higher than the one occurring in batch culture of the coccus on pH 6.5 and 7.0 and substrate concentrations of 15 and 20 g/l. In addition, the productivity of the system is strongly correlated with the substrate inflow rate which is dependent on the flow rate. For every set of pH and substrate concentration the optimum flow rate is different.

4.3 Mathematical Modelling of L.lactis on MBR

4.3.1 Introduction

Based on the unstructured kinetic mathematical algorithm developed for batch and continuous culture of *L.lactis*, an unstructured kinetic mathematical model was developed for theoretical predictions over the growth of *L.lactis* on MBR. A maintenance coefficient term was incorporated .The efficiency of the system was evaluated in terms of volumetric cell productivity (g/h). In addition, the model was developed using 2 methods for substrate feed, continuous substrate feed and fed-batch in order to compare the theoretical predictions done over the volumetric cell productivity (g/h). The model was tested against different substrate concentrations and flow rates varying between 2.5 to 20 g/l of substrate feed and 1 to 10 L/h flow rate in the optimum pH value for growth selected (pH 6.5).

4.3.2 Analysis

General Structure of the model

Describing the biomass formation rate and the biomass maintained in the system

The equations describing the biomass formation rate ($\frac{dx}{dt}, g/l$) is the following:

$$\frac{dx}{dt} = \mu * \mathrm{X} - k_d X \qquad \text{(Equation 3.33)}$$

where μ is the specific growth rate (h^{-1}) and X is the cell concentration (g/l).

A maintenance term ($k_d X$,g cells/g cells/h) is incorporated as the growth of the coccus is kept on stationary phase.

In the above equation μ is expressed by the following equation:

$$\mu = \frac{\mu \max * S}{Ks + S} / \frac{P}{Kp + P} \qquad \text{(Equation 3.34)}$$

where, μ is the specific growth rate (h^{-1}), μ max is the maximum specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and Ks is a constant of substrate consumption dependent on pH maintenance (g/l), X is the cell concentration (g/l), P is the end product concentration (g/l) and Kp is a constant of end product production (g/l).

On further analysing the μ the equation becomes:

$$\mu = \frac{(\mu \max * S) * (Kp + P)}{(Ks + S) * (P)} \qquad \text{(Equation 3.35)}$$

where, μ is the specific growth rate (h^{-1}), μ max is the maximum specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and Ks is a constant of substrate consumption dependent on pH maintenance (g/l), X is the cell concentration (g/l), P is the end product concentration (g/l) and Kp is a constant of end product production (g/l).

$$Xin = Xo + \frac{dx}{dt} \qquad \text{(Equation 3.36)}$$

where Xin (g/l) is the biomass maintained in the system and dx/dt (g/l) is the biomass formation rate during time

4.3.3 Describing substrate consumption rate during growth, the substrate feed rate and the substrate maintained within the system

The equation representing substrate accumulation ($\frac{ds}{dt}$, g/l) is directly linked to cell growth. A maintenance coefficient is also incorporated in the equation. The Monod equation then is linked to a yield coefficient for cell growth ($Y_{x/s}$, g/g) as follows:

$$\frac{ds}{dt} = \frac{-1}{Y_{x/s}} * \frac{dx}{dt} + k_d X \qquad \text{(Equation 3.37)}$$

On further analysing the equation becomes:

$$\frac{ds}{dt} = \frac{-1}{Y_{x/s}} * (\frac{\mu \max * S}{Ks + S}) / (\frac{P}{Kp + P}) + k_d X \qquad \text{(Equation 3.38)}$$

On further analysing the above equation:

$$\frac{ds}{dt} = \frac{-1}{Y^{x}/_{s}} * \left(\frac{(\mu \max * S) * (Kp + P)}{(Ks + S) * (P)}\right) + k_d X \qquad \text{(Equation 3.39)}$$

where $Y^{x}/_{s}$ is yield of biomass from substrate(g/g) μ max is the maximum specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and Ks is a constant of substrate consumption dependent on pH maintenance (g/l) , X is the cell concentration (g/l),P is the end product concentration (g/l) , Kp is a constant of end product production (g/l) and $k_d X$ is the maintenance coefficient.(g cells/g cells/h).

The substrate inflow rate (Sin, g/l) in the system is given by the following equation:

$$S_{in} = S_0 * F_s * t \qquad \text{(Equation 3.40)}$$

where Sin (g/l) is the substrate inflow rate per hour, So (g/l) is the initial substrate concentration within the nutrient, Fs (L/h) is the flow of substrate in the system and t is the time step (h).

The substrate outflow rate (Sout, g/l) in the system is given by the following equation:

$$Sout = Sin * \frac{dF}{dt} \qquad \text{(Equation 3.41)}$$

where Sout (g/l) is the substrate outflow rate per hour, Sin (g/l) is the substrate inflow in the system and $\frac{dF}{dt}$ is the flow rate of substrate per hour.

On further analysing the above equation the equation becomes:

$$S_{ins} = So + \frac{-1}{Y^{x}/_{s}} * \left(\frac{\mu \max * S}{Ks + S}\right) / \left(\frac{P}{Kp + P}\right) + k_d X - Sout + Sin$$

(Equation 3.42)

where $Y^{x}/_{s}$ is yield of biomass from substrate(g/g) μ max is the maximum specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and Ks is a constant of

substrate consumption dependent on pH maintenance (g/l) , X is the cell concentration (g/l),P is the end product concentration (g/l) , Kp is a constant of end product production (g/l) and $k_d X$ is the maintenance coefficient.(g cells/g cells/h), Sout (g/l) is the substrate outflow per time and Sin(g/l) is the substrate inflow is the system and So (g/l) is the initial substrate concentration within the nutrient.

On further analysing the above equation becomes:

$$S_{ins} = So + \frac{-1}{Y^{x}/_{s}} * (\frac{(\mu \max * S) * (Kp + P)}{(Ks + S) * (P)}) + k_d X - Sout + Sin$$

(Equation 3.43)

where $Y^{x}/_{s}$ is yield of biomass from substrate (g/g) μ max is the maximum specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and Ks is a constant of substrate consumption dependent on pH maintenance (g/l) , X is the cell concentration (g/l),P is the end product concentration (g/l) , Kp is a constant of end product production (g/l) and $k_d X$ is the maintenance coefficient.(g cells/g cells/h), Sout (g/l) is the substrate outflow per time and Sin(g/l) is the substrate inflow is the system and So (g/l) is the initial substrate concentration within the nutrient.

4.3.4 Describing the flow rate of nutrient in the system and the flow of nutrient in the system

The flow rate of nutrient during time ($\frac{dF}{dt}$, L/h) is described by the following formula:

$$\frac{dF}{dt} = \mu * F * t \qquad \text{(Equation 3.44)}$$

where μ is the growth rate (h^{-1}), F is the flow of the substrate (L/h) and t is the time step (h)

The flow of the substrate in the system (L/h) is given by the following equation:

$$Fs = F_0 + \frac{dF}{dt} \qquad \text{(Equation 3.45)}$$

where Fs is the flow of substrate in the system (L/h) F_0 is the initial flow in the system and $\frac{dF}{dt}$ (L/h) is the substrate flow rate of the system.

4.3.5 Describing the rate of Product Formation, product maintained in the system and product outflow rate

Product formation equation proposed is based over the simplified assumption that the rate of product formation is directly related to the rate of substrate consumption, through a yield coefficient $Y\,{}^{pLacticacid}/_{s}$ (g/g)

For lactic acid formation the proposed equation is the following:

$$\frac{dP(Lacticacid)}{dt} = -Y\,{}^{p(lacticacid)}/_{s} * \frac{ds}{dt} \quad \text{(Equation 3.46)}$$

where $\frac{dP}{dt}$ product formation rate (g/l) is, $Y\,{}^{pLacticacid}/_{s}$ is the yield coefficient of lactic acid production from substrate and $\frac{ds}{dt}$ is the substrate consumption rate (g/l)

On further analysing the equation becomes:

$$\frac{dP(Lacticacid)}{dt} = -Y\,{}^{p(lacticacid)}/_{s} * \{\frac{-1}{Y\,{}^{x}/_{s}} * \{(\frac{\mu\,\max * S}{Ks * S}) / (\frac{P}{Kp + P})\}\}$$

(Equation 3.47)

where $\frac{dP}{dt}$ is product formation rate(g/l), $Y\,{}^{pLacticacid}/_{s}$ is the yield coefficient of lactic acid production from substrate, $Y\,{}^{x}/_{s}$ is yield of biomass from substrate(g/g) μ max is the maximum specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and Ks is a constant of substrate consumption dependent on pH maintenance (g/l) , X is the cell concentration (g/l),P is the end product concentration (g/l) , Kp is a constant of end product production (g/l

On further analysing the equation becomes:

$$\frac{dP(Lacticacid)}{dt} = -Y\, p(lacticacid)/_{S} * \{\frac{-1}{Y\, x/_{S}} * \{\frac{(\mu \max * S) * (Kp + P)}{(Ks * S) * (P)}\}\}$$

(Equation 3.48)

where $\frac{dP}{dt}$ is product formation rate(g/l), $Y\, pLacticacid/_{S}$ (g/g) is the yield coefficient of lactic acid production from substrate, $Y\, x/_{S}$ is yield of biomass from substrate(g/g) μ max is the maximum specific growth rate (h^{-1}), S is the dissolved substrate concentration (g/l) and Ks is a constant of substrate consumption dependent on pH maintenance (g/l) , X is the cell concentration (g/l),P is the end product concentration (g/l) , Kp is a constant of end product production (g/l)

The rate on product maintained in the system is given by the formula:

$$P_{in} = \{Y\, p/_{S} * (So - Sin) - Pout\} \qquad \text{(Equation 3.49)}$$

where Pin is the rate of product maintained in the system, $pLacticacid/_{s}$ $id/_{S}$ (g/g) is the yield coefficient of lactic acid production from substrate, So (g/l) is the initial substrate concentration, Sin (g/l) is the substrate inflow and Pout (g/l) is the product outflow.

The rate of product outflow (Pout, g/l) is given by the formula:

$$Pout = Pin * F * t \qquad \text{(Equation 3.50)}$$

where Pout (g/l) is the product outflow, Pin (g/l) is the product maintained in the system, F (L/h) is the flux of nutrient in the nutrient, and t (h) is the time step.

The volumetric cell productivity of the system was evaluated according to the following equation both for fed-batch and continuous substrate feed

Volumetric Cell Productivity: Final x in the MBR (g) /Total Fermentation time(h)

(Equation 3.51)

4.3.6 Model Simulation

As previously discussed (section 4.3), the model was tested against different substrate feed concentrations and on different dilution rates over a range of pH on which growth of pH on which growth of *L.lactis* occurred.(section 3.4).Substrate feed concentrations varied between 2.5g/l to 20 g/l and fluxes were set between 1 to 10 L/h.(section 2.8.4) The efficiency of the system was evaluated according to the volumetric cell productivity(g/h) achieved on every test, increased feed continuously or doubling time as a function of μ.

Theoretical predictions were made over the volumetric cell productivity on the optimum pH set (pH 6.5) using several fluxes and substrate concentrations. All the conditions of the system were kept steady and all the numerical values of the parameters and the constants were kept the same used in the mathematical model for batch and continuous growth. Graphical representations of the optimum conditions are also included.

Figure 4.11 and 4.12 shows the results. Both feeding strategies produced high productivities compared with batch

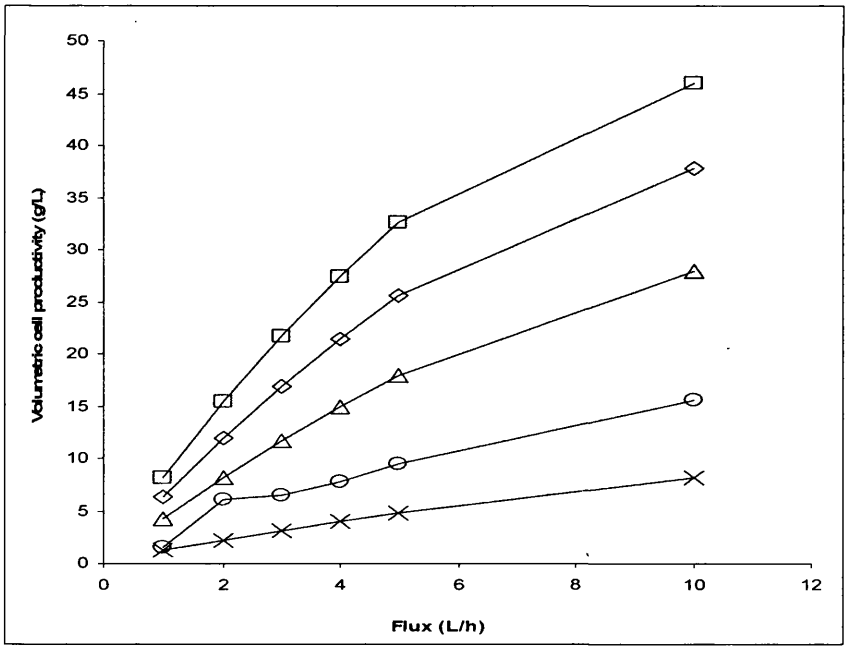


Figure 4.11: Theoretical predictions of volumetric cell productivity on different substrate concentrations of continuous growth of L.lactis on a MBR on different flux rates 2.5 g/l(x) 5 g/l(◊),10 g/l(⌂) , 15 g/l (☼) , 20 g/l(□)

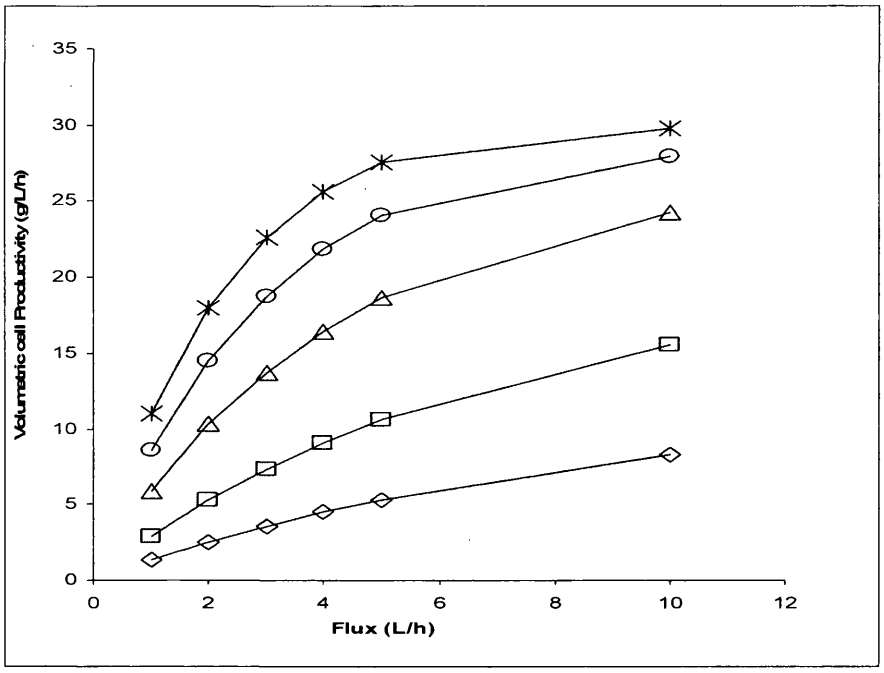


Figure 4.12: Theoretical predictions of volumetric cell productivity on different substrate concentrations of fed-batch growth of L.lactis on a MBR on different flux rates 2.5 g/l(◊), 5 g/l(□),10 g/l(△) , 15 g/l (☼) , 20 g/l(x)

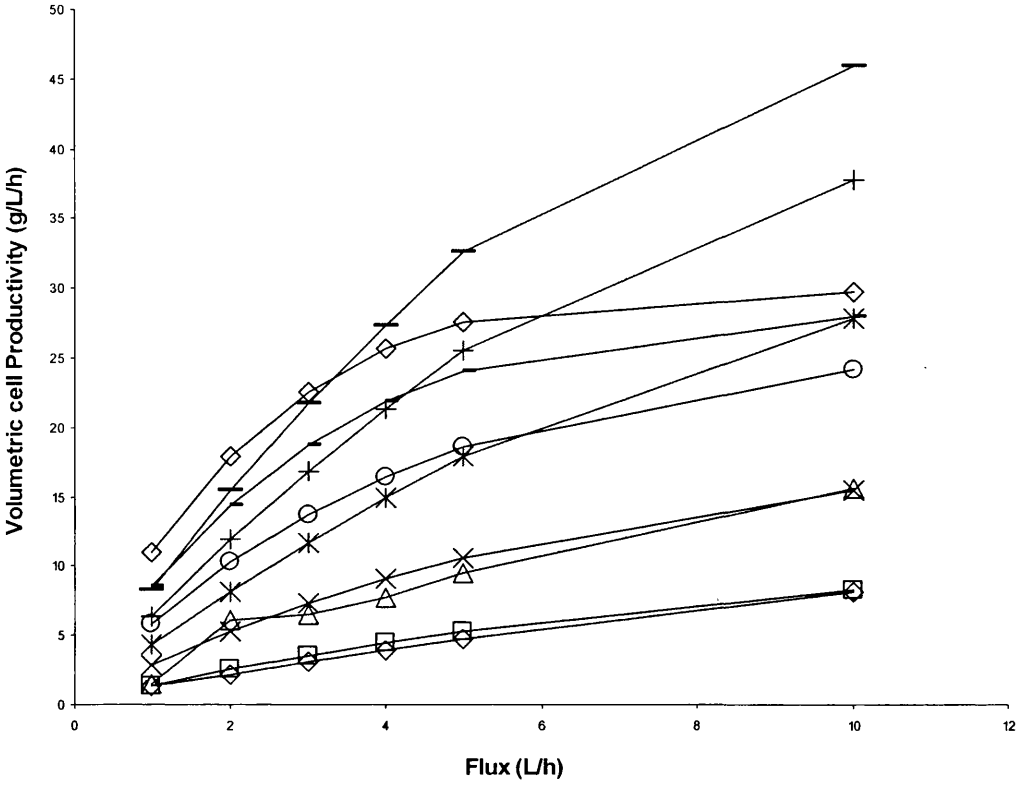


Figure 4.13: Comparison between the theoretical predictions of volumetric cell productivity on different substrate concentrations of fed-batch and continuous substrate feed on growth of L.lactis on a MBR on different flux rates. For continuous substrate feed the symbols are the following: 2.5 g/l(◊), 5 g/l(⌂)10 g/l (□), 15 g/l(+) , 20 g/l(-), for fed-batch substrate feed the symbols are the following: : 2.5(□)g/l, 5 g/l(*)10 g/l(☼),15 g/l(--) , 20 g/l (◊)

4.4 Discussion

4.4.1 Introduction

Three unstructured, unsegregated mathematical models were developed for the deeper understanding and investigation of the growth kinetics and volumetric cell productivity of *L.lactis* intensively cultured in 3 different systems of culture. (batch, continuous, MBR)

The models incorporated a maintenance coefficient term and a non competitive end product inhibition term and to confirm the model batch ate were obtained through previous investigation. The feasibility and the efficiency of the culturing systems were evaluated in terms of volumetric cell productivity (g/h).

4.4.2 Mathematical Modelling of Batch growth of L.lactis

The model developed for batch growth constitutes of 3 equations each one of them describing $\frac{dx}{dt}$, g/l, $\frac{ds}{dt}$, g/l, and $\frac{dP}{dt}$, g/l. All he mathematical terms incorporated in the equations were lined to the Monod kinetics model describing growth rate. The system was tested under a 10h operation incorporating different substrate concentrations (g/l). The maximum volumetric cell productivity (g/h) for batch culturing was 0.47 g/h and was achieved on 20 g/l substrate on pH 6.5. Although the mechanical construction and operation of a batch system is far less complicated comparing with other systems of culturing the volumetric cell productivity (g/h) is proven to be relatively small when compared with the theoretical predictions made by modelling the intensive propagation of *L.lactis* on continuous and on the MBR systems. Culturing in bath mode in this time circle was insufficient for high amounts of lactococcal biomass.

The batch system , although is preferable especially in industrial scale practise due to the mechanical simplicity of the apparatus used , results in simpler operation and due to the easier extraction methods of the desired end products.

4.4.3 Mathematical Modelling of Continuous culture of L.lactis

The model was further developed to predict theoretically the intensive culture of *L.lactis* n continuous mode s composed by 10 equations each one representing $\frac{dx}{dt}$, g/l, $\frac{ds}{dt}$, g/l, and $\frac{dP}{dt}$, g/l, Sin, Sout, Xin, Xout, Pin , Pout and Sins. All the equations where developed

based on the ones developed for the description of batch growth. The model was tested under a 1h circle of operation, under different substrate concentrations (g/l) and different flux rates (L/h).The maximum volumetric cell productivity (g/l/h) was 1.3 g/l/h at 20 g/l of continuous substrate feed in a dilution rate of 0.4 h^{-1} pH 6.5. When compared to the maximum volumetric cell productivity (g/h) achieved in the MBR system is much smaller.

A continuous system of intensive culture of *L.lactis* can offer higher yield of lactococcal biomass but its operation especially on a large scale is more complicated as there are more mechanical and physicochemical parameters to be considered.

Furthermore using a continuous system for intensive culture, due to simultaneous removal of inhibitory for growth end products ensures higher yields of biomass.

4.4.4 Mathematical Modelling of growth of L.lactis on MBR o continuous and fed-batch substrate feed operation

The mathematical algorithm developed for intensive culture of *L.lactis* on an MBR was comprised by 11 equations each one representing $\frac{dx}{dt}$, g/l, $\frac{ds}{dt}$, g/l, and $\frac{dP}{dt}$, g/l, Sin, Sout, Xin, Xout, Pin , Pout, Sins, Fs, $\frac{dF}{dt}$(L/h). The mode was tested under a 14h circle of operation and against different substrate concentrations (g/l) and flux rates (L/h) at the optimum pH 6.5 value. The substrate inflow (Fs, L/h) was modelled in 2 ways, continuously and fed-bath supply. The system's efficiency was evaluated in terms of volumetric cell productivity (g/h), with the volumetric cell productivity (g/h) for fed-batch supply of substrate being 28 g/l/hat flux rate of 10 L/h and for continuous supply, substrate being 45 g/h at flux rate of 10 L/h.

The volumetric cell productivity (g/l/h) grown on the MBR system is much higher when compared with the ones predicted from the models for batch and continuous mode of culturing.

In addition when comparing the volumetric cell productivity between the continuous and the fed-batch substrate support , the continuous substrate support was proven to be superior

4.4.5 Evaluating the Numerical accuracy of the Mathematical Models

The 3 unsegregated unstructured models were as previously .referred (sections 2.7-2.7.4) was developed n the commercially available Microsoft Office Excel 2003 software written on VBA language , on a spreadsheet.

The numerical accuracy of the numerical predictions given buy the models were evaluated in terms of standard deviation (σ^{-2}) assuming that the numerical values follow a normal distribution and in the case of batch growth modelling the fitting with the experimental values was evaluated in terms of the coefficient of determination (R^2). R^2 has values between 0 to 1, which become larger as the theoretical predictions given from the model fit with the experimental values (Shatland et al, 1989).The R^2 values for the batch growth model were 0.97to 0.99 suggesting a very good fitting with the experimental data.

4.4.6 Further Improvement of the Mathematical Models

The mathematical algorithms could be further improved through deeper investigation through testing against different substrate concentrations, flux rates , pH values of operation, time of operation and different initial biomass concentrations.

The mathematical models could also be developed in a simpler and more automated computer simulation program offering the same accuracy as the Excel 2003.

The following table demonstrates the volumetric cell productivity achieved on every mathematically developed system and the improvement of the productivity of the continuous culture and MBR over the batch system.

Culturing Systems	**Volumetric Cell Productivity (g/l/h)**	**Improvement over Batch system (times)**
Batch	0.4	-
Continuous	1.3	3.25
MBR Fed-Batch feed	28	70
MBR Continuous feed	45	112.5

Table 4.3 : Volumetric Cell Productivity of each system and improvement over batch growth

Chapter Five

Conclusions

5.1 Introduction

L.lactis as previously referred (section 1.0) is an important LAB , widely used as a natural acidifier in the dairy industry, nowadays. *L.lactis* is used in the form of starter culture , for inoculation of bulk quantities of milk. As such the need for a development of a strategy resulting in high ell density of the coccus is desirable.

Various reactor configuration have been investigated to give high volumes of cellular biomass produced. This objective was achieved through the investigation of the growth kinetics of L.lactis on 3 different culturing systems.(Batch, Continuous and MBR)

After confirming the accuracy and validity of the model , it was used to investigate the productivity of these systems under a variety of environmental conditions and flows.

The most productive system is the MBR which has factor of 20 increased productivity over batch and continuous growth and could justify the investigation of a more complex fermentation system.

An overall fulfilment of the objectives of this project were therefore achieved.

5.2 Production Systems used for L.lactis

For the intensive propagation of *L.lactis* 3 different production systems were investigated. The coccus was primarily grown in simple batch cultures without pH control, determining the nutritional needs of the coccus. The inhibition effects of the end products were also studied.

To explore further this system, the coccus was grown in a 2L, STR bioreactor incorporating continuous pH control. A simple mathematical model was developed for the deeper understanding of the growth kinetics of the coccus when cultured on a batch system. Through this model, theoretical predictions were made over the volumetric cell productivity f the system when using different substrate concentrations and inoculum size.

As the volumetric cell productivity of the batch system was relatively low (section 4.3.6) a continuous culture system of the coccus was investigated. A mathematical

model was developed to predict numerically the growth kinetics of the coccus. The model was tested against several substrate concentrations and flow rates and theoretical predictions were made over the volumetric cell productivity.

Continuous culture of the system offered higher biomass yields when compared to batch culture but the need for a more productive system was still present.
A mathematical model was then developed to predict the growth kinetics of L.lactis on an MBR system operated with continuous and fed-batch substrate feed.

Comparative studies were conducted between these systems. The selected parameter for comparison was the volumetric cell productivity achieved from every system on different substrate concentrations and for the case of continuous culture and MBR on different dilution and flow rates.
A comparative study was also conducted over the productivity f the MBR system when operated with continuous and fed-batch substrate feed.
These studies show that the MBR system proven to be highly productive offering higher yields of biomass in a shorter period of time especially when operated with continuous substrate feed.
Although the MBR system was proven to be highly productive, it construction and its handling are far more complicated, hen compared with the handling of a batch system of culturing. May mechanical parameters such as heat transfer, flux rates, membrane fouling, membrane surface charge and resistance, sterilisation of he mechanical apparatus and also of the growth media, cleaning and maintenance of the system have to be considered.
Additionally, despite the numerical predictions given over the performance of the system by the mathematical models developed the understanding of the microbial kinetics in the highly productive systems of continuous culturing and MBR demand further investigation. A more practical aspect of the system has to be obtained through realistic testing of the system as to have real time results over the growth kinetics of the coccus.

5.3 Further Work

5.3.1 Nutritional requirements of L.lactis

In order to produce high rates of cellular mass of *L.lactis* an economic liquid growth medium had to be synthesised. There are already commercially available media for growth of the *Lactococci* such as MRS broth and M17.The high cost though of those media do not allow their use in bulk quantities need for the development of the cellular biomass in industrial scale.

The medium should support the growth of the coccus in maximum yields incorporating all the chemicals compounds needed. The concentration of the carbohydrate, nitrogen and mineral sources had to be set in the optimum concentrations. In addition the effect of the pH of the medium over the growth had to determined.(section 3.4)

Combining the optimum concentrations of the above substances resulted n the composition of an optimised medium as used throughout the process.

The medium's effectiveness was tested in large scale combined with the use of the optimum pH values resulting in an amelioration of the growth rate of the coccus of 30%.

5.3.2 Inhibition Effects of End Products over the growth of L.lactis

L.lactis metabolism of glucose was determined as being purely homofermetative. As such, its growth is strongly inhibited by the produce during growth, lactic acid.

The inhibition effect of lactic acid over the biomass development had to be studied and the threshold values had to be defined. Furthermore the values for end product coefficient which would be used in the mathematical model had to be defined.

5.3.3 Growth Kinetics of L.lactis

Growth kinetics of *L.lactis* as previously referred (section 4.1- 4.4.6) was investigated through experimental procedures and theoretical predictions made by develop mathematical algorithms.

The equations for ds/dt, dp/dt, and dx/dt were interlinked with the Monod equation for growth rate. When the developed model for batch growth was tested against the experimental data obtained from the growth of *L.lactis* on a 2L STR, operated in batch mode, there was poor fitting as the inhibitory effect of lactic acid over growth could not be omitted.

When the end product inhibition term was incorporated in the model the fitting with the experimental data was successful. (R^2 value: 0.97 to 0.99)

The end product inhibition term developed was based on the non competitive models for product inhibition of Hinselwood and Taylor as the inhibition effect is caused only by lactic acid. The model was further developed incorporating a maintenance coefficient term which is proven to be very important when modelling the growth of the coccus on large scale.

The mathematical algorithm was further developed to model the growth of the coccus in continuous mode. Additional equations were constructed to model the biomass outflow, the biomass maintained in the system, the substrate inflow, outflow and the substrate maintained in the system, the product outflow and the product maintained in the system.

Theoretical predictions were made over the volumetric cell productivity (g/l/h) of the system on different substrate concentrations (g/l) and dilution rates (h^{-1}). Numerical predictions were made over the volumetric cell productivity (g/l/h) of the model on different dilution rates and substrate concentrations.

To investigate more the growth of the coccus on an MBR system a mathematical model was also made based on the equations developed for batch and continuous culturing. Additional equations were incorporated for flow rates, flux of the medium in the system. Numerical predictions were made over the volumetric cell productivity of the model on different flow rates (L/h) and substrate concentrations g/l) when the system was operated with continuous substrate feed (i.e. Appendix)

5.4 Further Development and Future Prospects

In order to investigate in more detail the productivity of the MBR system practical work obtaining experimental results should be performed. Enrichment of the system could be done by investigating the relationships between vessel tank and membrane module.

Another feeding strategy would be to use relatively diluted media for feeding through the MBR rapidly to obtain more rapid growth that then would be obtained more concentrated but the inhibition effect might be higher

Growth kinetics of the coccus could be also further developed with a better understanding of the physiological basis of the substrate uptake system and the conversion of carbohydrate sources into organic acids. The mathematical models used then could be transformed into segregated and structured predicting with higher accuracy the kinetics of the coccus.

The coccus could be also investigated as a potential candidate for the production of enzymes such as lactate dehydrogenase or β-galactosidase which are incorporated in its systems. Also the fermentation by the coccus of different carbohydrate sources could also be tested in order to investigate the production of any other chemicals producing other desirable flavour effects.

L.lactis incorporating the NICE system could be also used as a natural producer of the lantabiotic nisin a widely used antibiotic in the dairy industry.

Finally the coccus productivity of lactic acid could be further ameliorated as the need or high quality biopolymer in the industry is constantly augmenting.

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Appendix: Theoretical Predictions of the Volumetric Cell Productivity, Standard Curves for Dry Weight and HPLC, Mathematical Modelling Simulation (Batch, Continuous, MBR)

pH	Substrate Concentrations(g/l)	Volumetric Cell Productivity (g/l/h)
5.5	2.5	0.07
	5	0.1
	10	0.16
	15	0.21
	20	0.18
6.0	2.5	0.1
	5	0.15
	10	0.25
	15	0.35
	20	0.2
6.5	2.5	0.08
	5	0.11
	10	0.18
	15	0.24
	20	0.45
7.0	2.5	0.09
	5	0.14
	10	0.24
	15	0.33
	20	0.43

Table 1: Theoretical Predictions of Volumetric Cell Productivity in Batch System of *L.lactis*

Appendix 1

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Appendix: Theoretical Predictions of the Volumetric Cell Productivity, Standard Curves for Dry Weight and HPLC, Mathematical Modelling Simulation (Batch, Continuous, MBR)

pH	Substrate Concentrations(g/l)	Flow (L/h)	Volumetric Cell Productivity (g/l/h)	pH	Substrate Concentrations(g/l)	Flow (L/h)	Volumetric Cell Productivity (g/l/h)
5.5	2.5	0.1	0.003	6.5	2.5	0.1	0.05
		0.2	0.028			0.2	0.09
		0.3	0.013			0.3	0.10
		0.4	0.0032			0.4	0.047
		0.5	0.00065			0.5	0.007
	5	0.1	0.053		5	0.1	0.10
		0.2	0.061			0.2	0.18
		0.3	0.024			0.3	0.25
		0.4	0.005			0.4	0.17
		0.5	0.0087			0.5	0.024
	10	0.1	0.098		10	0.1	0.20
		0.2	0.010			0.2	0.39
		0.3	0.010			0.3	0.55
		0.4	0.007			0.4	0.48
		0.5	0.00004			0.5	0.05
	15	0.1	0.02		15	0.1	0.30
		0.2	0.023			0.2	0.59
		0.3	0.0055			0.3	0.85
		0.4	0.00055			0.4	0.73
		0.5	0.0004			0.5	0.06
	20	0.1	0.22		20	0.1	0.40
		0.2	0.15			0.2	0.78
		0.3	0.011			0.3	1.15
		0.4	0.0008			0.4	0.91
		0.5	0.00005			0.5	0.07
6.0	2.5	0.1	0.022	7.0	2.5	0.1	0.05
		0.2	0.018			0.2	0.09
		0.3	0.004			0.3	0.13
		0.4	0.0003			0.4	0.14
		0.5	0.00002			0.5	0..05
	5	0.1	0.048		5	0.1	0.095
		0.2	0.051			0.2	0.17
		0.3	0.009			0.3	0.26
		0.4	0.0007			0.4	0.32
		0.5	0.0004			0.5	0.11
	10	0.1	0.083		10	0.1	0.18
		0.2	0.64			0.2	0.36
		0.3	0.011			0.3	0.53
		0.4	0.0008			0.4	0.68
		0.5	0.00005			0.5	0.16
	15	0.1	0.19		15	0.1	0.27
		0.2	0.024			0.2	0.53
		0.3	0.0061			0.3	0.80
		0.4	0.000614			0.4	1.04
		0.5	0.00004			0.5	0.17
	20	0.1	0.26		20	0.1	0.36
		0.2	0.17			0.2	0.72
		0.3	0.013			0.3	1.07
		0.4	0.00009			0.4	1.4
		0.5	0.00005			0.5	0.18

Table 2: Theoretical Predictions of Volumetric Cell Productivity in Continuous Culture of *L.lactis*

Appendix 2

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Appendix: Theoretical Predictions of the Volumetric Cell Productivity, Standard Curves for Dry Weight and HPLC, Mathematical Modelling Simulation (Batch, Continuous, MBR)

pH	Substrate Concentrations(g/l)	Flow (L/h)	Volumetric Cell Productivity (g/l/h) Continous	pH	Substrate Concentrations(g/l)	Flow (L/h)	Volumetric Cell Productivity (g/l/h) Fed-Batch
6.5	2.5	1	1.33	6.5	2.5	1	1.31
		2	2.24			2	2.53
		3	3.13			3	3.59
		4	3.98			4	4.52
		5	4.82			5	5.33
		10	8.21			10	8.28
	5	1	1.56		5	1	2.88
		2	6.05			2	5.34
		3	6.53			3	7.39
		4	7.79			4	9.13
		5	9.45			5	10.65
		10	15.59			10	15.53
	10	1	4.33		10	1	5.86
		2	8.19			2	10.31
		3	11.65			3	13.75
		4	14.9			4	16.47
		5	17.96			5	18.63
		10	27.87			10	24.25
	15	1	6.39		15	1	8.56
		2	11.92			2	14.47
		3	16.84			3	18.72
		4	21.38			4	21.82
		5	25.59			5	24.04
		10	37.79			10	27.96
	20	1	8.23		20	1	11.01
		2	15.46			2	17.98
		3	21.68			3	22.62
		4	27.34			4	25.66
		5	32.55			5	27.53
		10	45.94			10	29.78

Table 3: Theoretical Predictions of Volumetric Cell Productivity in Continuous Culture of *L.lactis*

Appendix 3

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Appendix: Theoretical Predictions of the Volumetric Cell Productivity, Standard Curves for Dry Weight and HPLC, Mathematical Modelling Simulation (Batch, Continuous, MBR)

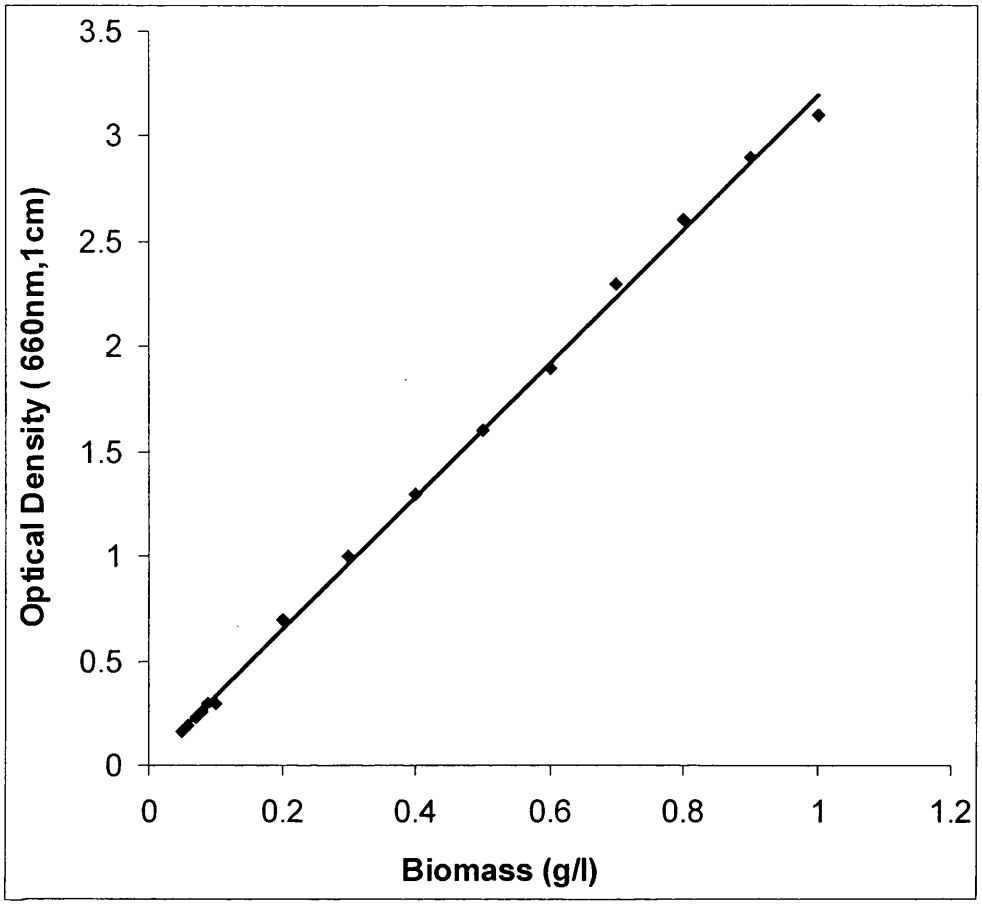

Figure 1: Standard Curve of determination of Dry Weight of *L.lactis*. This Curve was plotted by correlating the O.D. measurements and the dry weight of te coccus.Equation to determine theweight of te coccus in g/l was determined as following (Refer to 2.5.4)

Y= 0.21405X+0.00347

Y is described as the dry weight units (g/l) and X is for the O.D. units

An error range of equation was r=0.987802

Appendix 4

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Appendix: Theoretical Predictions of the Volumetric Cell Productivity, Standard Curves for Dry Weight and HPLC, Mathematical Modelling Simulation (Batch, Continuous, MBR)

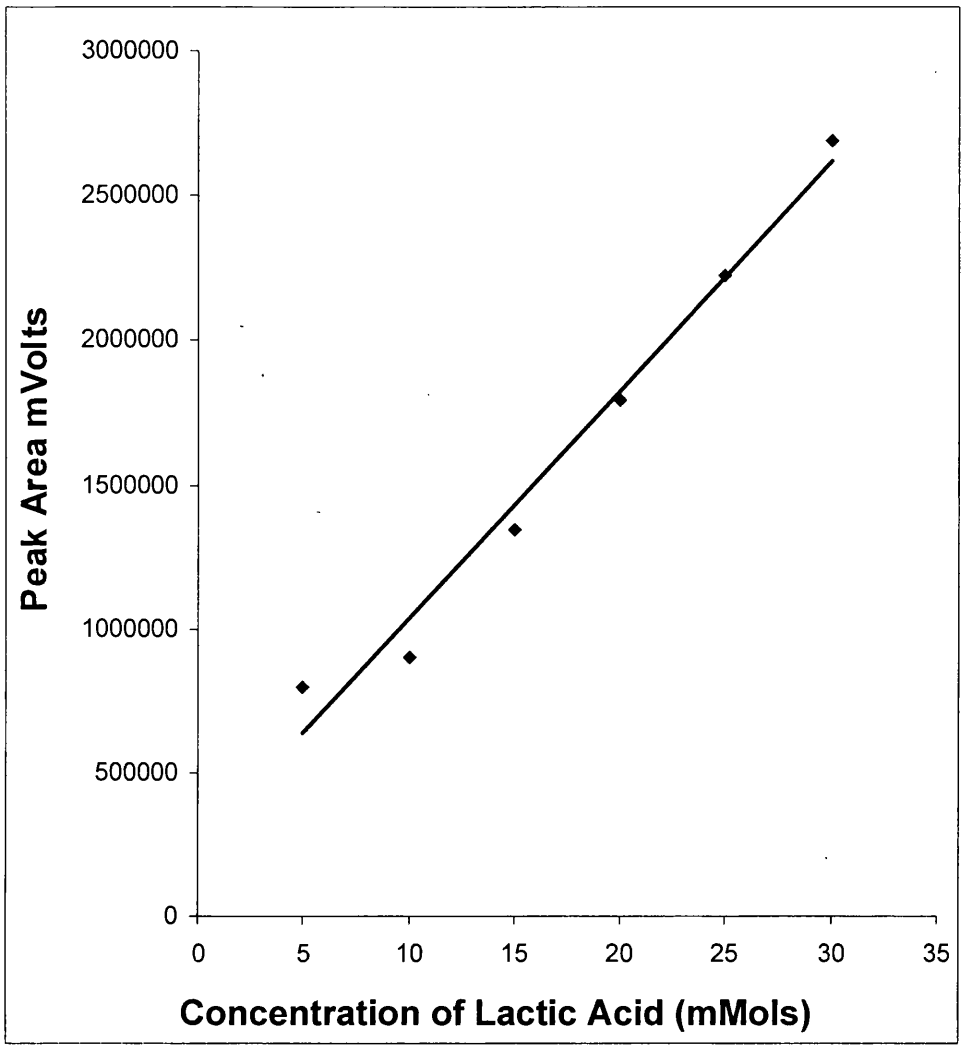

Figure 2: Standard Curve of determination of Lactic acid concentration. This Curve was plotted HPLC.Equation to determine acetic acid was determined as following (Refer to 2.5.4)

Y= 79277X+237815

Y is described as the area of the main peak and X is for the lactic acid concentration (mM)

An error range of equation was r=0.989891

Appendix 5

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Appendix: Theoretical Predictions of the Volumetric Cell Productivity, Standard Curves for Dry Weight and HPLC, Mathematical Modelling Simulation (Batch, Continuous, MBR)

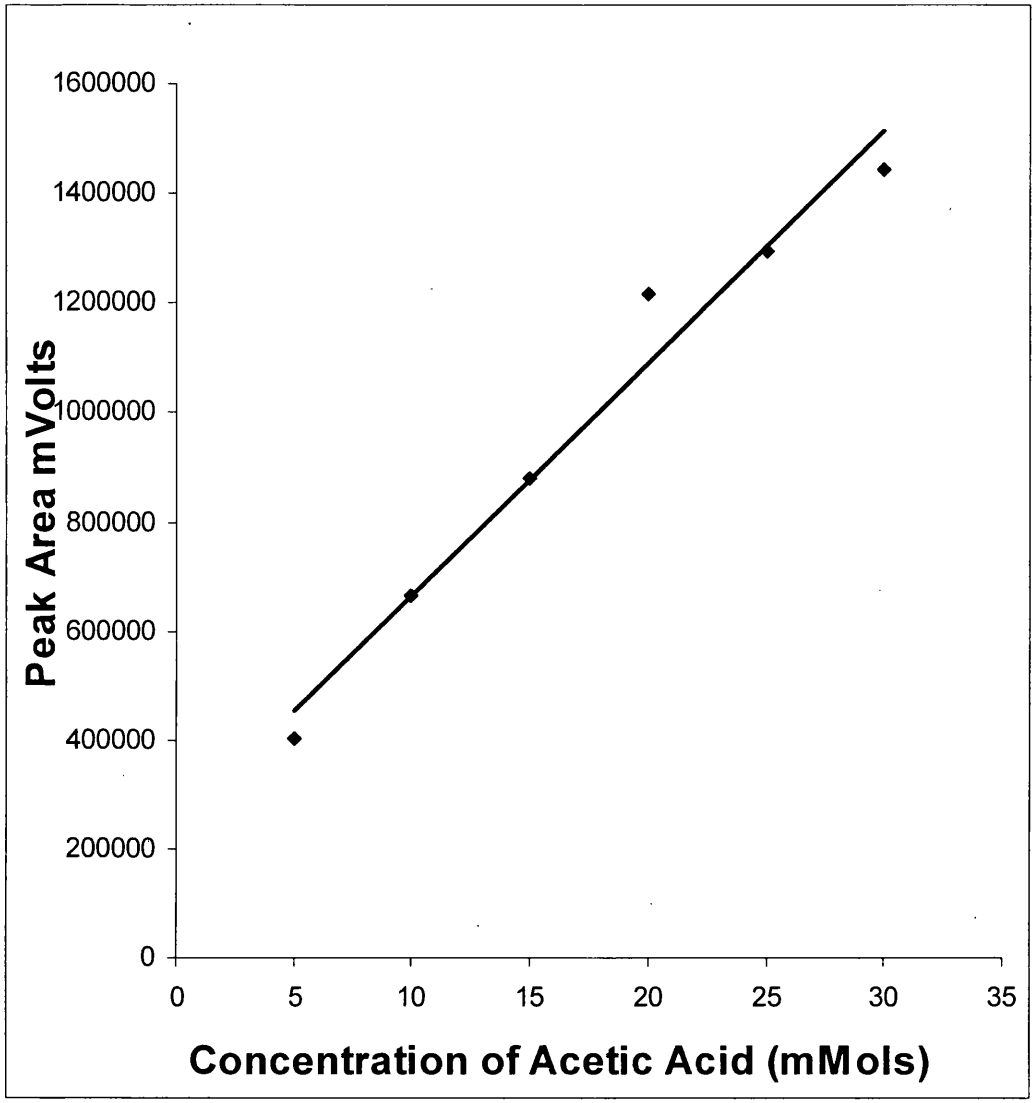

Figure 3: Standard Curve of determination of Acetic acid concentration. This Curve was plotted HPLC.Equation to determine acetic acid was determined as following (Refer to 2.5.4)

Y= 42457X+241361

Y is described as the area of the main peak and X is for the acetic acid concentration (mM)

An error range of equation was r=0.985447

Appendix 6

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Appendix: Theoretical Predictions of the Volumetric Cell Productivity, Standard Curves for Dry Weight and HPLC, Mathematical Modelling Simulation (Batch, Continuous, MBR)

pH 6.5 Batch Model

dx/dt=mu*x

mu= (mumaxS/(Ks+S))/(P/(Kp+P

ds/dt =(-1/Yx/s) dx/dt*x

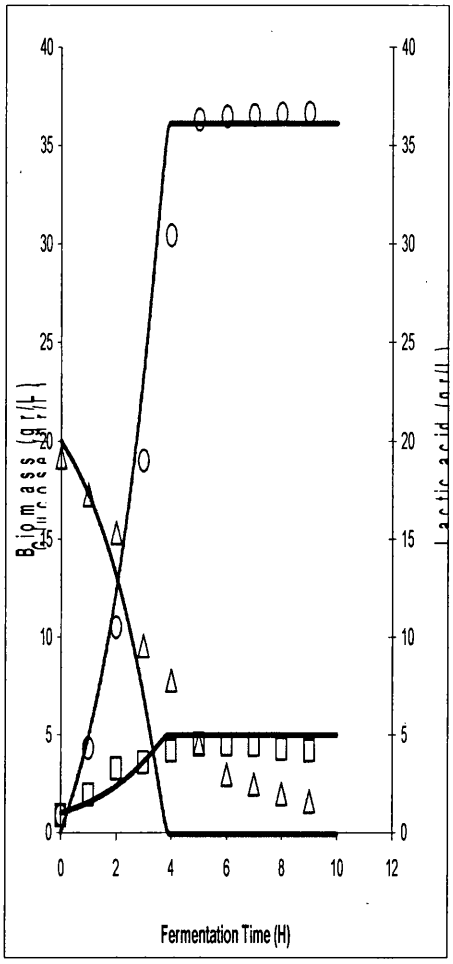
dp/dt=-Ys/p*S

time base 0.01

Volumetric cell productivity:Final Biomass/Total Fermentation Time

Volumetric cell Produc #ΔIAIP/0!

S	20 g/l
x	1 g/l
mu max	0.66 h-1
ks	0.471 g/l
Yx/s	0.2 g/g
Yp/s	1.8 mol/mol
kp	0.714 g/L
ms	0.057 gr/L



h	S	x	mu	dx/dt	ds/dt	dp/dt P
0	0	20	1	0.644815	0.006448	-0.03224 0
1	0.01	19.96776	1.006448	0.433191	0.006448	-0.03224 0.058033
2	0.02	19.93552	1.012896	0.433177	0.00436	-0.0218 0.116067
3	0.03	19.91372	1.017256	0.433167	0.004388	-0.02194 0.155305
4	0.04	19.89178	1.021644	0.433157	0.004406	-0.02203 0.194794
5	0.05	19.86975	1.02605	0.433148	0.004425	-0.02213 0.234452
6	0.06	19.84762	1.030476	0.433138	0.004444	-0.02222 0.27428
7	0.07	19.8254	1.03492	0.433128	0.004463	-0.02232 0.314278
8	0.08	19.80308	1.039383	0.433119	0.004483	-0.02241 0.354449
9	0.09	19.78067	1.043866	0.433109	0.004502	-0.02251 0.394792
10	0.1	19.75816	1.048367	0.433099	0.004521	-0.02261 0.435307
11	0.11	19.73556	1.052889	0.433089	0.00454	-0.0227 0.475997
12	0.12	19.71285	1.057429	0.433079	0.00456	-0.0228 0.516861
13	0.13	19.69006	1.061989	0.433068	0.004579	-0.0229 0.557901
14	0.14	19.66716	1.066568	0.433058	0.004599	-0.023 0.599116
15	0.15	19.64416	1.071168	0.433048	0.004619	-0.02309 0.640508
16	0.16	19.62107	1.075786	0.433037	0.004639	-0.02319 0.682078
17	0.17	19.59787	1.080425	0.433027	0.004659	-0.02329 0.723826
18	0.18	19.57458	1.085084	0.433017	0.004679	-0.02339 0.765753
19	0.19	19.55119	1.089762	0.433006	0.004699	-0.02349 0.80786
20	0.2	19.5277	1.094461	0.432995	0.004719	-0.02359 0.850147
21	0.21	19.5041	1.09918	0.432985	0.004739	-0.02369 0.892616
22	0.22	19.48041	1.103919	0.432974	0.004759	-0.0238 0.935267
23	0.23	19.45661	1.108678	0.432963	0.00478	-0.0239 0.9781
24	0.24	19.43271	1.113457	0.432952	0.0048	-0.024 1.021117
25	0.25	19.40871	1.118258	0.432941	0.004821	-0.0241 1.064319
26	0.26	19.38461	1.123078	0.43293	0.004841	-0.02421 1.107705
27	0.27	19.3604	1.12792	0.432919	0.004862	-0.02431 1.151278

Experimental Data

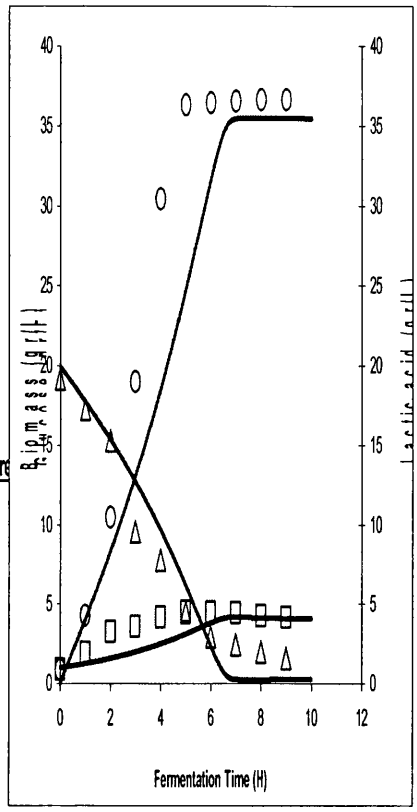
Hours	x	S	P	
0	0.895181	19.18	0.88	O.D.
1	1.96115	17.26	4.3	0.402
2	3.264715	15.33	10.5	0.9
3	3.58579	9.55	19	1.509
4	4.208675	7.77	30.43	1.659
5	4.538312	4.52	36.3	1.95
6	4.506205	2.98	36.46	2.104
7	4.456973	2.47	36.52	2.089
8	4.262188	2	36.61	2.066
9	4.208675	1.59	36.65	1.975

Appendix 7

Appendix: Theoretical Predictions of the Volumetric Cell Productivity, Standard Curves for Dry Weight and HPLC, Mathematical Modelling Simulation (Batch, Continuous, MBR)

substrate entering reactor:S*Flow*Time	flow	0.2	l/h	S	20	g/l
substrate leaving reactor: S in the reactor*Flow*Time	volume	1	l	x	1	g/l
substrate in the reactor: S+ (ds/dt) - S leaving+S entering	D		0.2 h-1	mu max	0.66	h-1
x leaving the reactor: X initial*Flow*Time b Xin the reactor*Flow*Time base				ks	0.471	g/l
X in the reactor: Xinit+dx/dt - X leaving				Yx/s	0.2	g/g
Product in the reactor: (Yp/s*(S-S in the reactor)-Prod.leav.in the reactor)		time base	0.01	Yp/s	1.8	mol/mol
Product leaving in the reactor: Product in the reactor* Flow* Time base				kp	0.714	g/L
Volumetric cell productivity:Final X leaving the reactor/Time base*Flow				ms	0.057	gr/L

Volumetric cell productivity: #TIMH!



1 Column	2 Column	3 Column	4 Column	5 Column	6 Column	7 Column	8 Column	9 Column	10 Column	11 Column
h	S in the re	S leaving	S entering	x in the re	x leaving t	mu	dx/dt	ds/dt	P	Product leaving the re
0	0	20	0.04	0.04	1	0.002	0.644815	0.006448	-0.03224	0	0
1	0.01	19.96776	0.039936	0.04	1.004448	0.002009	0.433191	0.006448	-0.03224	0.058033	0.000116
2	0.02	19.93558	0.039871	0.04	1.008887	0.002018	0.433177	0.004351	-0.02176	0.115834	0.000232
3	0.03	19.91396	0.039828	0.04	1.011221	0.002022	0.433167	0.00437	-0.02185	0.154648	0.000309
4	0.04	19.89228	0.039785	0.04	1.013569	0.002027	0.433158	0.00438	-0.0219	0.193593	0.000387
5	0.05	19.87059	0.039741	0.04	1.015922	0.002032	0.433148	0.00439	-0.02195	0.232549	0.000465
6	0.06	19.8489	0.039698	0.04	1.01828	0.002037	0.433139	0.0044	-0.022	0.271519	0.000543
7	0.07	19.8272	0.039654	0.04	1.020644	0.002041	0.433129	0.004411	-0.02205	0.310501	0.000621
8	0.08	19.80549	0.039611	0.04	1.023013	0.002046	0.43312	0.004421	-0.0221	0.349496	0.000699
9	0.09	19.78378	0.039568	0.04	1.025388	0.002051	0.43311	0.004431	-0.02215	0.388504	0.000777
10	0.1	19.76205	0.039524	0.04	1.027768	0.002056	0.4331	0.004441	-0.02221	0.427525	0.000855
11	0.11	19.74032	0.039481	0.04	1.030154	0.00206	0.433091	0.004451	-0.02226	0.46656	0.000933
12	0.12	19.71859	0.039437	0.04	1.032545	0.002065	0.433081	0.004462	-0.02231	0.505609	0.001011
13	0.13	19.69684	0.039394	0.04	1.034941	0.00207	0.433071	0.004472	-0.02236	0.544671	0.001089
14	0.14	19.67509	0.03935	0.04	1.037343	0.002075	0.433062	0.004482	-0.02241	0.583747	0.001167
15	0.15	19.65333	0.039307	0.04	1.03975	0.00208	0.433052	0.004492	-0.02246	0.622838	0.001246
16	0.16	19.63156	0.039263	0.04	1.042163	0.002084	0.433042	0.004503	-0.02251	0.661943	0.001324
17	0.17	19.60979	0.03922	0.04	1.044581	0.002089	0.433032	0.004513	-0.02257	0.701062	0.001402
18	0.18	19.588	0.039176	0.04	1.047005	0.002094	0.433023	0.004523	-0.02262	0.740196	0.00148
19	0.19	19.56621	0.039132	0.04	1.049435	0.002099	0.433013	0.004534	-0.02267	0.779345	0.001559
20	0.2	19.54441	0.039089	0.04	1.05187	0.002104	0.433003	0.004544	-0.02272	0.818509	0.001637
21	0.21	19.5226	0.039045	0.04	1.05431	0.002109	0.432993	0.004555	-0.02277	0.857688	0.001715
22	0.22	19.50078	0.039002	0.04	1.056756	0.002114	0.432983	0.004565	-0.02283	0.896883	0.001794
23	0.23	19.47895	0.038958	0.04	1.059208	0.002118	0.432973	0.004576	-0.02288	0.936093	0.001872
24	0.24	19.45712	0.038914	0.04	1.061665	0.002123	0.432963	0.004586	-0.02293	0.975319	0.001951
25	0.25	19.43527	0.038871	0.04	1.064128	0.002128	0.432953	0.004597	-0.02298	1.014561	0.002029
26	0.26	19.41342	0.038827	0.04	1.066596	0.002133	0.432943	0.004607	-0.02304	1.053819	0.002108

Experimental Data

Hours	x	S	P	
0	0.895181	19.18	0.88	O.D.
1	1.96115	17.26	4.3	0.402
2	3.264715	15.33	10.5	0.9
3	3.58579	9.55	19	1.509
4	4.208675	7.77	30.43	1.659
5	4.538312	4.52	36.3	1.95
6	4.506205	2.98	36.46	2.104
7	4.456973	2.47	36.52	2.089
8	4.262188	2	36.61	2.066
9	4.208675	1.59	36.65	1.975

Continuous mode

Appendix 8

Appendix: Theoretical Predictions of the Volumetric Cell Productivity, Standard Curves for Dry Weight and HPLC, Mathematical Modelling Simulation (Batch, Continuous, MBR)

pH 6.5 S 2.5 g/L F 1 l/h Model on the MBR CONTINUOUS FEED S starting 2.5

substrate entering reactor:S*Flow*Time

substrate leaving reactor: S in the reactor*Flow*Time

substrate in the reactor: S+ (ds/dt) - S leaving+S entering

x leaving the reactor: X initial*Flow*Time base& Xin the reactor*Flow*Time base

X in the reactor: Xinit+dx/dt - X leaving time base 0.002

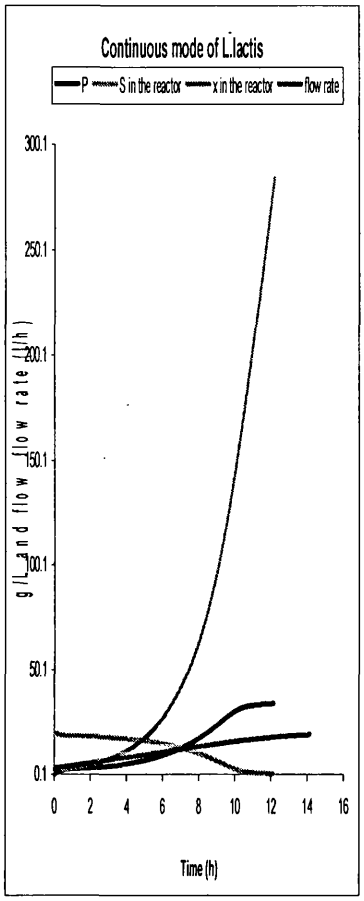
Product in the reactor: (Yp/s*(S-S in the reactor)-Prod.leav.in the reactor) D h-1 1

Product leaving in the reactor: Product in the reactor* Flow* Time base 1

Volumetric cell productivity:Final X leaving the reactor/Time base*Flow

Volumetric cell productivity: #ANAΦ! g/l/h

S	2.5 g/l
x	1 g/l
mu max	0.66 h-1
ks	0.475 g/l
Yx/s	0.2 g/g
Yp/s	1.8 mol/mol
kp	0.714 g/L
ms	0.057 g/g-1
flow	1 l/h
volume	1 l



h	flow rate	df/dt	S in the re	S leaving	S entering	x in the re	mu	dx/dt	ds/dt	P	Product leaving the reactor
0	0	1	0.001109	2.5	0.005	0.005	1	0.554622	0.001109	-0.00555	0	0
1	0.002	1.000761	0.000761	2.494454	0.002766957	0.005004	1.001109	0.380469	0.001109	-0.00555	0.009983	2E-05
2	0.004	1.001522	0.000761	2.491144	0.00498608	0.005004	1.002218	0.3804	0.000762	-0.00381	0.01592	3.19E-05
3	0.006	1.002282	0.000761	2.487353	0.004982277	0.005008	1.00298	0.380321	0.000762	-0.00381	0.022732	4.55E-05
4	0.008	1.003043	0.00076	2.483566	0.004978469	0.005011	1.003743	0.380242	0.000763	-0.00381	0.029535	5.92E-05
5	0.01	1.003803	0.00076	2.479785	0.00497466	0.005015	1.004506	0.380163	0.000763	-0.00382	0.036329	7.29E-05
6	0.012	1.004563	0.00076	2.476008	0.00497085	0.005019	1.005269	0.380084	0.000764	-0.00382	0.043112	8.66E-05
7	0.014	1.005323	0.00076	2.472238	0.004967039	0.005023	1.006033	0.380004	0.000764	-0.00382	0.049885	0.0001
8	0.016	1.006083	0.00076	2.468473	0.004963227	0.005027	1.006797	0.379925	0.000765	-0.00382	0.056649	0.000114
9	0.018	1.006843	0.00076	2.464713	0.004959413	0.00503	1.007562	0.379845	0.000765	-0.00383	0.063402	0.000128
10	0.02	1.007602	0.00076	2.460959	0.004955598	0.005034	1.008327	0.379766	0.000765	-0.00383	0.070146	0.000141
11	0.022	1.008362	0.000759	2.457211	0.004951783	0.005038	1.009092	0.379686	0.000766	-0.00383	0.07688	0.000155
12	0.024	1.009121	0.000759	2.453467	0.004947966	0.005042	1.009858	0.379606	0.000766	-0.00383	0.083604	0.000169
13	0.026	1.00988	0.000759	2.44973	0.004944148	0.005046	1.010624	0.379527	0.000767	-0.00383	0.090317	0.000182
14	0.028	1.010639	0.000759	2.445998	0.004940329	0.005049	1.011391	0.379447	0.000767	-0.00384	0.097021	0.000196
15	0.03	1.011398	0.000759	2.442271	0.004936509	0.005053	1.012158	0.379367	0.000768	-0.00384	0.103715	0.00021
16	0.032	1.012156	0.000759	2.43855	0.004932689	0.005057	1.012925	0.379287	0.000768	-0.00384	0.1104	0.000223
17	0.034	1.012915	0.000758	2.434835	0.004928867	0.005061	1.013693	0.379206	0.000768	-0.00384	0.117074	0.000237
18	0.036	1.013673	0.000758	2.431125	0.004925044	0.005065	1.014462	0.379126	0.000769	-0.00384	0.123738	0.000251
19	0.038	1.014431	0.000758	2.42742	0.004921221	0.005068	1.015231	0.379046	0.000769	-0.00385	0.130392	0.000264
20	0.04	1.015189	0.000758	2.423722	0.004917397	0.005072	1.016	0.378966	0.00077	-0.00385	0.137037	0.000278
21	0.042	1.015947	0.000758	2.420028	0.004913572	0.005076	1.016769	0.378885	0.00077	-0.00385	0.143671	0.000292
22	0.044	1.016704	0.000758	2.41634	0.004909746	0.00508	1.017539	0.378804	0.00077	-0.00385	0.150296	0.000305
23	0.046	1.017462	0.000757	2.412658	0.004905919	0.005084	1.01831	0.378724	0.000771	-0.00385	0.156911	0.000319
24	0.048	1.018219	0.000757	2.408981	0.004902092	0.005087	1.019081	0.378643	0.000771	-0.00386	0.163515	0.000333
25	0.05	1.018976	0.000757	2.40531	0.004898264	0.005091	1.019852	0.378562	0.000772	-0.00386	0.17011	0.000346
26	0.052	1.019733	0.000757	2.401644	0.004894436	0.005095	1.020624	0.378481	0.000772	-0.00386	0.176695	0.00036
27	0.054	1.02049	0.000757	2.397983	0.004890606	0.005099	1.021396	0.3784	0.000773	-0.00386	0.18327	0.000374
28	0.056	1.021247	0.000757	2.394329	0.004886777	0.005102	1.022169	0.378319	0.000773	-0.00386	0.189835	0.000387
29	0.058	1.022003	0.000756	2.390679	0.004882946	0.005106	1.022942	0.378238	0.000773	-0.00387	0.19639	0.000401
30	0.06	1.022759	0.000756	2.387035	0.004879115	0.00511	1.023715	0.378157	0.000774	-0.00387	0.202935	0.000415
31	0.062	1.023516	0.000756	2.383397	0.004875284	0.005114	1.024489	0.378075	0.000774	-0.00387	0.20947	0.000428
32	0.064	1.024272	0.000756	2.379765	0.004871452	0.005118	1.025263	0.377994	0.000775	-0.00387	0.215995	0.000442

Appendix 9

Appendix: Theoretical Predictions of the Volumetric Cell Productivity, Standard Curves for Dry Weight and HPLC, Mathematical Modelling Simulation (Batch, Continuous, MBR)

pH 6.5 S 2.5 g/L F 1 l/h STEPWISE SUBSTRATE FEED

substrate entering reactor:S*Flow*Time

substrate leaving reactor: S in the reactor*Flow*Time

substrate in the reactor: S+ (ds/dt) - S leaving+S entering

x leaving the reactor: X initial*Flow*Time base& Xin the reactor*Flow*Time base

X in the reactor: Xinit+dx/dt - X leaving

Product in the reactor: (Yp/s*(S-S in the reactor)-Prod.leav.in the reactor)

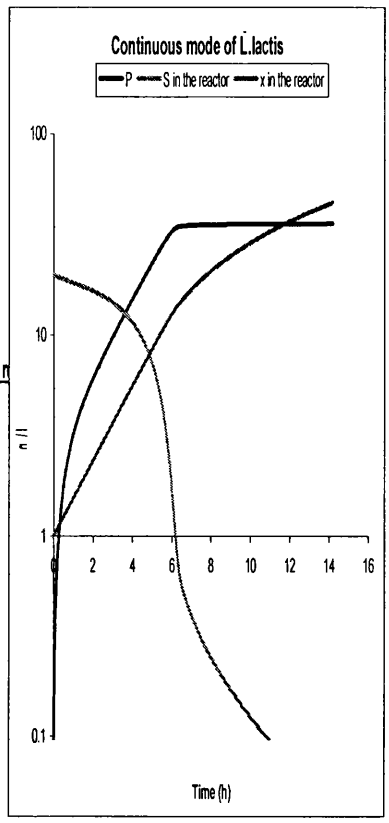
Product leaving in the reactor: Product in the reactor* Flow* Time base

Volumetric cell productivity :Final X leaving the reactor/Time base*Flow

Volumetric cell productivity: #TIMH! g/l/h

S	20	g/l	
x	1	g/l	
mu max	0.66	h-1	
ks	0.471	g/l	
Yx/s	0.2	g/g	
time base	0.002	Yp/s	1.8 mol/mol
D h-1	1	kp	0.714 g/L
		ms	0.057 g/g-1
		flow	1 l/h
		volume	1 l

1 COLUMN h	2 COLUMN S in the rea	3 COLUMN S leaving	4 COLUMN S entering	5 COLUMN x in the reactr	6 COLUMI x leaving t	7 COLUMI mu	8 COLUMI dx/dt	9 COLUMI ds/dt	10 COLUM P	11 COLUMN Product leaving the r
0	20	0.04	0.04	1	0.002	0.644815	0.00129	-0.00645	0	0
0.002	19.993552	0.0399871	0.04	1.001289629	0.002003	0.433202	0.00129	-0.00645	0.011607	2.32E-05
0.004	19.987117	0.03997423	0.04	1.002579258	0.002005	0.433199	0.000868	-0.00434	0.023167	4.63E-05
0.006	19.982805	0.03996561	0.04	1.003446779	0.002007	0.433197	0.000869	-0.00434	0.030905	6.18E-05
0.008	19.978496	0.03995699	0.04	1.004315412	0.002009	0.433195	0.000869	-0.00435	0.038645	7.73E-05
0.01	19.974192	0.03994838	0.04	1.005184792	0.00201	0.433193	0.00087	-0.00435	0.046377	9.28E-05
0.012	19.969893	0.03993979	0.04	1.006054922	0.002012	0.433192	0.000871	-0.00435	0.0541	0.000108
0.014	19.965599	0.0399312	0.04	1.006925801	0.002014	0.43319	0.000872	-0.00436	0.061814	0.000124
0.016	19.96131	0.03992262	0.04	1.007797429	0.002016	0.433188	0.000872	-0.00436	0.069519	0.000139
0.018	19.957025	0.03991405	0.04	1.008669809	0.002017	0.433186	0.000873	-0.00437	0.077216	0.000154
0.02	19.952745	0.03990549	0.04	1.00954294	0.002019	0.433184	0.000874	-0.00437	0.084904	0.00017
0.022	19.94847	0.03989694	0.04	1.010416823	0.002021	0.433182	0.000875	-0.00437	0.092583	0.000185
0.024	19.9442	0.0398884	0.04	1.011291459	0.002023	0.43318	0.000875	-0.00438	0.100254	0.000201
0.026	19.939935	0.03987987	0.04	1.012166848	0.002024	0.433178	0.000876	-0.00438	0.107917	0.000216
0.028	19.935674	0.03987135	0.04	1.013042991	0.002026	0.433177	0.000877	-0.00438	0.11557	0.000231
0.03	19.931419	0.03986284	0.04	1.013919889	0.002028	0.433175	0.000878	-0.00439	0.123216	0.000246
0.032	19.927167	0.03985433	0.04	1.014797542	0.00203	0.433173	0.000878	-0.00439	0.130852	0.000262
0.034	19.922921	0.03984584	0.04	1.015675951	0.002031	0.433171	0.000879	-0.0044	0.13848	0.000277
0.036	19.918679	0.03983736	0.04	1.016555117	0.002033	0.433169	0.00088	-0.0044	0.1461	0.000292
0.038	19.914442	0.03982888	0.04	1.01743504	0.002035	0.433167	0.000881	-0.0044	0.153711	0.000307
0.04	19.91021	0.03982042	0.04	1.018315721	0.002037	0.433166	0.000881	-0.00441	0.161314	0.000323
0.042	19.905982	0.03981196	0.04	1.01919716	0.002038	0.433164	0.000882	-0.00441	0.168909	0.000338
0.044	19.90176	0.03980352	0.04	1.020079359	0.00204	0.433162	0.000883	-0.00441	0.176495	0.000353
0.046	19.897541	0.03979508	0.04	1.020962317	0.002042	0.43316	0.000884	-0.00442	0.184073	0.000368
0.048	19.893328	0.03978666	0.04	1.021846036	0.002044	0.433158	0.000884	-0.00442	0.191642	0.000383
0.05	19.889118	0.03977824	0.04	1.022730516	0.002045	0.433156	0.000885	-0.00443	0.199203	0.000398
0.052	19.884914	0.03976983	0.04	1.023615758	0.002047	0.433154	0.000886	-0.00443	0.206756	0.000414



dx/dt=mu*x

mu= (mumaxS/(Ks+S))/(P/(Kp+P))

ds/dt =(-1/Yx/s) dx/dt

dp/dt=-Ys/p*ds/dt

Appendix 10

Nomeclature

Numerical Parameters

td : doubling time , h

μ : growth rate, h^{-1}

D : dilution rate, h^{-1}

Kp: a saturation constant of end product inhibition, g/l

Ks: a constant coefficient of substrate saturation, g/l

ms : maintenance coefficient , g/g/h

X : biomass , g/l

F : flux ,L/h

Yx/s : cellular yield coefficient ,g/g

Chemical Compounds

Mg^{2+} : Magnesium

H : Hydrogen

N^{2+} : Nitrogen

O_2 : Oxygen

CO_2 : Carbon dioxide

P : Potassium dioxide

Ca^{2+} : Calcium

ATP : Adenosine 5'-triphosphate

NAD : Nicotinamide adenine dinucleotide

NaCl : Sodium Chloride

KOH : Potassium Hydroxide

HCl : Hydrogen chloride

Appendix 11