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Bio-ethanol production from dairy industry wastes: Feasibility, Optimization and Modeling

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Preface

The present PhD project was born in Italy while I was having a stage in a dairy industry (*Agroalimentare Asso.La.C, Italy*) during my MSc thesis. The main objective of the stage was a rationalization of the dairy production processes aimed at identifying the main critical points.

One of the conclusions of the study was that one of the most expensive item in the industrial budget was due to the disposal of the dairy industrial wastes, which are mainly represented by the byproduct of cheese-production process, the so named *cheese whey*.

Therefore, with the aim of finding an alternative and sustainable solution to the problem (…and having a salary…), I applied for a PhD fellowship at the University of Calabria (*Rende, Italy*); *fate* wanted me to win the fellowship and therefore the games started.

The PhD started at the *Laboratory of Biotechnologies and Transport Phenomena* at the Department of Chemical Engineering (University of Calabria). Here, most of the experimental work was carried out and both process feasibility assessment and optimization were achieved. This part of the PhD was supervised by prof. Gabriele Iorio, prof. Vincenza Calabrò and engineer Stefano Curcio, thus, the first acknowledgement is addressed to them to have been valuable collaborators and supporters. A particular acknowledgement is to be addressed to both Francesco Caracciolo and Emanuele Ricca (*Hammanuè*) for the endless discussions which have both enlarged and increased my interest in Research.

The second part of the PhD, has been spent at the Technical University of Denmark (DTU) where, first, process modeling was performed at the Computer Aided Process Engineering Center (Department of Chemical and Biochemical engineering) in collaboration with assistant prof. Gurkan Sin and emeritus prof. John Villadsen whom invaluable suggestions made feasible this work. An acknowledgement goes to prof Rafiqul Gani who gave me the possibility to work in a so challenging environment and,

eventually, to associate professor Krist Gernaey for his support with *Matlab* programming.

Continuous-mode experimentation, instead, was carried out at the Department of System Biology (DTU) in collaboration with associate prof. Timothy Hobley who deserves my acknowledgement for his endless supply.

General introduction

The main byproduct of dairy industry is known with the general term *cheese whey*. Such a byproduct is produced in large amount in all over the world. In some countries cheese whey is further processed in order to obtain the so named *ricotta cheese* and, still, a large amount of cheese whey (now with different characteristics and called *ricotta cheese whey*) results from this stage as byproduct.

Cheese whey is to be considered an industrial waste. Although it contains valuable compounds (mainly lactose and proteins), its low concentration of milk constituents (6-7% of dry matter) makes it a waste, with high values of BOD and COD, that represents aserious environmental problem due to the need of its sustainable disposal.

Although several processes for cheese whey disposal have been developed, still, only about 50% of the worldwide cheese whey undergoes some treatment. Besides, much confusion reigns about the definition of the several kinds of cheese whey and it is very hard sometimes to even understand which substrate is actually treated in the process under consideration.

Cheese whey contains a relatively high amount of lactose (5%) which also represents the main part of the organic load of such a waste. This lactose content suggests the possibility to convert it into ethanol through anaerobic fermentation by a proper microorganism.

A process aimed at converting whey lactose (from now on the terms *whey lactose fermentation* and *cheese whey fermentation* will be used indifferently) into ethanol would imply several important benefits, first of all, a process like this would match two important requisites simultaneously, namely the disposal of the waste (cheese whey) and the production of the value-added product (ethanol). Moreover, cheese whey would represent a non-vegetable source for bio-ethanol production, which means that none of the problems related to foodstuffs-competitiveness and soil overexploitation would be involved.

The present study was mainly focused on the possibility to ferment ricotta cheese whey. This particular waste had never been given the due consideration and, although its production is now spread all over the world; it is often confused as just another kind of cheese whey but, rather, it should be considered a by-product of the process in which *sweet cheese whey* (term to indicate cheese whey that has not undergone any pretreatment) undergoes a particular process in order to get an additional product, i.e. *ricotta cheese* (such a matter will be exhaustively discussed in Chapter I).

In particular, this work assessed the possibility to process ricotta cheese whey in order to both dispose and valorize it by converting most of its organic content into ethanol.

Motivations and Objectives

The motivations of this PhD project are resumed as follows:

Only 50% of the cheese whey worldwide produced is processed, thus an alternative and sustainable solution is needed. The process cheese whey-to-ethanol in general has found rather few industrial applications which demonstrates that further efforts should be done to make this process attractive.

Data regarding the process ricotta cheese whey-to-ethanol are not available in the literature although in several Countries, such as Italy, the main byproduct of the dairy industry is actually ricotta cheese whey rather than other similar wastes. Furthermore, fermentation reaction lactose-to-ethanol is still not completely clear and a proper modeling could give a better insight of the mechanisms behind the global fermentation reaction.

By considering that the total amount of cheese whey produced worldwide is around 160 $Mt*year^{-1}$ it is possible to work out some calculation. If only the not-processed whey is considered, about 80 $Mt*year^{-1}$ are available, which means 4 $Mt*year^{-1}$ lactose. By considering 85% ethanol yield, 1.83 $Mt*year^{-1}$ bio-ethanol could be produced that would represent 2.3 Mm^3 bio-ethanol, namely the 3.5% of the total amount of ethanol produced worldwide in 2008.Now, it should be clear that this process is to be considered quite appealing.

The general objective of this thesis was to study ricotta cheese whey as an alternative non vegetable source for bio-ethanol production. This objective was achieved through the following steps:

1) Feasibility assessment through a preliminary experimentation in batch.

2) Data-driven modeling: Design of experiments and Optimization.

3) Knowledge-driven modeling: a Biochemically Structured Approach.

4) Continuous-mode experimentation and knowledge-driven modeling.

Structure of the thesis

The thesis is constituted by 5 chapters, which are structured as follows.

Chapter I basically contains the necessary background in order to understand the general characteristics and properties of the fermentation substrate that is considered in the present work, namely ricotta cheese whey. In this chapter an overview of the several kinds of cheese whey is given and most of the processes aimed at their disposal of and valorization are reviewed. This chapter should give a clear idea of the actual situation concerning the biotechnological potential of the dairy industry wastes.

Chapter II regards the feasibility assessment of ricotta cheese whey as substrate for bio-conversion into ethanol. In this chapter a comparison among the most common kinds of cheese whey is shown from the point of view of the fermentation performance. First, the actual growth of the microorganism in this particular substrate is assessed, then, a fermentation reaction is performed on three different substrates, namely ricotta cheese whey, sweet cheese whey and cheese whey permeate and the results are discussed in order to evaluate ricotta cheese whey potential for ethanol production.

Chapter III consists of a data-driven statistical-modeling study aimed at evaluating the actual effects of the operating conditions on the process and optimizing the fermentation reaction. A proper Design of Experiments (DOE) is planned and the experiments carried out, thus, the concerning results are analyzed and correlated by means of a statistical model which is capable to give the *best set* of operating conditions to carry out the fermentation reaction and, even more important, to predict fermentation performances at different operating conditions.

Chapter IV is still about modeling but, this time a knowledge-driven approach is adopted. Although a mathematical model was introduced in Chapter III, a more appropriate physically meaningful model was needed in order to have a better insight of the fermentation reaction. Therefore, in Chapter IV, a biochemically structured model is implemented and developed, then, used to represent three different batch sets of data.

Chapter V, eventually, concerns the assessment of the possibility to run the fermentation process in continuous configuration (chemostat mode). Five different dilution rates are tested on a lactose-based media and the experimental results are modeled in order to evaluate the true product yields. Material balances are applied to the system to verify the consistency of the experimental results.

All the chapters of this thesis, but the first one that represents a sort of introduction, either are or are going to be published on international scientific ISI-certified journals. Therefore, all chapters were written so as to make the reader able to understand them independently. Such a choice unavoidably pays some repetitions along the text, for instance the sections *Materials and Methods* that have common parts in the several chapters. On the other hand this structure facilitates a systematic reading approach and, for this reason, it was preferred to the classical monograph.

Chapter I

Cheese whey and its biotechnological potential

1.1 Introduction

Cheese whey is the main by-product of the dairy industry; it is constituted by the watery portion which remains during cheese-making process after cheese removal. Such a byproduct results in large amount, indeed, about 9-11 liters cheese whey are produced per kilogram cheese, thus representing 85-95% of the milk volume.

Cheese whey retains 55% of the milk nutrients where the most abundant are lactose (4.5-5%), soluble proteins (0.6-0.8%), lipids (0.4-0.5%), and mineral salts (8-10% of dried extract). Cheese whey salts include NaCl and KCl, calcium salts and others ,but the exact composition strongly depends on the particular cheese-making process that originated cheese whey. It also contains appreciable amounts of other components, such as lactic and citric acids, non-protein nitrogen compounds (urea and uric acid), B-group vitamins (Siso, 1996).

Cheese whey retains only 6-7% of the milk dry matter, thus it has always been considered a waste. An explanatory example is given by considering that a dairy farm processing 100 t of milk per day produces approximately in its effluent the same amount of organic products as would a town with 55000 residents (Sienkiewicz and Riedel, 1990). Cheese whey is indeed characterized by relatively high values of both BOD and COD, 30-50 g L^{-1} and 60-80 g L^{-1}, respectively, with lactose as the main responsible of such an organic load. Despite of its “poverty”, cheese whey still contains valuable components that suggest several processes aimed at its valorization.

1.2 Sweet cheese whey, cheese whey permeate and ricotta cheese whey

The term *cheese whey*(often *cheese* will be omitted in the following) has been used so far to indicate the general category represented by the watery byproduct that results from whatsoever cheese-making process. Actually, the situation is more complicated and a rational and systematic classification of the several kinds of cheese whey is rather difficult to carry out. However, two main categories can be generally recognized, namely acid (pH < 5) and sweet cheese whey (pH 6-7). Acid whey typically has higher ash and lower protein content than sweet whey and their use for alimentation is quite limited due to their acidic flavor and high saline content (Weetal et al., 1974, Kosikowski, 1979, Mawson, 1994).

Besides, another common byproduct, which actually comes from the satellite dairy industry, is *cheese whey permeate*. Such a byproduct (still a waste) comes from the ultrafiltration process practiced on sweet cheese whey and aimed at separating whey-proteins which are characterized by a very high biological value and a large potential in foodstuffs-market.

Ricotta cheese whey is the actual substrate used in most of the current work, therefore some clarification about it is due. Ricotta cheese whey owns to the category of *acid whey*, but, since this category includes all kinds of cheese whey that come from soft-cheese (cream and cottage cheese) productions, which often result in whey of different composition and characteristics, the specific name *ricotta cheese whey* will be kept along the text.

Ricotta cheese whey must be considered, rather than a classical kind of cheese whey, a byproduct of a further process which uses sweet cheese whey and other ingredients in order to produce a soft-cheese called *ricotta cheese*. Figure 1.1 depicts a simplified scheme of the entire process from milk to ricotta cheese.

Figure 1.1. Scheme of *ricotta cheese* production process.

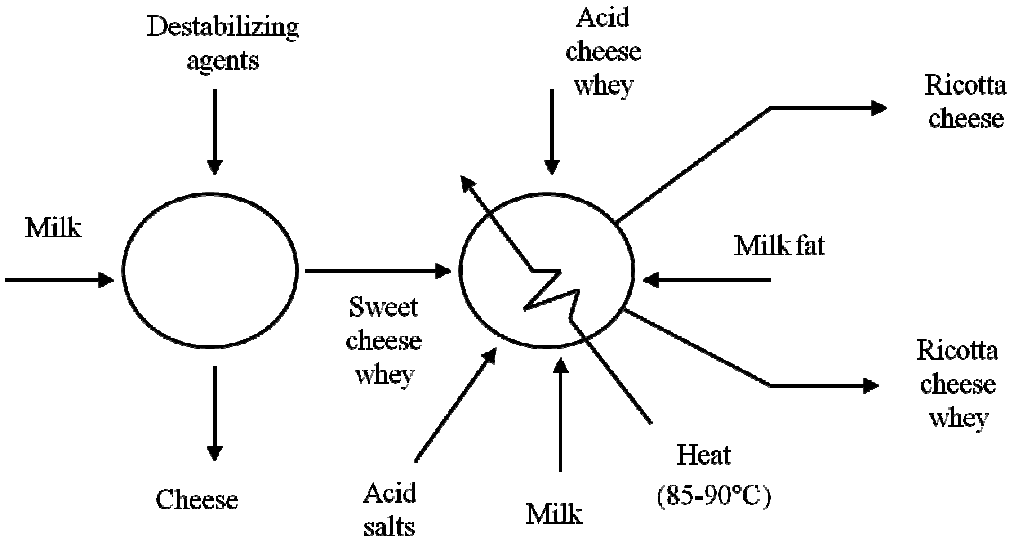


Figure 1.1 shows the following steps. First, milk is added of some agents that cause casein destabilization with consequent formation of cheese, then, after cheese removal, the watery part constitutes the resulting byproduct, i.e. sweet cheese whey. This byproduct is sent to another unit where it is mixed with acid whey, fresh milk (up to 10%), milk-fat (cream) and an acid solution of mixed salts. The so obtained mixture is kept at high temperature (85–90°C) for about 25 min to promote the precipitation of most of the whey proteins in order to get the cottage cheese known as *ricotta cheese*. Finally, the remaining watery portion is actually the so-called *ricotta cheese whey*.

Sweet cheese whey and ricotta cheese whey are characterized by different compositions and, most likely, different behaviors toward a certain process aimed at their valorization.

The average composition of sweet cheese whey (SCW), ricotta cheese whey (RCW) and cheese whey permeate (CWP), with respect to the main compounds, are reported in Table1.1.

Table 1.1. Average composition with respect to the main compounds in sweet cheese whey (SCW), ricotta cheese whey (RCW) and cheese whey permeate (CWP).

Substrate	Proteins [%]	Lactose [%]	Salts [%]	Organic acids [%]
SCW	0.15 – 0.22	4.8 – 5.0	1.0 – 1.3	0.20 – 0.25
CW	0.6 – 0.8	4.8 – 5.0	0.5 – 0.8	0.12 – 0.18
CWP	0.08 – 0.12	4.6 – 4.8	0.4 – 0.6	0.12 – 0.18

There are significant differences between the three substrates considered. The main difference is in the protein content: ricotta cheese whey has a particularly low protein content, as well as cheese whey permeate, if compared to sweet cheese whey; it is due to both the strong thermal treatment and the addition of acid salts that aided, together, protein flocculation. As far as cheese whey permeate concerns, proteins have been removed and what remains is basically lactose and salts. Such a difference in protein content will surely have a certain effect on a potential process aimed at valorize any deproteinized whey. Besides, ricotta cheese whey presents almost twice concentrations of salts and organic acids which, most likely, will affect fermentation performances.

Moreover, in addition to the chemical differences just highlighted above, physical differences are expected as well. The prolonged thermal treatment, for instance, that ricotta cheese whey went through can modify the structure of the few remaining proteins, thus changing their solubility. This is one of the reason, together with the particular acidity, because ricotta cheese whey is unsuitable for most of the food applications of sweet cheese whey and cheese whey permeate.

Eventually, it should be remarked that the intrinsic nature of ricotta cheese whey precludes several applications usually adopted to dispose of, totally or partially, sweet cheese whey. A fractionation procedure of the several compounds in sweet cheese whey, for instance, is one of the most common processes aimed at the disposal of or

valorization of this waste. The economics of this process is almost entirely sustained by the first step that consists of protein separation aimed at the food-integrators market. As a matter of fact, this step would not be feasible for ricotta cheese whey since the protein content is so small that it makes this step economically unsustainable.

An estimate of the total amount of whey (including all the typologies) produced globally is around 160 Mt*year^{-1} (OECD-FAO, 2008), therefore, an enormous quantity of by product that is to be disposed of. Currently, only about 50% of the cheese whey worldwide produced is processed into various food products (Becerra et al., 2001). With regard to the European Community, 45% is used directly in liquid form, 30% in the form of powdered cheese whey, 15% as lactose and delactosed by-products, and the rest as cheese whey-protein concentrates (Marwaha and Kennedy, 1988).

It is clear that an effective solution to the problem has become urgent, therefore, during the last thirty years several processes aimed at whey disposal and/or valorization have been developed. A brief review of these processes is given in the next section (1.3).

1.3 Cheese whey disposal and/or valorization processes

Cheese whey can be processed whole or may be first fractionated into rich-streams in compounds such as lactose or proteins. The main compounds in cheese whey and the relative amounts are reported in Table1.2.

Table 1.2. Mean composition of cheese whey.

Compound	[g L^{-1}]
Proteins	6-8
Peptides	0.83
Lipids	0.41
Lactose	50
Lactic acid	0.08
Citric acid	1.66
Monovalent cationic salts	1.66
Polyvalent cationic salts	1.01
Monovalent ammonium salts	0.92
Polyvalent ammonium salts	0.99

The choice of the process to be run strongly depends on the plant scale that produces whey (Modler, 1987). The most common processes aimed at disposing and/or valorizing cheese whey are reviewed in the following.

1.3.1 Direct utilization of cheese whey

Cheese whey can directly be used by adding it to drinking waters for farm animals, even though, excessive lactose and mineral proportions can limit whey consumption for this purpose (Sienkiewicz and Riedel, 1990). Another direct use of cheese whey is as agricultural fertilizer with the inconvenient of saline deposits formation that, after some years, affect land productivity. One of the most limiting factor in the direct use of cheese whey is represented by the very high cost for liquid whey transportation (Kosikowski, 1979).

1.3.2 Production of cheese whey powders (PCW)

One of the main problems in cheese whey valorization is that it tends to undergo microbial degradation. In order to preserve its quality and characteristics, it may be dried

and kept for a longer period of time (thus making easier transport operations).Among the most common PCW's are sweet-whey powders, demineralized whey powders, delactosed whey powders and deproteinized whey powders (Kosikowski, 1979; Yves, 1979; Anon, 1983; 1990). These powders are mainly used as dietary integrators for animal uses, although a small amount is also aimed at food for human utilize. Since often cheese whey conditions are not appropriate for human-food products (high mineral concentration and/or small protein/sugar ratio), several processes have been developed to adjust its condition (Coton, 1976; Coughlin and Charles, 1980; Anon, 1983; Marwaha and Kennedy, 1988).

1.3.3 Production of single-cell-proteins (SCP)

Production of biomass from cheese whey is perhaps the earliest application of this waste. Several processes have been developed for this purpose, such as *Vienna process* and *Bel process*(Moulin and Galzy, 1984). The first industrial application of biomass production from cheese whey is as earliest as 1958; the process is aimed at adding value to food-products by incorporating SCP. Cheese-whey permeate, for instance, is used to grow three yeast species in equilibrium*(K. lactis, K. fragilis, T. bovina).* Whole cheese whey is not used for this purpose because these microorganisms cannot metabolize its proteins; moreover, proteins promote yeast flocculation, which inhibits the fermentation process (Siso, 1996).

Yeasts are usually grown in continuous cultures over a period of more than 1 year at pH 3.5 and 38°C temperature so as to reduce the risk of contamination (Castillo, 1990). Cheese whey permeate is pasteurized at 80°C and high oxygen-transfer rates must be ensured to avoid the anerobic pathway toward ethanol formation (Mawson, 1994).Dried biomass yield on substrate is 50%. It contains about 50% proteins, essential amino acids, lysine and B-group vitamins (Yves, 1979).Such a biomass is mainly used as animal dietary supplement but also in human-foods (Olsen and Allerman, 1991).

Several microorganisms mutants have been produced to improve the nutritional characteristics of the biomass (Meyrath and Bayer, 1979; Pellòn and Hernàndez, 1986; Kitamoto and Nakahara, 1994; Moulin and Galzy, 1984) and several mixed cultures have been proposed as well (Carlotti et al., 1991a, 1991b; Kallel-Mhiri et al.,1994).

1.3.4 Production of whey protein concentrates (WPC)

Whey proteins are constituted by β-lactoglobulin (50%), α-lactalbumin (12%), immunoglobulins (10%), serum albumin (5%) and proteose peptones (0.23%).

The PER (protein efficiency ratio) value of whey proteins is high (3.4) compared to standard casein (2.8), and the proteins have a higher proportion of essential amino acids than casein (Evans and Gordon, 1980). Their biological value exceeds even that of whole egg protein (Siso, 1996).

Whey-protein-concentrates are usually obtained by means of tangential membrane filtration processes so as to reach a protein content of 30-60%. This process is the most common for cheese whey valorization and, almost every times, the first step of all other processes aimed at its valorization. The adoption of membrane filtration processes is generally convenient if compared with other separation techniques because of the particular proteins sensitivity toward thermal processes (Coton, 1976; Kosikowski, 1979; Evans and Gordon, 1980; Gardner, 1989).

Whey proteins are also used for other purposes than the direct addition to supplement foods. Protein-hydrolisates productions, for instance, produced by enzymatic processes have been considered in the literature (Moulin & Galzy, 1984; Gonzàlez-Tello et al., 1994a, 1994b; Margot et al.,1994). Such hydrolisates are constituted by peptide-mixtures with very interesting characteristics (Moulin and Galzy, 1984; Perea et al.,1993).

Whey protein concentrates can also be used indirectly in transformed food products (Kosaric and Asher, 1985; Kinsella and Whitehead, 1989; Mort and Foegeding, 1990) and in the production of iron proteinate, an antianemic preparation (Dalev, 1994).

On the other hand protein recovery does not solve the problem represented by the needed disposal of cheese whey, indeed, about70% of the total solids in cheese whey is represented by lactose.

Eventually, a process aimed at protein separation is not feasible if applied to ricotta cheese whey, indeed, the protein content is too low to make the protein recovery process economically sustainable.

1.3.5 Production of lactose and its derivatives

Lactose is the main compound in cheese whey (about 5%). It is usually obtained from cheese whey or cheese whey permeate (after ultrafiltration) by crystallization.

The main applications of lactose are, again, in food-products (as supplement for baby-foods) and in pharmaceuticals (as excipient). Lactose is characterized by high formability that makes it very useful in the sweets-market for cakes preparations and other similar applications. Nevertheless, the amount of lactose used for the above mentioned purposes is very small if compared with the available quantity from cheese whey (Coton, 1980), therefore, several processes aimed at its transformation in other value-added products have been developed (Shukla, 1975; Friend and Shahani, 1979; Hobman, 1984; Gekas and Lopez-Leiva, 1985; Champagne and Goulet, 1988; Jeong et al.,1991; Tin and Mawson, 1993; Nolan et al., 1994). All these processes are based on fermentation reactions of either lactose, directly, or glucose and galactose (the two monomers that constitute lactose).

Among the most common products there is galactose, which is obtained by lactose hydrolysis and selective glucose uptake by yeasts (Galzy and Moulin, 1976; Moulin et

al.,1977; Moulin and Galzy, 1984) to replace sorbitol (more expensive) in many products (Kosaric and Asher, 1985). Another product is lactosylurea that represents a non-protein nitrogen source to be used in animal food in order to avoid high levels of ammonia (Moulin and Galzy, 1984; Sienkiewicz and Riedel, 1990). Lactitol (4-β-galactopyranosyl-o-sorbitol) production is another common process used to obtain a product with a higher sweetening power or to produce lactitol-palmitate to be used in human nutrition. Eventually, lactose can be isomerized to lactulose (4-O-β-D-galactopyranosyl- o-fructose) that has a higher value due to its several possible uses in pharmaceutical industry (Dendene et al., 1994; Kozempel and Kurantz, 1994a, 1994b).

In general, lactose hydrolisates are more frequently used than lactose solutions since they have a stronger sweetening power and do not cause the common problems due to the difficult lactose digestibility. Moreover, a huge number of microorganisms are able to metabolize glucose and galactose, while direct uptake of lactose requires particular species. Therefore the hydrolysis significantly increases the number of bioproducts that can be obtained from cheese whey (Van Huyn and Decleire, 1982).For this reason lactose hydrolysis has become an important process for which two main procedures have been developed, namely acid and enzymatic hydrolysis (Kosaric & Asher, 1985). Enzymatic hydrolysis is the preferred one. It is performed with the enzyme β-galactosidase that is usually isolated from few species of yeast and microfungi (*K. lactis,K. fragilis, A.niger*and *A. oryzae*) (Coughlin and Charles, 1980; Gekas and Lopez-Leiva, 1985; Machado and Linardi, 1990).

Enzyme produced by Microfungi is excreted outside the cell so making the enzyme recovery quite easy; the problem is that microfungal enzyme production is aided by acid pH thus limiting its application to acid whey only. For lactose in sweet cheese whey, instead, intracellular yeast hydrolysisis used, even though, in this case, the recovery of the enzyme β-galactosidase is very expensive because of the needed extraction of this enzyme from the cells (Shukla, 1975; Coughlin and Charles, 1980; Fenton, 1982; Greenberg and Mahoney, 1982; Moulin and Galzy, 1984; Gekas and Lopez-Leiva, 1985; Gonzalez and Monsan, 1991; Stredansky et al., 1993). In order to avoid this problem,

direct utilization of the whole cell has been proposed (Van Huyn and Decleire, 1982; Van Huyn and Decleire, 1985).

Lactose hydrolysis in cheese whey is almost always performed in heterogeneous-phase processes, by adopting particular procedures in order to immobilize the enzyme on specific supports. (Coughlin and Charles, 1980; Trevan, 1980; Baret, 1982; Gekas and Lopez-Leiva, 1985; Piesecki et al., 1993; Siso et al., 1994).Very interesting is the use of β-galactosidase in two-phase aqueous systems that avoids the loss of activity during the immobilization step (Chen and Wang, 1991).

Much attention during lactose hydrolysis must be paid to the possibility of polymerization of galactose or lactose with formation of oligosaccharides that remarkably lower the hydrolysis yields (Guy and Bingham, 1978; Gekas and Lopez-Leiva, 1985).

As far as lactose-permease system of the microorganism is concerned, lactose transportation through the cell membrane is the limiting step (Dickson and Barr, 1983; Joshi et al.,1987, 1989). Several procedures in order to improve membrane permeability have been developed (Decleire et al., 1986; 1987; Gowda et al., 1991; Siso et al., 1992; Siso and Suarez Doval, 1994).

1.3.6 Biogas

A gas mixture mainly constituted by methane, can be produced by anaerobic digestion of cheese whey (actually always fermented together with other substrates such as cattle manure).Several kinds of anaerobic digesters are in literature (Mendez et al.,1989; Yan et al., 1989; Mawson, 1994) and practically all the existing plants aimed at biogas production from biomasses (mainly cattle manure) can be fed with mixtures of cheese whey and other biomasses. With the only use of cheese whey as substrate, loading rates of30 kg COD/m^3 per day have been successfully treated with COD removal efficiencies

up to95% (Kemp and Quickenden, 1989). However, downstreams of this process are generally not suitable for pouring into water streams.

1.3.7 Other compounds

Maybe one of the most studied processes regarding cheese whey, other than the others mentioned above, is glycerol production. It has been proposed as an alternative process to the organic synthesis (Rapin et al., 1994).

Calcium magnesium acetate can be also produced by anaerobic fermentation as deicer (Yang et al., 1992).Other products that are worth to be mentioned are compounds such as organic acids (acetic, propionic, lactic, lactobionic, citric, gluconic and itaconic), vitamins (BI2 and B2), amino-acids (glutamic, lysine, threonine) (Hobman, 1984; Blanc and Goma, 1989; Nielsen et al.,1990; Fairbrother et al.,1991; Chiarini et al., 1992; Colomban et al.,1993; Fournier et al.,1993; Norton et al.,1994).

Last, but not least, ethanol production from lactose is to be considered; since this process is the object of the present thesis, it is treated separately in the next section (1.4).

1.4 Ethanol production from cheese whey

1.4.1 Introduction

Ethanol production from biomasses of every kind has received much attention in recent years and several processes aimed at its production have been developed and optimized. On the other hand, nowadays, nearly all bio-ethanol is obtained by fermentation of vegetable biomasses, essentially sugar cane and cereals, thus contributing to the observed increase of foodstuffs price and causing all the discussions about soil overexploitation and use of fertilizers.

It is, then, necessary to identify alternative renewable and non-vegetable sources for bio-ethanol production. Therefore, biomasses that come from process waste streams may be particularly attractive for bio-ethanol production, for example cheese whey.

Cheese whey contains a high amount of lactose (5%) which also represents the main part of the organic load of cheese whey. Such a lactose content suggests the possibility to convert it into ethanol through an anaerobic fermentation by means of a proper microorganism thus achieving a double target, cheese whey disposal and value-added product production.

Cheese whey fermentation into ethanol has been considered for a long time, since at least 1940s to our knowledge (Whittier, 1944; Rogosa et al, 1947; Webb and Whittier, 1948). The global reaction, often reported in literature, is as follows (Reaction 1.1).

$$C_{12}H_{22}O_{11} + H_2O \xrightarrow{yeast} 4CH_3CH_2OH + 4CO_2 \qquad (1.1)$$

In words, one molecule of lactose gives four molecules of ethanol which, on a mass basis means that 0.538 g ethanol are theoretically produced per each gram of lactose consumed. Actually, as it will be discussed later on, the reaction framework is more complicated, but, for the moment, that is enough to represent the bio-conversion reaction. The process is an alcoholic fermentation thus is to be carried out in anaerobic conditions at appropriate operating conditions of pH, agitation rate and so forth.

1.4.2 Cheese whey-to-ethanol: state of the art

During the last years plenty of studies concerning lactose (whey lactose, actually) fermentation have been published. The many parameters of the process(bio-reactor configuration, initial lactose concentration, microorganism type etc.) make very difficult

to present a systematic review of the several studies in the literature. A first classification can be done with regard to the particular microorganism exploited to achieve the fermentation process.

Although lactose is easily metabolized in aerobic conditions by many microorganisms, this is not true in anaerobic conditions. Mainly, four *categories* of microorganisms have been used to achieve lactose fermentation, namely *Kluyveromyces marxianus*, *Kluyveromyces fragilis*, *Candida pseudotropicalis* and *Saccharomyces cerevisiae*. At this point, before continuing with the discussion, some considerations must be done. Most of the works in the literature were presented with the above cited names for the microorganisms but, in a "recent" taxonomic study (Kurtzman and Fell, 1998) the first three categories were reported all as synonyms of *K. marxianus*, so, now *K. fragilis* is included in *K. marxianus* spcies and *C. pseudotropicalis* is recognized as the anamorph form of *K. marxianus*. However, to keep easy to understand the several comparisons, the original nomenclature used in the relative works will be used hereafter. *S. cerevisiae*, instead, has been mainly used for hydrolysates (glucose and galactose) since it is not suitable to directly ferment lactose. Nevertheless, the construction of lactose-consuming *S. cerevisiae* strains has been attempted by protoplast fusion, expression of heterologous β -galactosidases secreted to the extracellular medium or simultaneous expression of the permease and β-galactosidase of *K. lactis*.

Kluyveromyces species and *S. cerevisiae* differ mainly for glucose repression which inhibits galactose utilization. Indeed, not all *K. lactis* strains show glucose repression or, at least, the effect is less pronounced than in *S. cerevisiae*. It holds also for *K. marxianus* species, which are mostly adapted to environments containing lactose and galactose (Gancedo, 1998; Rubio-Texeira, 2005).

Candida pseudotropicalis (called also *C. Kefyr*) has shown high efficiency for lactose fermentation into ethanol and several studies have been performed aimed at characterizing the behavior of such a microorganism with regard to anaerobic fermentation performances, such as in extractive fermentation (Jones et al, 1993) and in

fermentation-pervaporation systems (Shabtai and Mandel, 1993). Probably, the most interesting studies on lactose-to-ethanol fermentation by *C. pseudotropicalis* have been performed by Ghaly and El-Taweel. The effect of microaeration was investigated in batch configuration (Ghaly and El-Taweel, 1995a),the effect of nutrients addition to cheese whey was assessed (Ghaly and El-Taweel, 1995b) as well as how the initial lactose concentration can affect the fermentation performance (Ghaly and El-Taweel, 1995b). Furthermore, a continuous-mode experimentation at different dilution rates was carried out (Ghaly and El-Taweel, 1997a) and the data used to develop a kinetic model (Ghaly and El-Taweel, 1997b).

Kluyveomyces species has been extensively studied for several biotechnological purposes.*K. lactis*, for instance was studied in comparison to *S. cerevisiae* as "non-conventional yeast" (Breunig et al., 2000). *K. lactis* is not commonly used for ethanol production, although it has been exploited for other biotechnological applications such as the production of heterologous proteins (van Ooyen et al., 2006) from cheese whey (Maullu et al., 1999). This yeast is capable to metabolise lactose due to the presence of a lactose permease (encoded by the LAC12 gene) and a β-galactosidase (LAC4 gene) (Rubio-Texeira, 2006). β-galactosidase hydrolyses lactose into glucose and galactose. Intracellular glucose can enter glycolysis while galactose follows the Leloir pathway (Breunig et al., 2000; Schaffrath and Breunig, 2000; Rubio-Texeira, 2005).

K. marxianus has recently received much attention regarding its biotechnological potential, motivated by some advantages that it has in comparison to *K. lactis* (Ribeiro et al., 2007). *K. marxianus* has been used in a wide range of biotechnological applications (Fonseca et al., 2008).

Most of the works concerning lactose/whey fermentation into ethanol involves the use of *Kluyveromyces* species such as *K. marxianus* and *K. fragilis* (Gawel and Kosikowski, 1978; Janssens et al., 1983; Vienne and von Stockar, 1985; Kamini and Gunasekaran, 1987; Grubb and Mawson, 1993; Dale et al., 1994; Zafar et al., 2005; Silveira et al., 2005; Ozmihci and Kargi, 2007c, d, e).

The common thing in all these works is the intolerance of the microorganism to both high lactose concentrations(> 75g L^{-1}) and high ethanol concentration (> 25 g L^{-1}). The mechanisms behind these phenomena is still not completely clear but it seems that both osmotic effects on the cells and low ethanol tolerance be the reasons. The problem of high lactose concentration inhibition can be circumvented by adopting a fed-batch configuration, while higher ethanol tolerance can be obtained with improved strains by means of the so-called *evolutionary engineering* (Wisselink et al., 2009).

Whey nutrients can play a key-role in lactose fermentation. Far better results, for instance, were obtained by supplying the media with ergosterol and Tween 80 (Janssens et al., 1983), indeed, in strictly anaerobic conditions, the added lipids were, most likely, incorporated in the cell membrane, which is in accordance with the importance of sterols and unsaturated fatty acids for yeast fermentative performance and ethanol tolerance(Casey and Ingledew, 1986; You et al., 2003; Aguilera et al., 2006; Guimarães et al., 2006;). The role of such lipids (ergosterol) is to suffice for the complete lack of oxygen (strictly anaerobic conditions) which should be present in a minimum amount for the biosynthesis of membrane lipids. Therefore, the oxygen is extremely important in lactose fermentation processes and its effect has been extensively studied (Castrillo and Ugalde, 1993; Castrillo et al., 1996; Breunig et al., 2000; Goffrini et al., 2002; Snoek and Steensma, 2006).

Eventually, perhaps the most important characteristic of *K. marxianus* strains is its capability to grow and ferment at elevated temperatures, which results in a reduction cost of the cooling operation on a large scale. Ethanol production from lactose has been obtained with a particular strain (IMB3) at 45 °C (Brady et al., 1994, 1995; Brady et al., 1997; Kourkoutas et al., 2002a).

S. cerevisiae is undoubtedly the most studied yeast ever. Among its main advantages, it should be remarked the capability to grow fast, high ethanol tolerance and good fermentative performance in general. Hundreds of studies have been carried out on this microorganism (Antoni et al., 2007; Cot et al., 2007). Besides the well known

physiology, one of the main reason because it is often preferred for industrial applications is that it may grow very well in anaerobic conditions which makes the industrial fermentation process much easier (Snoek and Steensma, 2007) and, moreover, its biomass can be used as animal feed so as avoiding further disposal processing downstream (Bai et al., 2008).

Although all these advantages and despite that it can take up galactose through Leloir pathway, *S. cerevisiae* is not able to metabolize lactose. For this reason most of the works concerning ethanol production from cheese whey by *S. cerevisiae* involve two steps, a first one where lactose is hydrolyzed and a second one where the mixture glucose-galactose is fermented. However, such a process does not seem to be convenient, the pre-hydrolysis is an expensive step that involves the use of β-galactosidase and, moreover, glucose repression effect must be taken into account. This effect can slow down the reaction and cause a diauxic growth where glucose is preferentially fermented before galactose (O'Leary et al., 1977; Mehaia and Cheryan, 1990; Gancedo, 1998). The problem of catabolite-repression was overcome with the production of resistant- mutants (Bailey et al., 1982; Terrell et al., 1984). The use of a co-immobilized biocatalyst (in calcium alginate)was proposed for whey fermentation as well (Hahn-Hägerdal, 1985; Roukas and Lazarides, 1991). Similar biocatalysts have been used in other works, for instance, in simultaneous hydrolysis-fermentation with permeabilized *K. marxianus* cells as the source of ß-galactosidase, so obtaining better results than the direct *K. marxianus* fermentation (Rosenberg, 1995).

Concluding this first section where the several studies have been reviewed with specific regard to the characteristics of the microorganism used to perform the fermentation, another important distinction can be done in function of the particular process configuration.

Most of the studies in the literature have been carried out in batch configuration (Gawel and Kosikowski, 1978; Janssens et al., 1983; Bothast et al., 1986; Grubb and Mawson, 1993; Castrillo et al., 1996; Kourkoutas et al., 2002a; Longhi et al., 2004; Silveira et al.,

2005; Zafar et al., 2005; Kargi and Ozmihci, 2006; Zafar and Owais, 2006; Ozmihci and Kargi, 2007a, e).

Several other studies have considered the continuous configuration (Linko et al., 1981; Cheryan and Mehaia, 1983; Janssens et al., 1984; Hahn-Hägerdal, 1985; Gianetto et al., 1986; Kleine et al., 1995; Ozmihci and Kargi, 2007b, c, 2008; Teixeira et al., 1990) and the fed-batch configuration as well (Ferrari et al., 1994; Grba et al., 2002; Ozmihci and Kargi, 2007d).

In order to keep a logical path through the thesis, the most remarkable points and results obtained in both batch and continuous studies cited above will be discussed and compared, when relevant, later on in the text.

1.4.3 Cheese whey to ethanol: industrial applications

Only a few companies, in Ireland, New Zealand and United States are currently operating a cheese whey-to-ethanol fermentation process.

Carbery Milk Products (*Cork, Ireland*) started its ethanol production in 1978 by adopting a batch fermentation process where, at present, about 11 thousand tons per year ethanol are produced from whey permeate. Eleven batch reactors are used, the biomass is re-circulated and a continuous distillation train is operated downstream the reactors. The fermentation lasts up to 20 hours. *Carbery*'s ethanol production was mainly for potable purposes (pharmaceuticals and food) but, since 2005 ethanol for both E85 and E95 fuels has been produced.

Anchor Ethanol (*Auckland, New Zealand*) is operating three batch plants with a total production of 17 million liters ethanol per year by processing cheese whey permeate. Eight different grades of ethanol are produced, from potable alcohol for beverages to pure alcohol for bio-fuels production. Whey permeate is concentrated up to 8% lactose

and a batch fermentation is run for about 24 h thus obtaining ethanol concentrations around 4%, then, the fermentation broth is processed at different degrees.

Golden Cheese (*Corona, California*) is disposing of its cheese whey via a protein recovery step and then a batch whey permeate fermentation to produce an alcoholic *beer* with a process similar to the *Carbery*'s one. Another plant has been built and operated in Wisconsin, USA, but no data have been found in the Literature.

Eventually, the *Dansk Gaerings* process was developed in Denmark in the 1970s to produce ethanol from cheese whey permeate by means of a continuous fermentation process but, to our knowledge, it has never materialized into an industrial process.

Chapter II.

Batch fermentation of ricotta cheese whey: feasibility

The aim of the present chapter is to investigate the feasibility of bio-ethanol production by batch fermentation of ricotta cheese whey. Such a substrate could represent an effective non-vegetable source for renewable energy production. The microrganism used to carry out the fermentation process was the yeast *Kluyveromyces marxianus*. Preliminary experiments, performed in aerobic conditions on different volumes of ricotta cheese whey, have shown the actual growth of the yeast. The fermentation experiments were carried out, in anaerobic conditions, on three different substrates: ricotta cheese whey, sweet cheese whey and deproteinized whey. The experimental data have demonstrated the process feasibility: ricotta cheese whey is an excellent substrate for fermentation and exhibits better performance with respect to both sweet cheese whey and deproteinized whey. Complete lactose consumption, indeed, was observed in the shortest time (13 h) and with the highest ethanol yield (89% of the theoretical value).

2.1 Introduction

Nowadays, nearly all bio-ethanol is obtained by fermentation of vegetable biomasses, essentially sugar cane and cereals; thus contributing to the observed increase of foodstuffs price. It is, therefore, necessary to identify alternative renewable and non-vegetable sources for bio-fuels production. Ricotta cheese whey could potentially fit this requirement and may potentially represent an interesting fermentation substrate owing to its main characteristics, namely the significant content of fermentable sugar and its low cost, as determined by the fact that – as a waste – it requires a proper (and costly) treatment, which prevents from serious environmental problems. The relatively high content of lactose does indeed suggest the possibility of bio-conversion into ethanol, according to the overall Reaction1 (reported in section 1.4.1).Such a stoichiometry predicts a theoretical yield equal to 0.538 g ethanol per gram lactose consumed.

In the scientific literature, only few papers dealt with ricotta cheese whey and its possible utilization; none of them, however, identified ricotta cheese whey as a potential source for bio-ethanol production. Several authors, instead, considered both sweet cheese whey and cheese whey permeate as potential substrates for ethanolic fermentation.

Gawel and Kosikowski (1978) assessed concentrated cheese whey permeate (24%) as batch fermentation substrate by means of *K. fragilis*; 3 L bottles were used in anaerobic conditions and final ethanol concentrations of 80 g L^{-1} were obtained with 0.2 g*$(L*h)^{-1}$ ethanol productivity.

Mahmoud and Kosikowski (1982) adopted the same microorganism and the same substrate with reduced ash and performed a batch fermentation in a 14 L bioreactor obtaining ethanol productivity of 0.6 g*$(L*h)^{-1}$.

Cheryan and Mehaia (1983) checked several reactor configurations with *K. fragilis* in enriched media(5% lactose); batch ethanol productivity of 3 g*$(L*h)^{-1}$was obtained.

Janssens et al. (1983) used deproteinized cheese whey powder added with 0.5% peptone and 15% lactoseobtaining2 g*$(L*h)^{-1}$ethanol productivity by using 1 L stirred flasks.

Gunasekaran and Kamini (1991), in batch and with *K. fragilis*, fermented a complete media (20% lactose) obtaining 0.74 $g*(L*h)^{-1}$ ethanol productivity but only 55 $g\ L^{-1}$ ethanol. The same authors immobilized the yeast in calcium alginate and performed the batch fermentation obtaining ethanol productivity of 0.88 $g*(L*h)^{-1}$ and higher ethanol concentrations (63 $g\ L^{-1}$). Eventually, the same authors, immobilized in calcium alginate a mix of *K. fragilis* and *Z. mobilis* and, in the same conditions obtained an ethanol productivity of 1.0 $g*(L*h)^{-1}$ and 72 $g\ L^{-1}$ ethanol concentration.

Ryu et al. (1991) fermented a semi-synthetic medium (20 % lactose) in a 20 L batch bio-reactor by using *K. fragilis*, and obtained 2.1 $g*(L*h)^{-1}$ethanol productivity with 72 $g\ L^{-1}$ ethanol concentration.

Rosenberg et al. (1995) obtained 0.52 $g*(L*h)^{-1}$ ethanol productivity by means of *K. marxianus* in a 5 L batch bioreactor; the substrate was deproteinized whey enriched with yeast extract and salts (6.5% lactose).

Ghaly and El-Taweel (1995a) adopted *C. pseudotropicalis* as microorganism and fermented lactose-added cheese whey (10-20%) in a 5 L batch bioreactor so obtaining 0.7-1.0 $g*(L*h)^{-1}$ ethanol productivity and 40-45 $g\ L^{-1}$ ethanol concentrations.

The same microorganism was used by Szczodrak et al. (1997) to ferment both a semi-synthetic medium (12% lactose) and deproteinized whey (10% lactose) obtaining 1.2 and 0.85 $g*(L*h)^{-1}$ ethanol productivity, respectively; the reactors were actually shake flasks.

Grba et al. (2002), by using *K. marxianus* fermented deproteinized whey added with yeast extract and salts (10% lactose) and obtained, in a 2 L batch bioreactor, an ethanol productivity of 3.1 $g*(L*h)^{-1}$.

Silveira et al. (2005) performed the fermentation of whey permeate solution (17% lactose), by *K. marxianus*, in a 1 L batch stirred flask in both hypoxic and anoxic conditions obtaining 1.0 and 1.5 $g*(L*h)^{-1}$ ethanol productivity, respectively.

Kargi and Ozmhici (2006) with the same microorganism, in shake flask, fermented cheese whey powder solutions (15% lactose) achieving 0.4 $g*(L*h)^{-1}$ ethanol productivity.

Eventually, a mix of *K marxianus* and *S. cerevisiae* was immobilized in calcium alginate and concentrated cheese whey solution (10% lactose) was fermented in anaerobic shake flask (Guo et al, 2010); ethanol productivity equal to 0.88 $g*(L*h)^{-1}$ was obtained.

As it results clear from this brief review, none of the works in the literature have been concerned about ricotta cheese whey.

The work presented in this chapter is intended to investigate the possibility of using ricotta cheese whey as a source for bio-ethanol production, evidencing the differences existing between ricotta cheese whey and other kinds of substrates, namely sweet cheese whey and deproteinized whey. Batch fermentation experiments were performed by *K. marxianus*, evaluating the time evolutions of lactose, ethanol and biomass concentrations, thus obtaining preliminary indications on the influence of the actual substrate on the system performance.

2.2 Materials and Methods

2.2.1 Controlled bio-reaction system

A controlled batch bio-reactor, consisting of a 1.5 liter autoclavable plexiglas cylinder (*Applikon, Holland*), was used to perform the present experimental study. The main operating parameters (pH, O_2 concentration, temperature, agitation and foam level) were monitored by a set of sensors and controlled by means of an *ADI 1030* Shelf-top controller.

2.2.2 Yeast Strain

Lactose bio-conversion experiments were performed by a yeast, i.e. *Kluyveromyces marxianus var. marxianus CBS 397*, isolated at the *Centraalbureau voor Schimmercultures* (*Utrecht, the Netherlands*). This yeast was selected for its particular performances toward lactose fermentation (see chapter I). The yeast, initially freeze dried, was revived suspending the microorganism by pouring it into a cylinder containing 1-2 mL of sterile water and, then, shaking and storing the suspension at 20°C for 12 h.

2.2.3 Maintenance culture

Kluyveromyces marxianus was maintained in a generic yeast medium having the following composition: agar 10 g L^{-1}, lactose 20 g L^{-1}, bactopeptone 10 g L^{-1}, yeast extract 5 g L^{-1}. The culture was sterilized in an autoclave at 121°C for 30 min, then it was poured on *Petri* dishes for solidification and, eventually, the yeast inoculum was spread on the surface and incubated at 20°C for 48 h. At growth completed, the dishes were kept at 4°C.

2.2.4 Inoculum medium

The inoculum medium was prepared with a single colony withdrawn from the *Petri* dishes and incubated in a *GRANT OLS 200* thermostated bath, maintained for 12 h at a temperature of 37°C with an orbital shaking velocity of 150 rpm. In all the experiments 100 mL of medium were poured in a 300 mL sterile flask. Each of the used materials, before performing this stage, was autoclaved at 121°C for 30 min. The inoculum medium was constituted by lactose, 50 g L^{-1}, bactopeptone, 10 g $L^{-1}$and yeast extract, 5 g L^{-1}.

2.2.5 Fermentation medium

Three kinds of fermentation medium were used, i.e. ricotta cheese whey, sweet cheese whey and deproteinized whey in order to assess the performance of *K. marxianus* with respect to bio-ethanol yield. All the tested raw materials came from the same lot of cow milk, originally designed to *mozzarella cheese* production; both ricotta cheese whey and sweet cheese whey considered in the present paper represented, respectively, by-product and raw material of the same production cycle aimed at ricotta cheese obtainment. The deproteinization of sweet cheese whey was performed by ultrafiltration (UF) through a cellulose membrane, *Nadir C005 Filtration*, having a nominal molecular weight cut-off of 5000 Da. The UF system was operated at a 2 bar trans-membrane pressure with a feed flow rate of 2 $L*min^{-1}$. It is worthwhile to remark that each of the comparisons hereafter presented was performed on samples not subjected to any other pre-treatment, but those normally carried out in the production plant. All the samples, kindly provided by a local dairy industry, *Agroalimentare Asso.La.C.*(*Italy*) were stored at4°C; each fermentation test, however, was performed within 6 h from the production time. The average compositions of ricotta cheese whey, sweet cheese whey and deproteinized whey are reported in table1 (see section 1.2).

2.2.6 Analytical methods

The samples were periodically withdrawn from either the flasks or the bio-reactor in aseptic conditions in order to determine, by HPLC, the time evolution of lactose and ethanol concentrations. A 0.1% (v/v) phosphoric acid solution was used as mobile phase at a flow rate of 0.5 $mL*min^{-1}$. A 50x4.6 mm *Supelcogel* pre-column, a 300x7.8 mm *SupelcogelC-610* column and a refractive index detector, *Jasco RI 930*, constituted the experimental equipment. Biomass was evaluated by *BactoScan FC* (*Foss Integrator, Denmark*) an instrument capable to determine, on the basis of an optical method, the number of cells contained per milliliter of solution. The amount of cells, on a mass basis,

was obtained multiplying the cells concentration by 303 ng per cell (Ghaly and El-Taweel, 1995a; b).

2.2.7 Experimental protocol

A set of preliminary aerobic tests was carried out in order to assay the actual growth of *K. marxianus* in ricotta cheese whey. The microbial growth experiments were performed withdrawing a single colony from a *K. marxianus* culture, contained in a *Petri* dish, and then inserting this colony in a flask containing a known volume of ricotta cheese whey. Four volumes of ricotta cheese whey were investigated, i.e. 50, 75, 100 and 150 mL. The volume range was chosen according to the consideration that the amount of fermentation starter should be in the order of 10% the fermentation medium which, on a typical laboratory scale, is in the range 0.5-1.5 L.

The flasks were placed in a *GRANT OLS 200* thermostated bath and maintained for 12 h at 37°C temperature with an orbital shaking velocity of 150 rpm. A 100 µL sample was collected, every hour, from the bulk and poured in 25 mL of a 2% sodium citrate solution and eventually analyzed to obtain the amount of biomass formed. The above-described steps were performed in aseptic conditions by instruments and tools previously autoclaved at 121°C for 25 min.

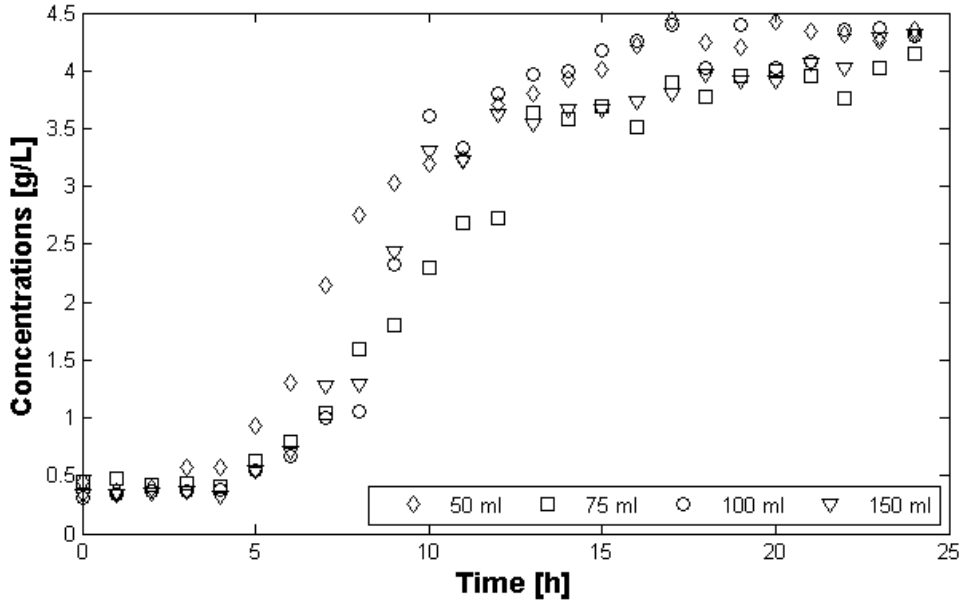
Anaerobic fermentation experiments lasted 18 h and were carried out starting1L fermentation medium with 100 mL inoculum. Each experiment was repeated twice to assess data reproducibility; the average concentrations of lactose, ethanol and biomass were reported versus time, together with an “error bar” indicating the maximum variation of each measured point from the corresponding calculated mean value. Operating conditions were as follows: temperature 37°C, stirrer velocity 200 rpm, pH 5, dissolved O_2-level ranging between 0 and 0.2%. The pH of the fermentation broth was controlled by means of a 6N sodium hydroxide solution. Two samples of fermentation broth were withdrawn, every hour, during the experiment: a 100 µL sample was destined to

microorganism quantification, as described for growth experiments, a 1 mL sample was, instead, centrifuged at 5000 rpm for 15 min, filtered through a 0.45 µm filter and finally sent to the HPLC for assaying the evolution of both lactose and ethanol concentration.

2.3 Results and Discussion

Figure 2.1 shows biomass concentrations versus time resulting from the experiments of biomass growth in ricotta cheese whey.

Figure 2.1. Time evolution of average biomass concentrations during aerobic fermentation of ricotta cheese whey (T = 37 °C, orbital shaking velocity=200 rpm).

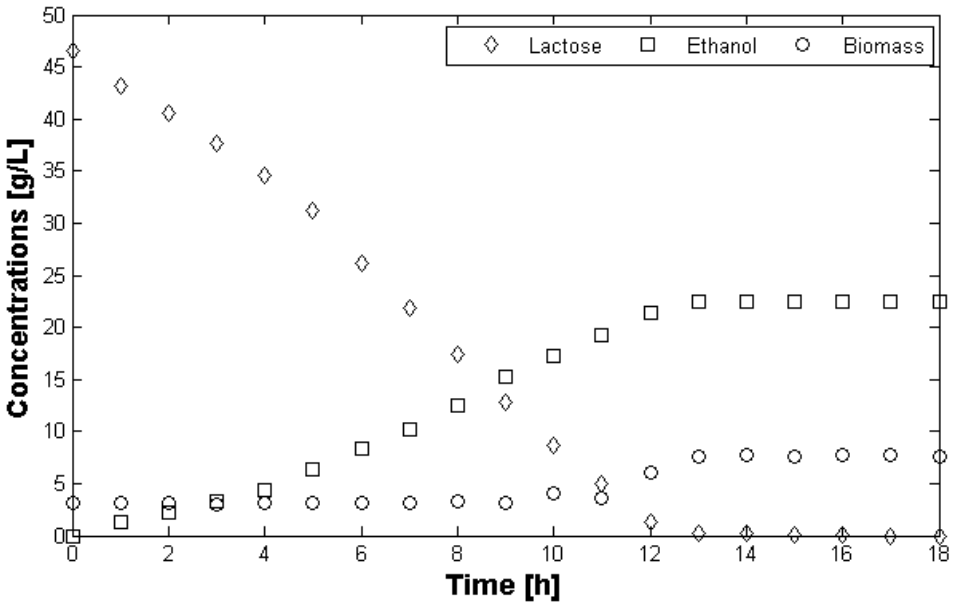


For each considered volume, the typical growth phases characterizing a batch cultivation are shown; it is worthwhile to observe that after a lag-phase of 3.5 h the linear growth phase takes over and after 17 h from the beginning of the experiment, the so-called stationary phase starts. These results are of crucial importance to demonstrate the actual

growth of *K. marxianus* in ricotta cheese whey and to prove that, in the considered range, volume does not significantly affect the system behavior. However the actual working environment of the microorganism, in order to produce ethanol, is anoxic (or hypoxic) therefore the behavior can change significantly, indeed, this experiment must be considered merely as a proof that ricotta cheese whey is not an hostile environment for *K marxianus.*

Figures 2.2-2.4 show the experimental results regarding the fermentation of ricotta cheese whey, sweet cheese whey and cheese whey permeate, respectively.

Figure 2.2. Anaerobic fermentation of ricotta cheese whey. Time evolution of lactose, ethanol and biomass concentrations (T = 37 °C, orbital shaking velocity=200 rpm, pH = 5, O2 = 0 – 0.2 %)



As far as ricotta cheese whey fermentation is concerned (Figure 2.2), lactose consumption goes to completion within 13 h only, i.e. much earlier than it was reported for sweet cheese whey fermentation for instance (Zafar and Owais, 2005). A remarkable result is the achieved ethanol concentration, 22.44g L^{-1}, corresponding to a final yield

equal to 89% of the theoretical one. Finally, it is worthwhile to observe the relatively low biomass growth, probably due to the low protein concentration.

Figure 2.3 depicts the results concerning sweet cheese whey fermentation. As compared to figure 2, a higher biomass concentration, related to the existence of an exponential phase starting after 2 h, is achieved; this phenomenon is to be ascribed to the characteristics of sweet cheese whey that, being richer in nutrients, allows an improved growth for the microorganism. The higher yeast growth, however, corresponds to a lower ethanol yield, which is equal to about 83% of the theoretical one in the final stage of the experiment. It can be also observed that complete lactose consumption is attained only after 18 h, namely 5 h later than what it was measured, in the same conditions, with ricotta cheese whey; finally, ethanol can be detected after 5 h, thus suggesting that process dynamics is delayed of about 4–5 h. This behavior can be ascribed to several phenomena occurring in the reaction medium; at the beginning, the microorganism follows the respiratory cycle rather than the anaerobic fermentation, at least until oxygen concentration becomes a limiting factor (during the initial stage of reaction, since no inert gas has been introduced in the reactor, oxygen concentration is equal to the equilibrium concentration detectable before yeast addition). When, at this point, 15 g L^{-1} biomass are formed, the ethanol yield is unavoidably reduced, since a certain amount of lactose had been consumed to allow the respiratory cycle. It remains to be explained why this tendency, for sweet cheese whey, to exploit the small amount of oxygen in the bulk instead of taking directly the anaerobic pathway (which, however, is still an hypothesis).This question would require a further experimentation which is beyond the scopes of this work, since the aim was to demonstrate the actual technical feasibility of ricotta cheese whey fermentation.

Figure2.3. Anaerobic fermentation of sweet cheese whey. Time evolution of lactose, ethanol and biomass concentrations (T = 37 °C, orbital shaking velocity=200 rpm, pH = 5, O2 = 0 – 0.2 %)

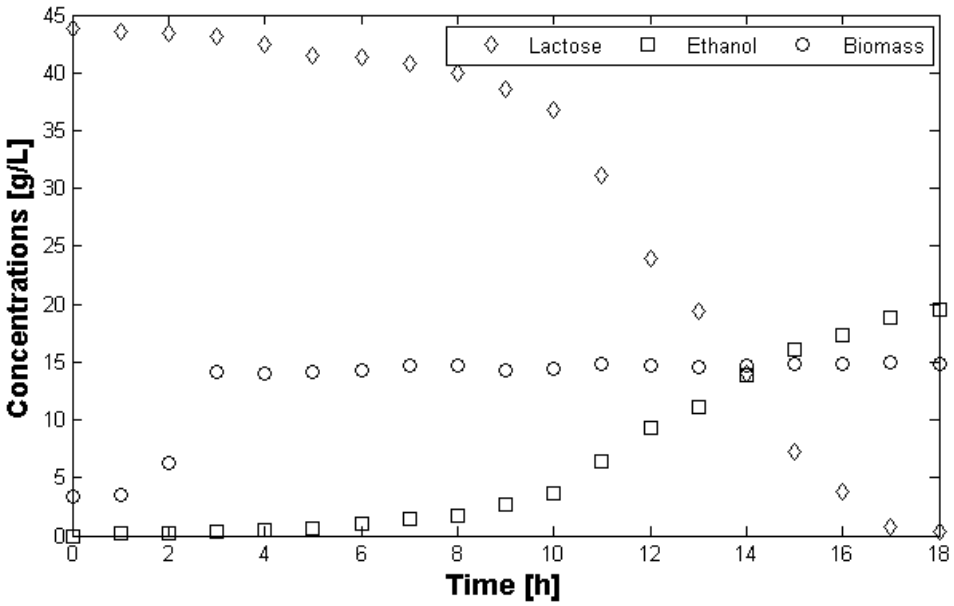


Figure 2.4. Anaerobic fermentation of cheese whey permeate. Time evolution of lactose, ethanol and biomass concentrations (T = 37 °C, orbital shaking velocity=200 rpm, pH = 5, O2 = 0 – 0.2 %)

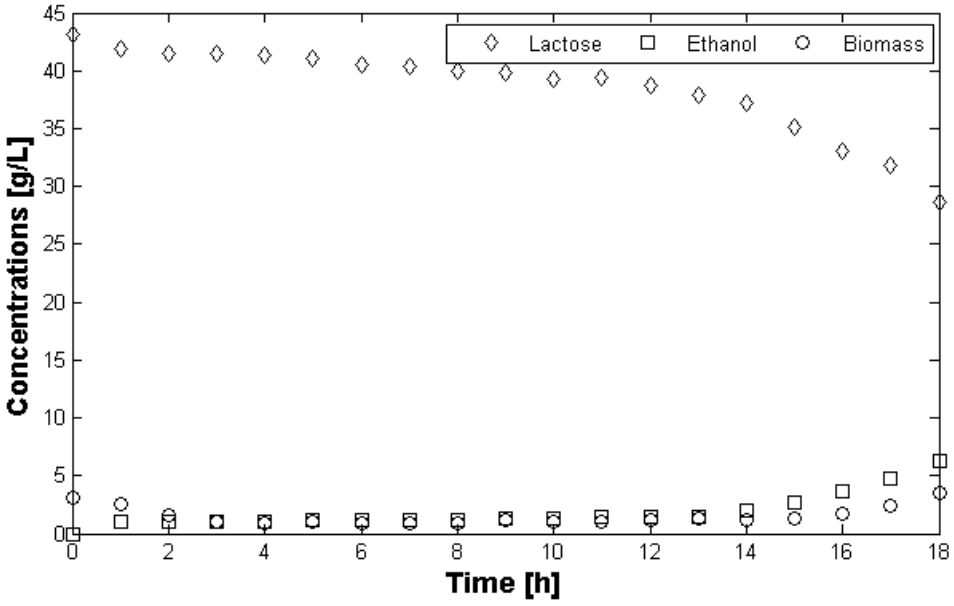


Figure 2.4 shows the behavior of cheese whey permeate as fermentation substrate. Lactose consumption does not occur within the considered time interval; the reason could be somewhat ascribed to the very low protein content (see table 1) which does not allow the microorganism to produce the molecules actually necessary to perform the fermentation process. Protein concentration in cheese whey permeate is, in fact, about a half of that in ricotta cheese whey. Besides, the two substrates have different concentrations of both salts and organic acids, which do might affect the process performance. A deeper experimental analysis is however necessary to better ascertain the reasons of a such a different behavior. As a matter of fact, cheese whey permeate, therefore, can be regarded as a poor fermentation substrate, as compared to both ricotta cheese whey and sweet cheese whey. It should be remarked, however, that such results may be affected by the actual procedure adopted to obtain the deproteinization (i.e. the ultrafiltration).

Another important observation should be done; the rates of both ethanol formation and lactose consumption(see the slopes of the curves in figures 2.2 and 2.3) are higher when ricotta cheese whey is the fermentation medium, thus suggesting that such a substrate actually may be an effective and promising non-vegetable source for renewable energy production. Indeed, ricotta cheese whey productivity ensured an ethanol yield of 89% with a final ethanol concentration equal to 22.44g L^{-1} which means an ethanol productivity of 1.73g*$(L*h)^{-1}$.The result obtained from this experimental study is quite remarkable, the achieved productivity is the highest if compared to the results reported in the literature (see section 2.1). Higher ethanol productivities were obtained only with enriched media (3 g*$(L*h)^{-1}$) (Cheryan and Mehaia, 1983), with peptone-added-cheese whey powder solutions (2 g*$(L*h)^{-1}$(Janssens et al., 1983) and semi-synthetic media (2.1 g*$(L*h)^{-1}$) (Ryu et al., 1991); in the present study, instead, ricotta cheese whey was fermented without any pre-treatment, not even any sterilization process.

2.4 Conclusions

In this chapter, the technical feasibility of ricotta cheese whey fermentation was demonstrated. It was shown that ricotta cheese whey represents an excellent substrate since it allows attaining an ethanol yield of 89%of the theoretical one, without any optimization procedure performed on the operating conditions. Besides, complete lactose consumption was observed after only 13 h (remarkable if compared to 18 h for sweet cheese whey), therefore, ricotta cheese whey ensured the achievement of the best performance ($1.73 g*(L*h)^{-1}$) even if such a performance is compared to the results reported in the literature (see the introduction to this chapter).

Ricotta cheese whey is to be considered as a substrate completely different from traditional sweet cheese whey and, most likely (a further experimentation should be done before any statement),different from deproteinized whey as well. Thus, ricotta cheese whey may represent a valid alternative (and integrative) source to produce bio-ethanol.

The study brought to the formulation of many questions regarding the reasons of the different fermentation performance with different, but still very similar, substrates. Such a matter, despite it is beyond the scope of the present study, should definitely be investigated.

Eventually, a more detailed investigation on the influence of fermentation parameters such as temperature, agitation velocity, pH and initial lactose concentration on ethanol yield could give a better understanding of the fermentation process of ricotta cheese whey and, most probably, allow to obtain better results in terms of ethanol yield as well.

In other words a proper modeling of the process is needed in order to achieve the fermentation at the *best* conditions and to gain a better understanding of the phenomena hidden behind the process. Such a need will be addressed in the next chapters.

Chapter III

Data-driven modeling: Design of Experiments and Optimization by Response Surface Methodology

A Central Composite Design (CCD) was performed to 1) evaluate the effects of four factors, i.e. temperature (T), pH, agitation rate (K) and initial lactose concentration (L), on batch fermentation of ricotta cheese whey and 2) optimize the fermentation process. Anaerobic batch fermentation experiments were carried out by using the yeast *Kluyveromyces marxianus*. After a preliminary experimental analysis, the chosen factors values were 32 and 40°C for T, 4 and 6 for pH, 100 and 300 rpm for K, 40 and 80 g L^{-1} for L.

Response Surface Methodology (RSM) was used to optimize the fermentation process and an empirical model was used to fit the experimental data. The best set of operating conditions resulted to be T = 33 °C, pH = 5.4, K = 195 rpm and L = 40 g L^{-1} and the model ensured both a good fitting of the observed data and good prediction performance.

3.1 Introduction

The work reported in this chapter consists of a first part where an experimental analysis aimed at characterizing the batch fermentation of ricotta cheese whey was performed and a second part where the data were modeled.

An experimental design was planned and the experiments carried out. Afterwards, the experimental data were interpreted, correlated and modeled by *Response Surface Methodology* (RSM).

RSM was adopted in several studies. It was applied successfully to determine the optimum physiological condition for which the maximum rate of acetic acid production occurred from partial acidogenesis of swine wastewater (Hwang et al., 2001).

Cladera-Olivera et al. (2004) performed a factorial design to optimize the bacteriocin production by *Bacillus licheniformis* P40.

The same methodology was also used by Aktas et al. (2006), who considered four factors, i.e. pH, temperature, whey powder concentration and total ammonium salts concentration, to carry out the optimization of lactose utilization in aerobic fermentation of deproteinized whey by *K. marxianus.*

RSM was also applied to assess an electrochemical treatment of deproteinated whey as alternative treatment method (Guven et al, 2007).

Dragone et al. (2008) evaluated the effects of three factors, i.e. initial lactose concentration, temperature and inoculum concentrations, on cheese whey permeate fermentation by means of a Central Composite Design (CCD) and optimized the operating parameters by RSM.

Dagbagli and Goksungur (2009) investigated the production and optimization of β-galactosidase using synthetic medium by *Kluyveromyces lactis* by using the same technique to evaluate the effects of the fermentation parameters.

Eventually, de Lima et al. (2010) carried out two RSM involving central composite designs to evaluate the effect of cheese whey, corn steep liquor, ammonium sulphate, temperature and pH control on lactic acid fermentation by *Lactobacillus* sp. (LMI8) isolated from cassava flour wastewater.

The work reported in this chapter is aimed at two main purposes: 1) to investigate the effects of the main operating parameters on the process and 2) to build an empirical model capable to provide the "best set" of operating conditions to run the process. Therefore, first, a preliminary analysis is reported where the ranges of the values of the operating conditions are chosen and, afterwards, a complete central composite design (CCD) is carried out and the results correlated and modeled by RSM.

3.2 Design of Experiments: a Central Composite Design

A proper design of experiments is the best starting point to have a quantification of the effects of the several factors on a process. Several kinds of design can be planned and the choice of the best one is sometimes tough. As far as fermentation reactions concern, the main operating parameters are undoubtedly temperature (T), agitation rate (K), initial lactose concentration (L) and pH. Thus, being the factors four (a quite high number), the choice of two levels for each factor is most likely the best choice for a screening analysis like the one presented hereby (Box et al., 1978).

Therefore, the choice was to plan a Central Composite Design (CCD) by considering four factors, namely the ones cited above, with two levels for each factor. The CCD was thus constituted by 2^k+2k+2 runs, where k was the number of considered factors (4 in the specific case). The three terms were: 1) an un-replicated factorial portion with two levels for each factor (2^k runs), 2) a set of axial points constituted by runs identical to the central point except for one factor, which assumes values both below and above the median of the two factorial levels (2k runs), 3) a central point replicated twice necessary to improve the precision of the experiments (2 runs). Therefore, by considering four

factors, k = 4, a total number of 26 experiments followed. The CCD was performed to evaluate both single and interaction effects of both first and second order. To evaluate these effects a proper response function must be chosen in order to have an optimal parameter as representative of the fermentation performance. The response function was defined as in Equation 3.1.

$$RF = \left(\frac{1}{0.538}\frac{gethanolformed}{glactoseconsumed}\right) \cdot \left(\frac{glactoseconsumed}{ginitiallactose}\right) \quad (3.1)$$

where the first term is the ethanol fractional yield (ethanol yield calculated with respect to the theoretical value) and the second term is the lactose conversion, thus, the resulting response function is a sort of ethanol yield based on the initial amount of lactose in the fermentation medium.

In order to choose the two levels for each factor a preliminary experimental analysis was carried out. Actually, L and pH values were deduced from the literature and set up equal to both values 4 and 6 for pH (the range 4-6 is the optimal for yeasts) and 40 and 80 g L^{-1} for L (40 g L^{-1} is the *natural* lactose concentration of cheese whey in the worst case and 80 g L^{-1} holds in the hypothesis whey lactose is pre-concentrated two times). Temperature and agitation rate ranges, instead, were determined from the experimental results presented later on.

Preliminary experiments consisted of four fermentation reactions at different temperatures, i.e. 34, 37, 40 and 45°C, and three further reactions, performed at different agitation rates, namely 100, 200 and 300 rpm. Initial lactose concentration and pH were fixed at 50 g L^{-1} (about) and 5, respectively. Each run was performed in duplicates and the maximum variation of each measured point never exceeded 5% of the corresponding calculated average value.

Performances regarding these preliminary experiments were evaluated by determining the fractional yield, y_{et}, calculated as in Equation 3.2.

$$y_{et} = \frac{gethanolformed}{glactoseconsumed} \cdot \frac{100}{0.538} \quad (3.2)$$

Once chosen the factors levels (from the preliminary analysis) and performed the CCD (calculating the RF from the experiments by Equation 3.1), data were used to quantify the effects of the factors on the process performance (represented by the RF defined as inEquation3) by performing the test of variance (ANOVA test).

$$RF = a_0 + \sum_{i=1}^{4} a_{ii}x_i + \sum_{i=1}^{4} a_{ii}^2 x_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} a_{ij}x_i x_j \quad (3.3)$$

Here a_0 was the constant coefficient, x_i were the non coded variables, a_{ii}'s (i = 1-4) and a_{ii}^2's were the coefficients corresponding to linear and quadratic terms, respectively, and, finally, a_{ij}'s (i and j = 1-4) were the second order interaction coefficients. R^2 coefficient was determined to assay the quality of the polynomial model, whereas its statistical significance was checked by the F-test. It is worthwhile remarking that all runs of the CCD were carried out randomly. The present analysis was performed by means of the commercial software *Statgraphics Plus 5.1* (*Virginia, USA*).

Equation 3.3 was used to build the response surfaces as function of the factors and, by maximizing this function response function, the fermentation reaction was optimized.

It is worth observing that, in batch processes rather than in continuous ones, optimization procedure is far more complicated due to the time-varying nature of these processes; indeed, the optimal values of the factors require to be adjusted with time. Since the

fulfillment of a rigorous dynamic optimization (Srinivasan et al, 2001) is beyond the scopes of this study, the time was set at a definite value, chosen on the basis of the results collected during the preliminary experiments and represented by the shortest time (t = 18 h) necessary to obtain complete lactose consumption. Therefore, Equation 3.3 evaluated at t = 18 h, was the actual objective function to be maximized in order to achieve fermentation optimization.

3.3 Materials and Methods

3.3.1 *Controlled bio-reaction system*

A controlled batch bio-reactor, consisting of a 1.5 liter autoclavable plexiglas cylinder (*Applikon, Holland*), was used to perform the present experimental study. The main operating parameters (pH, O_2 concentration, temperature, agitation and foam level) were monitored by a set of sensors and controlled by means of an *ADI 1030* Shelf-topcontroller.

3.3.2 Yeast Strain

Lactose bio-conversion experiments were performed by a yeast, i.e. *Kluyveromyces marxianus var. marxianus CBS 397*, isolated at the *Centraalbureau voor Schimmercultures* (*Utrecht, the Netherlands*). This yeast was selected for its particular performances toward lactose fermentation (see chapter I). The yeast, initially freeze dried, was revived suspending the microorganism by pouring it into a cylinder containing 1-2 mLof sterile water and, then, shaking and storing the suspension at 20°C for 12 h.

3.3.3 *Maintenance culture*

Kluyveromyces marxianus was maintained in a generic yeast medium having the following composition: agar 10 g L^{-1}, lactose 20 g L^{-1}, bactopeptone 10 g L^{-1}, yeast extract 5 g L^{-1}. The culture was sterilized in an autoclave at 121°C for 30 min, then it was poured on *Petri* dishes for solidification and, eventually, the yeast inoculum was spread on the surface and incubated at 20°C for 48 h. At growth completed, the dishes were kept at 4°C.

3.3.4 Inoculum medium

The inoculum medium was prepared with a single colony withdrawn from the *Petri* dishes and incubated in a *GRANT OLS 200* thermostated bath, maintained for 12 h at a temperature of 37°C with an orbital shaking velocity of 150 rpm. In all the experiments 100 mL of medium were poured in a 300 mL sterile flask. Each of the used materials, before performing this stage, was autoclaved at 121°C for 30 min. The inoculum medium was constituted by lactose, 50 g L^{-1}, bactopeptone, 10 g $L^{-1}$and yeast extract, 5 g L^{-1}.

3.3.5 Fermentation medium

The fermentation medium was ricotta cheese whey. All the samples came from the same lot of cow milk, originally designed to *mozzarella cheese* production. It is worthwhile to remark that each of the comparisons hereafter presented was performed on samples not subjected to any other pre-treatment, but those normally carried out in the production plant. All the samples, kindly provided by a local dairy industry, *Agroalimentare Asso.La.C.*(*Italy*) were stored at 4°C; each fermentation test, however, was performed within 6 h from the production time.

3.3.6 Analytical methods

The samples were periodically withdrawn from either the flasks or the bio-reactor in aseptic conditions in order to determine, by HPLC, the time evolution of lactose and ethanol concentrations. A 0.1% (v/v) phosphoric acid solution was used as mobile phase at a flow rate of 0.5 $mL*min^{-1}$. A 50x4.6 mm *Supelcogel* pre-column, a 300x7.8 mm *SupelcogelC-610* column and a refractive index detector, *Jasco RI 930*, constituted the experimental equipment. Biomass was evaluated by *BactoScan FC* (*Foss Integrator, Denmark*) an instrument capable to determine, on the basis of an optical method, the number of cells contained per milliliter of solution. The amount of cells, on a mass basis, was obtained multiplying the cells concentration by 303 ng per cell (Ghaly and El-Taweel, 1995a; b).

3.3.7 Experimental protocol

Anaerobic fermentation experiments lasted 18 h and were performed starting 1 L fermentation medium with 100 mL inoculum. Each experiment was repeated twice to assess data reproducibility; the average concentrations of lactose, ethanol and biomass were reported versus time. The pH of the fermentation broth was controlled by means of a 6N sodium hydroxide solution.

Two samples of fermentation broth were withdrawn, every hour, during the experiment: 1) a 100 µL sample was poured in 25 mL of a 2% sodium citrate solution and eventually analyzed to obtain the amount of biomass formed and 2) a 1 mL sample was, instead, centrifuged at 5000 rpm for 5 min, filtered through a 0.45 µm filter and finally sent to the HPLC for assaying the evolution of both lactose and ethanol concentration.

3.4 Results and discussion

The preliminary analysis on various temperatures provided the results shown in Table 3.1.

Table 3.1. Preliminary analysis results at different temperatures: 34°, 37°, 40° and 45°C (pH = 5, K = 200 rpm).

Run	Temperature [°C]	Initial lactose concentration [g L^{-1}]	Final biomass concentration [g L^{-1}]	Time of complete lactose consumption [h]	Ethanol Yield at 18 h [-]	Ethanol formed at 18 h [g L^{-1}]
1	34	46.58	6.61	14	90	22.48
2	37	47.63	6.55	13	85	21.75
3	40	49.25	4.95	> 24 h	69	17.49
4	45	47.11	2.61	> 24 h	8	2.02

All the runs were performed in duplicates and indicated that the maximum variation of each measured point from the corresponding calculated mean value was always less than 5%. Runs performed at 34 and 37°C resulted in high ethanol yields, 90% and 85%, respectively, with complete lactose depletion achieved in 14 and 13 h only; the operating temperatures of 40 and 45 °C, with ethanol yields equal to 69 and 8%, respectively, instead, both appeared to be too high since complete lactose consumption was not achieved even after 24 h.

The results obtained at different agitation rates are reported in Table 3.2.

Table 3.2. Preliminary analysis results at different agitation rates: 100, 200 and 300 rpm (pH = 5, T = 37 °C).

Run	Agitation rate [rpm]	Initial lactose Concentration [g L^{-1}]	Final biomass concentration [g L^{-1}]	Time of complete lactose consumption [h]	Ethanol Yield at 18 h [-]	Ethanol concentration at 18 h [g L^{-1}]
5	100	47.36	7.36	23	72	16.81
6	200	47.63	6.55	13	85	21.75
7	300	47.16	15.53	23	60	14.71

The highest ethanol yield was 85% at 200 rpm (this run is actually run 2 reported in table 2, it was renamed to highlight the variable factor, i.e. the agitation rate), while yields equal to 72% and 60% were attained at agitation rates of 100 and 300 rpm, respectively. These results are consistent with the corresponding biomass concentrations; in fact, the 100 rpm run showed a lower final biomass concentration (7.36 g L^{-1}), as compared to the 300 rpm run, which exhibited a final biomass concentration of 15.53 g L^{-1}. Actually, a higher agitation rate, aided the microorganism growth pathway instead of the ethanol fermentation process, thus resulting in a lower amount of substrate available to attain the conversion into ethanol. It is worthwhile remarking that , although the stirrer was always submerged in the bulk, no tools aimed at keeping anaerobic conditions were used (such as sparging nitrogen in the bulk) so, the increase in biomass concentration at higher values of the agitation rate may be ascribed to the major oxygenation of the yeast as determined by the stirrer. On the other hand, the dissolved oxygen concentration in the broth was constant for all the fermentation time and equal to 0-0.2%. Further investigations are actually necessary in order to better understand this effect; anyway, as far as this work is concerned, it is evident that, in the tested range, the best agitation rate that allowed achieving the highest ethanol yield was the intermediate one, i.e. 200 rpm.

On the basis of the above-described results, the values of the remaining two factors temperature (T) and agitation rate (K) were set up at 32 and 40°C for *T* and 100 and 300 rpm for *K* (which actually are the typical ranges of operation in similar fermentation processes).

It should be remarked that, although these results provided some indications about the influence of the considered factors on the fermentation performance, the actual effect of any parameter cannot be quantified and characterized without a proper and rigorous experimental analysis, such as the one planned and presented later on in the text.

Table 3.3 shows the operating conditions characterizing each of the 26 experiments resulting from the Design of Experiments and the corresponding values of the RF (calculated by Equation 3.1).

Runs 1^{st} and 26^{th} are the replicated central point. Runs from 2^{nd} to 17^{th} represent the factorial portion and, eventually, runs from 18^{th} to 25^{th} represent the axial points.

CCD data were elaborated in order to assess the effects of the factors on the response function (representative of the process); the results are summarized in Table 3.4 where the ANOVA test for the response surface quadratic model (Equation 3.3) is reported. The F-value 10.07 indicates that the model is significant; likewise, the 0.8355 value of the adjusted R squared is sufficiently good. The signal/noise ratio, i.e. 9.777, which indicates that the model could be used to investigate the design space. Nevertheless, the predicted R squared value of 0.5832 is not as close to the 0.8355 adjusted R-squared as it was expected and this is probably due to a large block effect.

At this point some improvement of the model can be achieved by observing closer Table 3.4. Indeed, by considering the effects statistically significant at a 95% confidence level, only the parameters that showed P-values less than 0.05 are to be taken into account in the model, whereas the other parameters are actually undistinguishable from the noise.

Table 3.3. Central composite design (CCD). Factors are temperature (T), pH, agitation rate (K) and initial lactose concentration (L). The factors levels are 32° and 40°C for T, 4 and 6 for pH, 100 and 300 rpm for K, 40 and 80 gL^{-1} for L. In the last column the obtained response values are shown.

Run	Temperature [°C]	pH	Agitation rate [rpm]	Initial lactose concentration[$g\ L^{-1}$]	Response function[-]
1	36	5	200	60	0.88
2	32	4	100	40	0.65
3	40	4	100	40	0.35
4	32	6	100	40	0.80
5	40	6	100	40	0.50
6	32	4	300	40	0.38
7	40	4	300	40	0.08
8	32	6	300	40	0.78
9	40	6	300	40	0.48
10	32	4	100	80	0.35
11	40	4	100	80	0.05
12	32	6	100	80	0.75
13	40	6	100	80	0.35
14	32	4	300	80	0.33
15	40	4	300	80	0.03
16	32	6	300	80	0.72
17	40	6	300	80	0.42
18	28	5	200	60	0.77
19	44	5	200	60	0.15
20	36	3	200	60	0.18
21	36	7	200	60	0.28
22	36	5	0	60	0.05
23	36	5	400	60	0.15
24	36	5	200	20	0.84
25	36	5	200	100	0.66
26	36	5	200	60	0.88

Table 3.4.Analysis of variance for response function with all effects. ANOVA test.

Source	Sum of squares	Degrees of freedom	Mean square	F Value	P Value
T: temperature	0.582817	1	0.582817	42.79	0.0000
pH	0.322017	1	0.322017	23.64	0.0005
K: agitation rate	0.006017	1	0.006017	0.44	0.5200
L: initial lactose concentration	0.079350	1	0.079350	5.83	0.0344
T^2	0.161001	1	0.161001	11.82	0.0055
T-pH	0.000625	1	0.000625	0.05	0.8343
T-K	0.000625	1	0.000625	0.05	0.8343
T-L	0.000625	1	0.000625	0.05	0.8343
pH^2	0.411491	1	0.411491	30.21	0.0002
pH-K	0.021025	1	0.021025	1.54	0.2399
pH-L	0.009025	1	0.009025	0.66	0.4329
K^2	0.604128	1	0.604128	44.35	0.0000
K-L	0.021025	1	0.021025	1.54	0.2399
L^2	0.009673	1	0.009673	0.71	0.4173
Total error	0.149833	11	0.013621		
Total (corr.)	2.07042	25			
F-value of the model: 10.07 Adjusted R-squared value: 0.8355 Predicted R-squared value: 0.5832					

The significant terms, as shown in Table3.4, are T, pH, L, T^2, pH^2 and K^2 (marked in bold). Therefore, by eliminating the other terms from the model (except K to support hierarchy as requested from the methodology (Box et al., 1978)), the ANOVA test reported in Table 3.5 was obtained.

Table 3.5. Analysis of variance for response function with all significant effects. ANOVA test.

Source	Sum of squares	Degrees of freedom	Mean square	F Value	P Value
T: temperature	0.582817	1	0.582817	49.38	0.0000
pH	0.322017	1	0.322017	27.28	0.0001
K: agitation rate	0.006017	1	0.006017	0.51	0.4844
L: initial lactose concentration	0.079350	1	0.079350	6.72	0.0184
T^2	0.160228	1	0.160228	13.57	0.0017
pH^2	0.448878	1	0.448878	38.03	0.0000
K^2	0.676378	1	0.676378	57.30	0.0000
Total error	0.212457	18	0.011803		
Total (corr.)	2.07042	25			
F-value of the model: 22.49 Adjusted R-squared value: 0.8575 Predicted R-squared value: 0.6687					

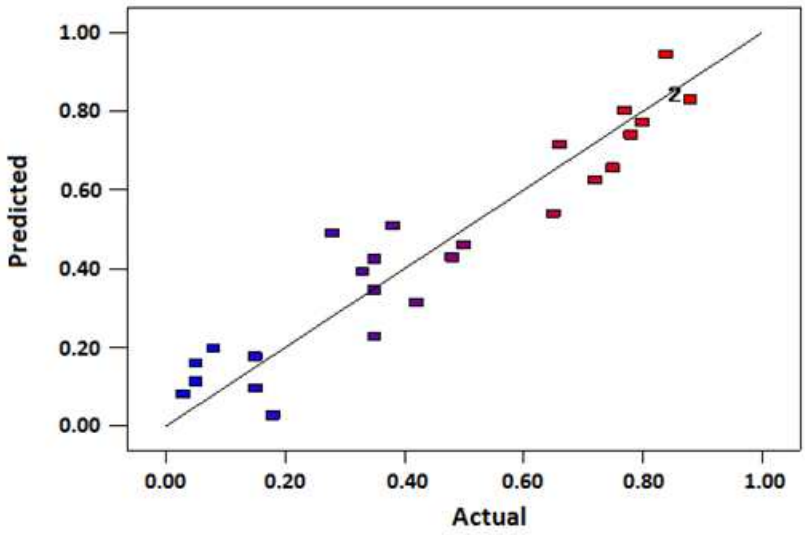
ANOVA test regarding the new model (where negligible effects were eliminated), indicates a F-value of 22.49, which makes the model much more significant than the previous one (F-value: 10.07). Moreover, a better signal/noise ratio was obtained, i.e. 15.233, and the predicted R-squared value of 0.6687 is in reasonable agreement with the adjusted value of 0.8575.

The related empirical polynomial model is reported in Equation 3.4.

$$RF = -9.33 + 0.34T + 1.54pH + 6.85 \cdot 10^{-3}K - 2.87 \cdot 10^{-3}L - 5.33 \cdot 10^{-3}T^2 + -0.14pH^2 - 1.75 \cdot 10^{-5}K^2 \quad (3.4)$$

Figure 3.1 shows the values predicted by the model versus the observed data evidencing a fairly reliable correlation (R-squared equal to 0.96) especially considering the large ranges of the factors considered in the optimization procedure.

Figure 3.1. Predicted versus actual values of the response function.



Figures 3.2-3.4 show the effects of the factors on the RF. Figure 3.2a exhibits a strong response surface dependence on both T and pH; indeed RF changes its value from about 0.35, at 40 °C and pH 4, to about 0.60 at the same T and pH 6. A similar behavior can be observed passing from 40 to 32 °C at the same pH of 4. Moreover, a good system behavior corresponding to a RF of 0.88, is obtained at 32 °C and pH 6. Figure 3.2b reports the response surface versus pH and K. It is evident that a pH value around 5.5 improves the fermentation process. The worst conditions were achieved at pH 4 and at agitation rate of both 100 and 300 rpm.

Figure 3.2. a) Response surface as a function of pH and temperature (T). b) Response surface as a function of pH and agitation rate (K). The other two factors were set up at the intermediate values, i.e. K = 200 rpm and L = 40 g L^{-1} in (a) and T = 36°C, L = 60 g L^{-1} in (b).

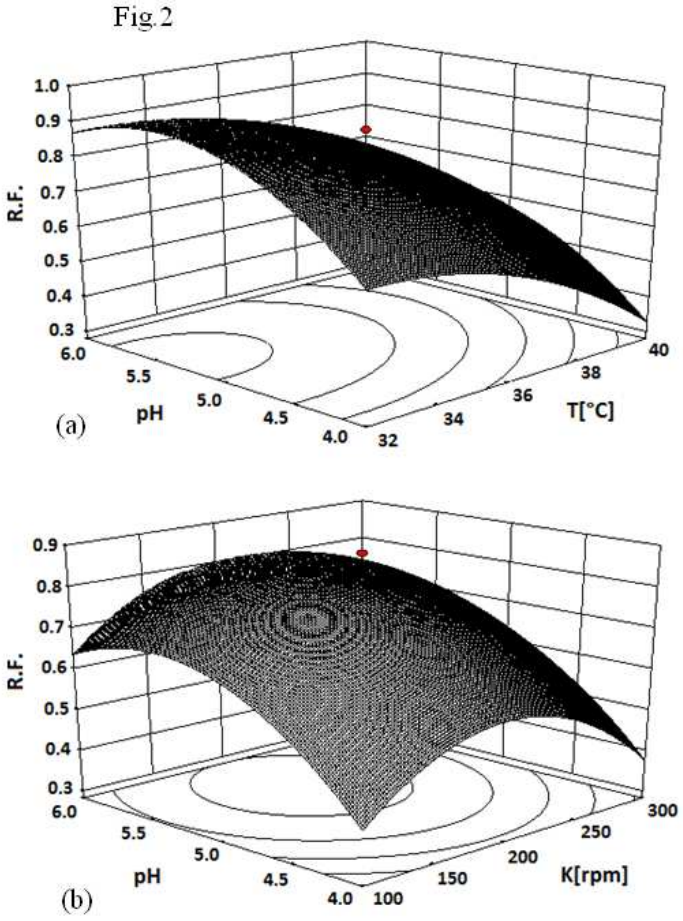


Figure 3.3a shows the effects of the factors pH and L on the RF; a relatively weak effect of L and a stronger effect of pH can be noted. Figure 3.3b, showing the RF dependence on both T and K, confirms the strong dependence on temperature, that was capable to determine a variation of the RF from about 0.5 to about 0.75 when T was decreased from 40 °C to 32 °C, at the same stirring rate of 100 rpm. As far as the influence of K on the RF is concerned, a relatively weak effect is observed even though an intermediate value, i.e. 200 rpm, improves the ethanol formation.

Figure 3.3. a) Response surface as a function of pH and initial lactose concentration (L). b) Response surface as a function of temperature (T) and agitation rate (K). The other two factors were set up at the intermediate values, i.e. K = 200 rpm and T = 36 °C in (a) and L = 40 g L^{-1}, pH = 5 in (b).

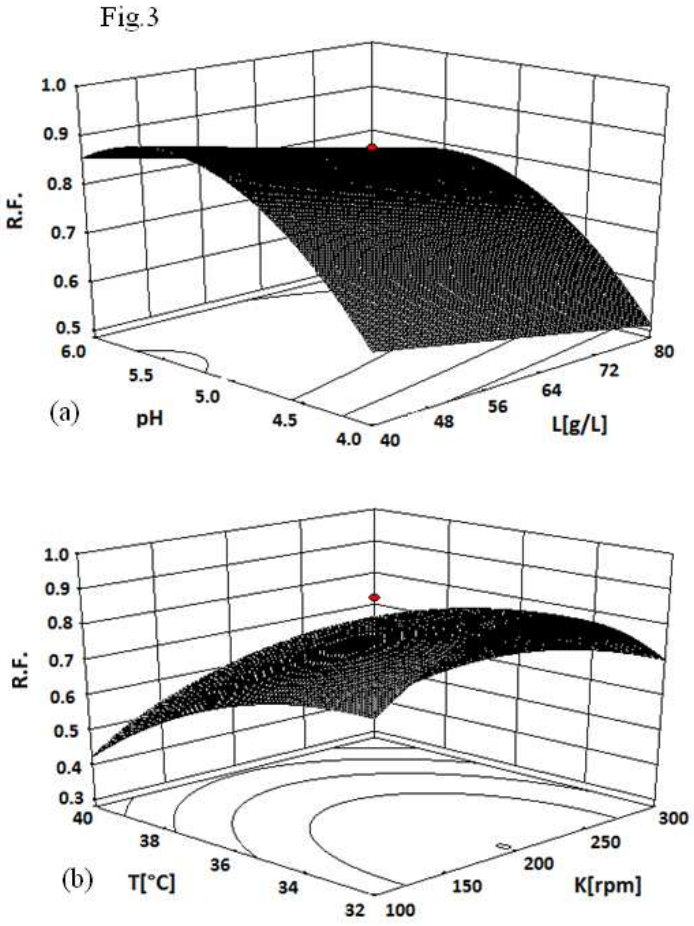
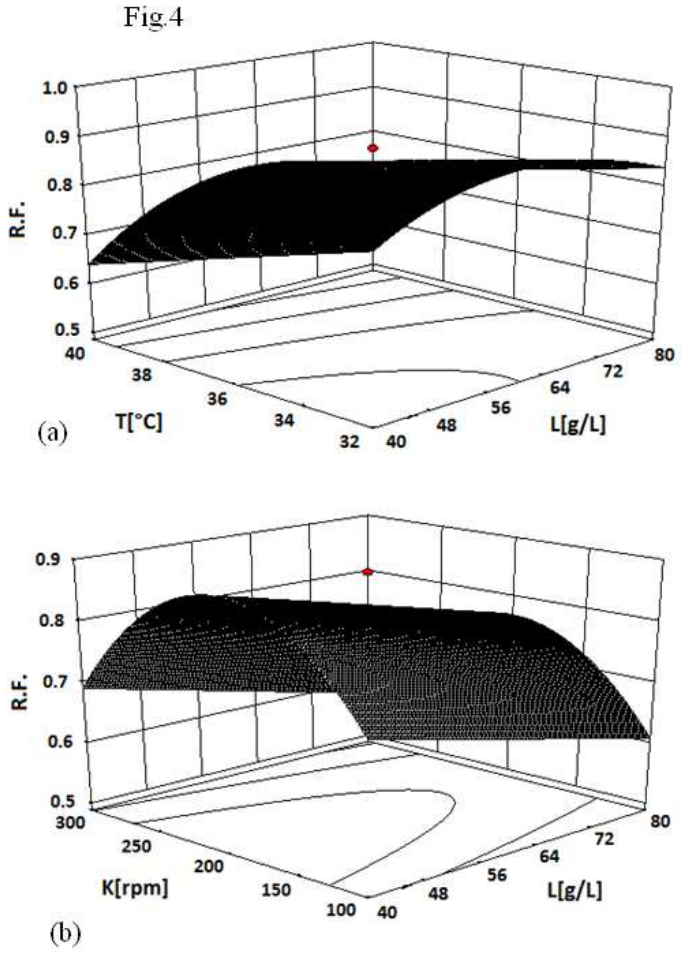


Figure 3.4a presents the response surface versus L and T; it strengthens the conviction that ricotta cheese whey fermentation process is enhanced by relatively low T values. Factor L has a weak effect on the RF, even though better results were achieved with the lowest value of L, i.e. about 40 g L^{-1}. Figure 3.4b, presenting the response surface performance as a function of both L and K, confirms that it should be advisable to use intermediate K values and low L values.

Figure 3.4. a) Response surface as a function of temperature (T) and initial lactose concentration (L). b) Response surface as a function of agitation rate (K) and initial lactose concentration (L). The other two factors were set up at the intermediate values, i.e. K = 200 rpm and T = 36 °C in (a) and T = 36 °C, pH = 5 in (b).



The obtained results require some further considerations. RSM provides the "best" set of operating conditions as the following: T = 33 °C, pH = 5.4, K = 195 rpm and L = 40 g L^{-1}, with a correspondent predicted value of the RF of 0.981 (the optimization was strictly performed in the considered range of the factors and possible extrapolations are to be avoided).

Considering that ricotta cheese whey is usually characterized by a pH ranging between 4.5 and 5.0, in which the model provided a R.F. equal, respectively, to 0.864 and 0.958, it is evident that pH has a notably effect on the final ethanol yield. Therefore, it is strongly advisable to operate the fermentation only after a preliminary pH adjustment (likely economically inconvenient) or, actually, just after ricotta cheese whey production, since this substrate tends to acidify very easily (see chapter I). Another remarkable information could be derived from the results regarding the estimation of optimum lactose concentration; indeed, a value of 40 g L^{-1} is definitely close to the initial average lactose concentration of ricotta cheese whey (48-50 g L^{-1}). At an initial lactose concentration equal to 48 g L^{-1} the model would provide a RF of 0.958, thus suggesting not increasing the initial lactose concentration by any kind of pre-treatment. Nevertheless, it is worthwhile observing that a higher ethanol concentration flowing out the reactor would significantly reduce both separation and purification costs. Considering, for instance, a maximum lactose concentration equal to 75 g L^{-1} the model would provide a slightly lower RF, equal to about 0.88, which, however, could be economically more convenient if it resulted in an actual higher final ethanol concentration (and a reasonable amount of lactose converted).

Furthermore, another aspect of the study performed in this chapter should be remarked, the optimum results are strongly dependent on the particular response function chosen to optimize the fermentation reaction. Indeed, the choice of an ethanol yield based on the initial lactose concentration may push the optimum value of the initial lactose concentration (meant now as a parameter) toward low values.

Eventually, some validation tests were performed on the model. In particular, the experimental data regarding the preliminary analysis presented above were used to compare the experimental results with the predicted performance by the model.

The experimental RF can be easily calculated from the data reported in both Tables 3.2-3.3; the predicted RF can be, instead, calculated with Equation 3.4 substituting the values of the factors correspondent to the run under consideration.

Such a comparison is reported in Table 3.6 for runs 1, 2. 3, 5 and 7 (run 4 was excluded since outside the range of validity of the model and run 6 is not reported since it being actually identical to run 2).

Table 3.6. Application of the quadratic response model to the runs 1, 2 (identical to 6), 3, 5 and 7 of the preliminary experimentation (tables 2 and 3).

Run in the preliminary analysis	Actual RF calculated from the experiments	Predicted RF from the model	Deviation of the predicted value from the actual value [%]
1	0.90	0.92	2.22
2	0.85	0.82	3.53
3	0.66	0.59	10.60
5	0.66	0.66	0
7	0.58	0.63	8.62

The results shown in Table 3.6 validate the model in the ranges of the factors considered. Indeed, model predictions for runs 1, 2 and 5 result extremely good with deviations smaller than 4%. Predictions concerning runs 3 and 7, instead, though still acceptable, are worse and the reason is most likely due to the fact that such runs are in correspondence of the extreme values of the ranges; indeed, run 3 is the one performed at T = 40°C, i.e. the upper value of temperature range, and run 7 was carried out at K = 300 rpm, i.e. the upper value of agitation rate range.

3.5 Conclusions

A central composite design based on the analysis of 26 experiments, involving the anaerobic fermentation of lactose contained in ricotta cheese whey was performed.

The effects of four factors, i.e. temperature, pH, agitation rate and initial lactose concentration were estimated. After having evaluated the ANOVA test on the complete

quadratic model, all the negligible effects were eliminated in order to improve the model predictive performance. A response surface quadratic model was obtained as a function of the only significant effects, i.e. T, pH, L, K, T^2, pH^2 and K^2. ANOVA test showed a 22.49 F-value which made the model rather significant. Nevertheless, an adjusted R squared value of 0.8575, testified a good model correlation performance, with a predicted R squared value of 0.6687 in reasonable agreement with the adjusted value.

Afterwards, RSM was applied to optimize ricotta cheese whey fermentation with respect to the values of the main operating factors. The optimization provided the best set of operating parameters, namely 33°C for temperature, 5.4 for pH, 195 rpm for agitation rate and 40 g L^{-1} for initial lactose concentration.

Finally, the developed model was validated by checking the agreement between its predictions and the experimental results collected during the preliminary analysis. A much satisfactory agreement was observed with deviations of the predictions lower than 4%.

Eventually, some critics on the limitations of the model should be remarked. First, the model should be used strictly in the ranges of the factors considered. On the other hand the considered ranges were quite wide thus, this should not be a serious problem. Second, the model is an empirical one, therefore no physical meaning at all is included in its structure, which means that the model, although very useful for predictions to avoid further experimentation, cannot give a better insight of the fermentation process. For the last reason, it is author's belief that it is necessary to develop a physically meaningful model in order to better understand the mechanisms that play important roles behind the observable phenomena. This is the topic of the next chapter.

Chapter IV

Knowledge-driven modeling: a Biochemically Structured Approach

Anaerobic fermentation of lactose into ethanol by *K. marxianus* was studied both experimentally and theoretically. Three different batch runs at different operating conditions were performed on samples of ricotta cheese whey. In order to model the experimental data, the metabolic pathways through which lactose is converted into the several products involved was considered and the model developed on this metabolic structure. The resulting biochemically structured model provided 1) a fairly good fitting performance as demonstrated by the estimated parameters analysis and 2) an excellent insight of the fermentation reaction, since it was based on both stoichiometric and thermodynamic principles.

4.1 Introduction

Ethanol fermentation processes have recently been the object of much attention from the scientific community due to the intense debate on renewable energy, particularly with respect to bio-fuels production technologies (Lin and Tanaka, 2006). Ethanol is obtained by anaerobic fermentation of sugars by an appropriate microorganism. A number of different substrates as well as several microorganisms have been tested to achieve ethanol fermentation. One substrate that is very interesting is lactose, which is the main sugar in milk and represents an enormous underutilized waste product from all the different kinds of cheese whey which are produced by the dairy industry.

Lactose can, under proper operating conditions, be either hydrolyzed and fermented or directly fermented (Carvalho et al, 1990; Berruga et al, 1997) giving ethanol as the main product (Mawson, 1994; Siso, 1996; Sansonetti et al, 2009).

Many mathematical models have been developed attempting to describe fermentation reactions. These models were developed largely for the purpose of quantitatively describing the experimental data, as well as to support fermentation reactor design and operation. Both structured (Esener et al, 1982; Lei et al, 2001) and unstructured models (Nielsen and Villadsen, 1992) have been developed. In the current work, the attention will be focused on unstructured models, for reasons explained below.

Particular attention has been paid to the kinetics of the fermentation processes, thus many mathematical equations have been proposed in order to describe the concentrations of the different species involved (Lee and Rogers, 1983; Lee et al, 1983; Dourado et al, 1987, Bailey and Ollis, 1986). For example, a rational and exhaustive comparison of the main kinetic forms used to describe sugar consumption, product formation, product inhibition and biomass growth was provided by Starzak et al. (1994) by considering sucrose as carbon source. Furthermore, the kinetics of batch fermentation of lactose in cheese whey powder solutions was considered by Ozmihci and Kargi (2007) by using a modified Monod expression to take into account the inhibition effect of substrate at high concentrations. A similar kinetic model, modified in order to include also the effect of

ethanol inhibition, was used by Zafar et al. (2005) for fermentation of solutions simulating cheese whey. A rather complete kinetic model for lactose fermentation in raw cheese whey, published by Ghaly and El Taweel (1994), takes into account substrate limitation, substrate inhibition, ethanol inhibition and cell death as well. A different approach was developed by Wang and Bajpai (1997a; 1997b); they used a cybernetic model to represent the microbial kinetics in the fermentative production of ethanol from cheese whey. A statistical thermodynamic approach was used by Tan et al. (1994) to describe the microbial growth obtaining a more general model including several widely used expressions as special cases.

Most of these studies are recognizable for the use of unstructured models which, although successfully applied to several fermentation processes, involve some limitations due to the lack of structure, which results in the lack of tools for describing the fermentation reaction, mainly in dynamic conditions. A great limitation is that these models are mainly constituted by empirical equations which may be very useful as data fitters but reveal next to nothing about the mechanisms hidden behind the observed phenomena. Nevertheless, the value of reliable and predictive models in biochemical engineering is becoming vital as “white biotechnology” becomes more mainstream (Villadsen, 2007); therefore, further efforts are required to provide models capable of providing a better understanding of the mechanisms that regulate the fermentation process. To this end, an interesting approach to model the fermentation reactions, often referred to as a “biochemically” or “metabolically structured approach”, was introduced as early as the 1980s by Roels and co-workers (1980; 1983). Simply (an exhaustive explanation will be given later on) it is based on the consideration of the main reactions taking place in a working microorganism, namely anabolic, catabolic, polymerization and maintenance reactions. Several examples of this approach are present in the literature.

Krzystek and Ledakowicz (1997) set up a biochemically structured model by adapting the metabolic pathways provided by Oura (1972) for use of sucrose as carbon source, and

compared the experimental yields with the ones predicted by the model obtaining a good agreement with a 0.986 correlation coefficient.

A stoichiometric analysis of *K. fragilis* growth on lactose, under aerobic conditions, was performed by the same authors (Krzystek and Ledakowicz, 2000) by applying the same principles and the calculated true biomass yield coefficients were correlated with the values resulting from the balance analysis of stoichiometric equations.

A kinetic modeling of Poly-(b-hydroxybutyrate (PHB)) production was carried out by van Leeuwen et al. (1997) who determined the maximum yield of biomass on PHB and biomass on substrate by adopting the same approach. Finally, another example performed to show a practical application of such a bioenergetics principles, was provided by Heijnen et al. to model the penicillin fermentation (1979).

With this background in mind, in the present chapter, a biochemically structured model was built to describe the conversion of lactose into ethanol by *K. marxianus* under anaerobic conditions. In particular, the aim of the work was to develop and evaluate a model capable to provide physically meaningful yield estimates as well as a sound basis for a better understanding of the fermentation process.

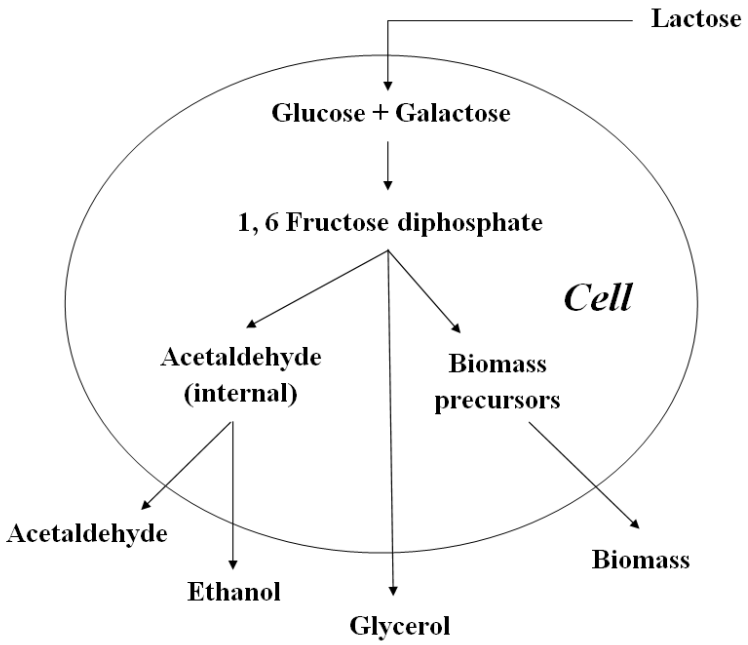
4.2 Theoretical background and modelling

4.2.1 Metabolic pathways

Ethanol fermentation is a typical example in which the carbon source consumption cannot be divided into two different processes, namely biomass growth and ethanol formation. Ethanol is a by-product of the microbial metabolism and is thus directly associated to biomass growth. Therefore, a natural choice would be the adoption of a black box model in which all the processes involved are lumped into one single reaction (Nielsen et al, 2003).On the other hand, with such an approach, the species yields would

be constants (thus, without taking into account any variation in the yields due to the maintenance) and their values based on the material balances only leading to a large limitation on the descriptive potential of the model. Instead, the biochemically structured approach is based on the main metabolic pathways which are involved in the carbon flux from the substrate to the products. Basically, these processes can be divided into: 1) anabolic reactions in which the formation of biomass precursors is achieved, usually with the consumption of a certain amount of energy (ATP) and the production of reducing equivalents (indicated as NADH in the text). 2) Catabolic reactions in which ATP is produced at the expense of a certain amount of substrate. 3) Polymerization of biomass precursors into active biomass with an extra consumption of ATP and 4) maintenance-associated ATP consumption. In order to identify the particular reactions involved in lactose fermentation, it is necessary to consider the metabolic structure involved in the fermentation reaction. Lactose is mainly converted into biomass, ethanol, glycerol, carbon dioxide and acetaldehyde. The metabolic structure is depicted in Figure4.1. It must be noted that batch experiments, reported later on, have shown that the excreted acetaldehyde is present in negligible amounts, therefore it will not be considered in the model development. Furthermore, compounds in trace amounts such as pyruvate, acetate and succinate will be neglected in the model as well.

Figure 4.1. Metabolic structure considered in the model for anaerobic bioconversion of lactose by *K. marxianus*.



There are two other processes that play key roles in the fermentation reactions that should be considered in the model, i.e. polymerization of biomass precursors and biomass maintenance. If lactose is the only carbon source in the substrate, the stoichiometry of each metabolic pathway is given in Table 4.1.

Table 4.1. Metabolic reactions involved in the model for anaerobic lactose fermentation by *K. marxianus*. Each reaction is written per unit of C-mol as indicated by Roels (1980). The rates of reaction q_i are expressed in [(C-mol)*(L*h)$^{-1}$].

Process	Stoichiometry
I. Formation of biomass* precursors	$(1+\delta_x)CH_{11/6}O_{11/12} + 0.2NH_3 + \delta ATP \xrightarrow{q_x'} CH_{1.8}O_{0.5}N_{0.2} + \delta_x CO_2 + 2(\delta_x - 0.05)NADH + \left(\frac{5-13\delta_x}{12}\right)H_2O$
II. Ethanol formation	$1.5CH_{11/6}O_{11/12} + 0.125H_2O \xrightarrow{q_e} CH_3O_{0.5} + 0.5CO_2 + 0.5ATP$
III. Glycerol formation	$CH_{11/6}O_{11/12} + \frac{1}{12}H_2O + \frac{1}{3}ATP + \frac{1}{3}NADH \xrightarrow{q_g} CH_{8/3}O$
IV. ATP consumption for polymerization of biomass*	$CH_{1.8}O_{0.5}N_{0.2} + K \cdot ATP \xrightarrow{q_x} \frac{1}{n}(CH_{1.8}O_{0.5}N_{0.2})_n$
V. ATP consumption for maintenance	$ATP \xrightarrow{r_{ATP}} maintenancepuposes$

*Biomass composition is assumed to be $CH_{1.8}O_{0.5}N_{0.2}$ (Roels, 1983).

ATP consumption for both polymerization and maintenance results in a net ATP consumption rate, R_{ATP} [(mol ATP)*(L*h)$^{-1}$], which can be written as the sum of two terms, one growth-associated (IV), proportional to q_x, and one due to the maintenance (V); this last process is assumed to be linear with the biomass concentration X [(C-mol)*L^{-1}] (Stouthamer and Bettenhaussen, 1973) as reported in Equation 4.1 where m[(mol ATP)*(C-mol biomass*h)$^{-1}$]represents the maintenance coefficient for biomass and K [(mol ATP)*(C-mol biomass)$^{-1}$] is the metabolic coefficient for biomass polymerization.

$$R_{ATP} = -Kq_x - mX \tag{4.1}$$

Process I contains two metabolic coefficients, δ_x [(C-mol substrate)*(C-mol biomass)$^{-1}$] that represents the amount of carbon lost as carbon dioxide and δ [(mol ATP)*(C-mol

biomass)$^{-1}$] which is the amount of ATP consumed in the anabolic formation of biomass precursors that, in general, depends on the culture conditions.

4.2.2 Model development

By assuming pseudo steady-state conditions for biomass (metabolic intermediates at pseudo steady-state), the conservation balances on ATP, reducing equivalents and biomass can be set up, as reported in Equations 4.2, 4.3 and 4.4.

$$-\delta q'_x + 0.5 q_e - \frac{1}{3} q_g - R_{ATP} = 0 \quad (4.2)$$

$$(2\delta_x - 0.1) q'_x - \frac{1}{3} q_g = 0 \quad (4.3)$$

$$q_x{}' - q_x = 0 \quad (4.4)$$

The ranges of the values of the metabolic coefficients δ_x and δ are: δ_x [0.08, 0.14] (C-mol lactose)*(C-mol biomass)$^{-1}$ (Nielsen et al, 2003); it is often the value $\delta_x = 0.095$ (C-mol lactose)*(C-mol biomass)$^{-1}$ (Roels, 1983). The amount of ATP consumed for the formation of biomass precursors, δ, is more difficult to be specified; a value equal to 0.051 (mol ATP)*(C-mol biomass)$^{-1}$ can be assumed as reported in other cases (Roels, 1980; 1983).

The metabolic coefficient for biomass polymerization, K, can be taken in the range 1.5 – 2.5 (mol ATP)*(C-mol biomass)$^{-1}$, particularly, a value equal to 2 (mol ATP)*(C-mol biomass)$^{-1}$ was experimentally found by Roels (1983), a value equal to 1.5 (mol ATP)*(C-mol biomass)$^{-1}$ was determined by Verduyn et al. (1991) and, finally, a value equal to 1.75 (mol ATP)*(C-mol biomass)$^{-1}$ was used by Krzystek and Ledakowicz (2000). Eventually, an empirical value of 2.42 (mol ATP)*(C-mol biomass)$^{-1}$ was used

for the total ATP consumption for biomass formation (precursor formation plus polymerization) for anaerobic fermentation of *S. cerevisiae* (Nielsen et al, 2003) and, in the same text, better results were obtained with a total value equal to 1.8 (mol ATP)*(C-mol biomass)$^{-1}$. By performing a substrate balance and solving the system constituted by Equations2, 3 and 4, the expressions for substrate consumption, ethanol and glycerol formation can be obtained as in Equations 4.5, 4.6 and 4.7.

$$q_g = 0.270 q_x \tag{4.5}$$

$$q_e = 4.282 q_x + 2mX \tag{4.6}$$

$$(-q_s) = 7.790 q_x + 3mX \tag{4.7}$$

In these equations, the rates of consumption/production of the compounds are obtained as functions of the biomass growth rate and the maintenance coefficient (an exhaustive discussion on the meaning of these terms will be given later on). In order to complete the model, a kinetic form for biomass growth needs to be defined. To this end, a modified version of the expanded Monod expression is used (Equation 4.8).

$$q_x = \frac{1}{1+e^{t_d - t}} \frac{\mu_m C_s}{C_s + K_{ss}} \frac{K_e}{C_e + K_e} X \tag{4.8}$$

The equation is constituted by three terms, the first one is a delay function (Vanrolleghem et al, 2004; Sin et al, 2008) necessary in order to take into account the presence of the lag-phase of the microorganism; the second term is the well known Monod expression for the specific growth rate μ and the last term is a function which

takes into consideration a possible ethanol inhibition effect. The meaning and units of all the symbols are reported in *section 2.3*.

4.2.3 Model parameters

The model's parameters are constituted by the kinetic parameters introduced with Equation8, i.e. μ_m, K_{ss}, K_e, t_d and the metabolic parameter m introduced in Equation 4.1.

The term t_d [h] is the delay time to be considered so as to take into account the lag phase of the microorganism. The value of t_d was chosen by means of experimental observations, and it does not need to be estimated. The parameter μ_m [h^{-1}] is the maximum specific growth rate. The parameter K_{ss} [C-mol*L^{-1}] is the limiting substrate concentration, assumed to be the concentration at which the specific growth rate is half its maximum value. The parameter K_e [C-mol*L^{-1}] is the inhibiting ethanol concentration and m [(mol ATP)*(C-mol biomass*h)$^{-1}$] is the specific maintenance requirements for ATP.

4.3Materials and Methods

4.3.1 Yeast strain and inoculum preparation

Lactose fermentations were performed with yeast *Kluyveromyces marxianus var. marxianus CBS 397*, obtained from the *Centraalbureau voor Schimmercultures (Utrecht, the Netherlands)*. It was maintained in a sterile generic yeast medium having the following composition: agar, 10 g L^{-1} (*Fluka, Italy*) lactose, 20 g L^{-1} (*Fluka, Italy*), peptone (from casein), 10 g L^{-1} (*code 82303,Fluka, Italy*), and yeast extract, 5 g L^{-1}(*code*

70161,Fluka, Italy). The yeast inoculum was spread on the surface of the agar plates, incubated at 20 °C for 48 h and stored at 4 °C.

The inoculum was prepared by introducing a single colony into 100 mL of sterile medium in a 300 mL Erlenmeyer flask. The inoculum medium contained lactose, 50 g L^{-1}, peptone, 10 g $L^{-1}$and yeast extract, 5 g L^{-1}. The flasks were incubated in a *GRANT OLS 200* thermostated bath for 12 h at 37 °C with an orbital shaking velocity of 150 rpm.

4.3.2 Fermentations in a controlled bio-reactor.

A controlled batch bioreactor, consisting of a 1.5 L autoclavable plexiglass cylinder (*Applikon, Holland*), was used. The main operating parameters (pH, O_2 concentration, temperature, agitation and foam level) were monitored by a set of sensors and controlled by means of an *ADI 1030* s*helf – top* controller. pH was continuously adjusted by adding 2N NaOH and no relavant foam was formed so the addition of antifoam was not needed. The agitation was provided by an impeller connected to a stirrer speed controller, *ADI 1032*. The fermenter and all probes and connections were sterilized (without the fermentation medium) before use.

The fermentation medium consisted of non-sterilised ricotta cheese whey only and was kindly provided by *Agroalimentare Asso.La.C.* (*Italy*). It consisted of (% w/v): proteins (0.15 – 0.22), lactose (4.8 – 5.0), salts (1.0 – 1.3), organic acids (0.20 – 0.25). The whey was not sterilized prior to fermentation and this was possible since it contained a very low microorganism concentration due to the thermal treatment involved in the ricotta cheese production process (Sansonetti et al, 2009).

Three different types of fermentation (in duplicate) were carried out at different operating conditions as reported in Table 4.2.

Table 4.2. Operating conditions for the fermentations conducted. Instrumentation and procedures were the same in all cases.

Run	Temperature [°C]	Agitation rate [rpm]	pH	Initial lactose concentration [g L^{-1}]
1	34	200	5	46.6
2	37	200	5	47.7
3	33	195	5.4	44.0

These conditions were chosen for developing the model based on previous work in which the optimal conditions for ethanol yield were studied (Sansonetti et al., 2010). All the fermentations were performed under self anaerobic conditions and were started by adding 100 mL of inoculum to 1 L of whey in the fermenter. Each experiment was replicated twice and the maximum deviation of each correspondent value from the mean value did not exceed 5 %.

4.3.3 Analytical methods

Samples for determination of lactose and ethanol were immediately centrifuged at 5000 rpm (7000 g), then filtered through a 0.5 µL filter and analyzed by HPLC. A *Supelcogel* (*USA*) 50x4.6 mm pre-column, *Supelcogel* C-610 300x7.8 mm column and a refractive index detector (*Jasco RI 930*) were used. A 0.1% v/v phosphoric acid solution was used isocratically as mobile phase at a flow rate of 0.5 mL min^{-1}.

Samples for biomass determination (100 µL) were immediately diluted in 25 mL of 2% sodium citrate solution, to avoid undesirable osmotic effects on the cells. A BactoScan FC (*Foss Integrator, Denmark*) was then employed, which determines the number of cells per unit volume of solution. The amount of biomass, on a mass basis, was obtained multiplying the cell concentration by 303 ng $cell^{-1}$ (Ghaly ad El Taweel, 1994).

4.3.4 Parameter estimation, confidence intervals and uncertainty

The model has been implemented in Matlab (*The Mathworks, Natick, MA, USA*). The system of ordinary differential equations was solved by means of the Runge-Kutta-Feldberg algorithm (ode45).

A non linear least squares method was used for the parameter estimation by using as objective function the sum of squared errors between the measurements and their corresponding model predictions (Seber and Wild, 1989):

$$J(\boldsymbol{\theta}) = \sum_{k=1}^{N} \sum_{t=1}^{M} \left(\frac{y_{k,meas}(t) - y_k(t,\boldsymbol{\theta})}{\sigma_{k,t}} \right)^2 \qquad (4.9)$$

$J(\boldsymbol{\theta})$ is the sum of squared errors, *N* stands for the total number of variables measured, e.g. lactose, biomass, ethanol etc., *M* is the total number of measurements of each variable, $y_{k,meas}(t)$ refers to measured values of the variables at time *t*, $y_k(t, \boldsymbol{\theta})$ refers to predicted values of the variables at time *t* with a given set of parameter values $\boldsymbol{\theta}$; finally, $\sigma_{k,t}$ refers to the standard deviation of the measurement errors at time *t* for the variable *k*. The standard deviation considered in the algorithm was set up at the value corresponding to the maximal deviation of the measurements from the average value obtained during the experiments at each time instant, which was equal to 5%. The objective function (4.9) was minimized by using the trust-region based nonlinear minimization algorithm in Matlab (*fminsearch* function).

The confidence intervals of the estimated parameters were determined by means of a linear approximation of the covariance matrix of parameter estimators, $\boldsymbol{COV}(\boldsymbol{\theta})$(Omlin and Reichert, 1999), as reported in Equation 4.10, where P is the total number of estimated parameters and the other symbols as defined earlier.

$$\boldsymbol{COV(\theta)} = \frac{J(\boldsymbol{\theta})}{NM-P}\left(\frac{\partial \boldsymbol{y_k}}{\partial \boldsymbol{\theta}}\right)^T \begin{pmatrix} \frac{1}{\sigma_{1,1}^2} & 0 & 0 \\ 0 & \ddots & 0 \\ 0 & 0 & \frac{1}{\sigma_{k,NxM}^2} \end{pmatrix} \left(\frac{\partial \boldsymbol{y_k}}{\partial \boldsymbol{\theta}}\right) \qquad (4.10)$$

The confidence interval of the estimated parameters at (1-α) confidence level is given by:

$$\boldsymbol{\theta_{1-\alpha}} = \boldsymbol{\theta} \pm \sqrt{diag\left(\boldsymbol{COV(\theta)}\right)} \cdot t\left(N-M, \frac{\alpha}{2}\right) \qquad (4.11)$$

where$t\left(N-M, \frac{\alpha}{2}\right)$ is the t-distribution value corresponding to the α/2 percentile with N – M degrees of freedom.

The linear correlation between two parameters, R_{ij}, is given by:

$$R_{ij} = \frac{COV(\theta_i, \theta_j)}{\sqrt{\sigma_{\theta_i}^2 \sigma_{\theta_j}^2}} \qquad (4.12)$$

The confidence interval of the predictions is calculated using the covariance matrix of estimated parameters. First, the covariance matrix of predictions is approximated using linear error propagation (Omlin and Reichert, 1999) as follows:

$$\boldsymbol{COV(y_k)} = \left(\frac{\partial \boldsymbol{y_k}}{\partial \boldsymbol{\theta}}\right) COV(\theta) \left(\frac{\partial \boldsymbol{y_k}}{\partial \boldsymbol{\theta}}\right)^T \qquad (4.13)$$

Then, the confidence interval of the predictions at (1-α) confidence level is given by:

$$\boldsymbol{y_{k,1-\alpha}} = \boldsymbol{y_k} \pm \sqrt{diag\left(\boldsymbol{COV}(\boldsymbol{y_k})\right)} \cdot t\left(N - M, \frac{\alpha}{2}\right) \quad (4.14)$$

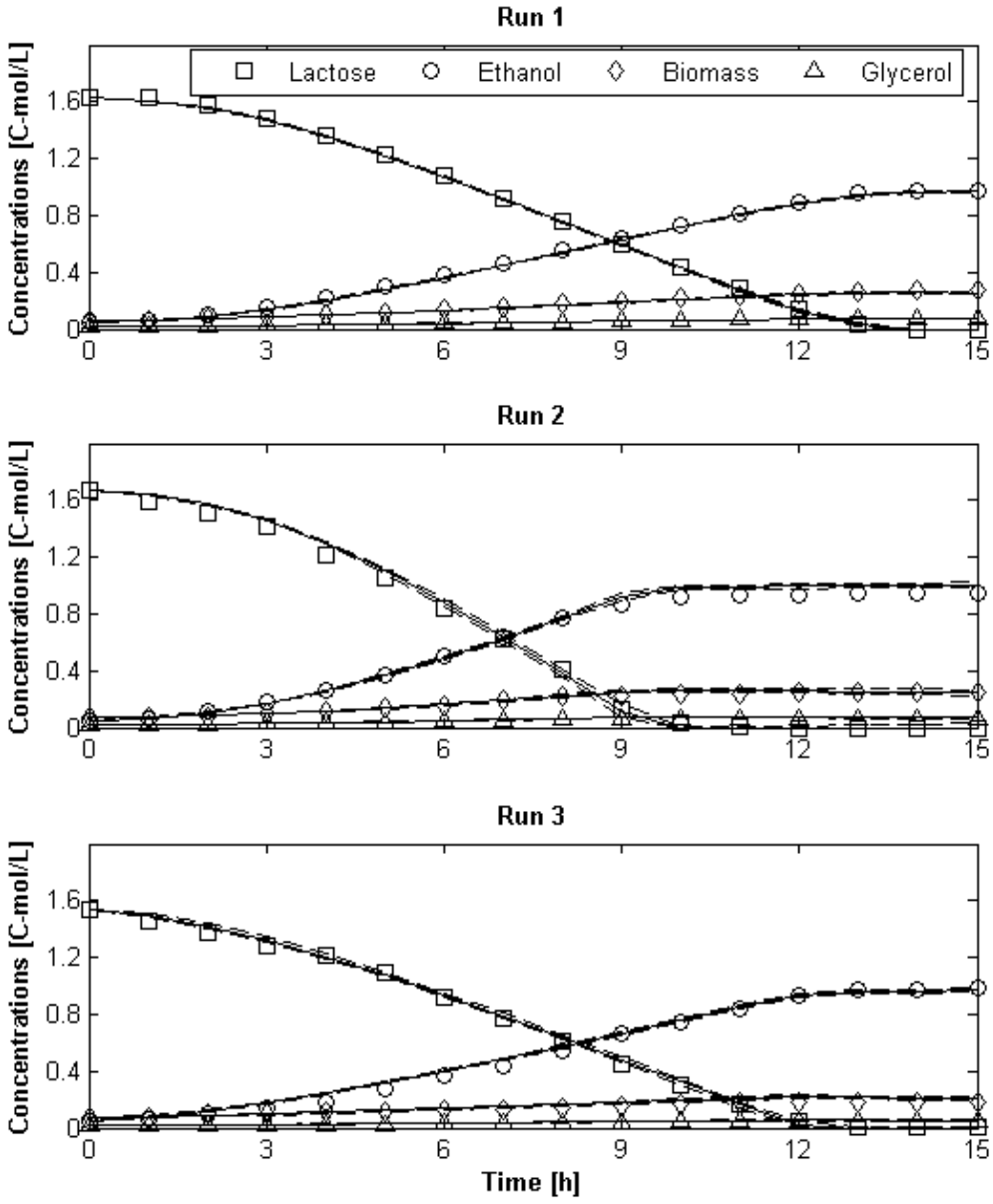
The above mentioned methods for the calculation of parameter estimation errors and the correlation matrix were implemented in *Matlab* as m-file scripts.

4.4 Results and discussion

4.4.1 Experimental data

The anaerobic fermentations, represented by runs 1, 2 and 3 (Table 4.2), provided three sets of experimental data in terms of lactose, ethanol, biomass and glycerol concentrations versus time. The experimental results are represented by the data points in Figure 4.2 and summarised in Table 4.3.

Figure 4.2. Lactose, ethanol, biomass and glycerol concentrations versus time for Runs 1, 2 and 3 at the operating conditions reported in table2. The results showed are averaged values of two replicates where the deviation from the mean value was always less than 5%.



For fermentation 1 (34^{o}C) a lag phase of 1.9 h was followed by complete lactose consumption within 14 h leading to the formation of approximately 6.6 g L^{-1} biomass, 22.5 g L^{-1} ethanol and 2.4 g L^{-1} glycerol. The trends were the same for all fermentations, with lag phase of similar length but fermentation time being longer at 33^{o}C and shorter at 37^{o}C. Interestingly, the ethanol yield, as a percentage of the theoretical, was highest (95%) in run 3. Ethanol yield, expressed for instance as gram of ethanol formed per gram of lactose consumed, is extremely important for the evaluation of the fermentation performance. This was calculated based on biochemical reaction II (Table4.1)which gives a theoretical yield of 0.538 g ethanol formed per g lactose. The best performance (i.e. highest percentage yield) was obtained in run 3, which is consistent with the optimal set of operating conditions predicted by the empirical model developed in our previous work (Sansonetti et al, 2010).

Table 4.3. Summary of experimental results corresponding to Figure 4.2.

Run*	Ethanol [g L^{-1}]	Glycerol [g L^{-1}]	Biomass [g L^{-1}]	Initial lactose concentration [g L^{-1}]	Ethanol yield [%]	Complete lactose consumption time [h]
1	22.48	2.36	6.61	46.58	90	14
2	21.75	2.01	6.55	47.63	85	11
3	22.68	1.28	4.41	43.99	95	15

*See Table 2 for a summary of fermentation conditions.

The best performance, obtained in run 3 (ethanol yield 95%), is most likely due to the slightly higher pH (5.4) and lower temperature (33^{o}C),which is evidently less suitable for biomass formation (μ_{max} = 0.167 h^{-1} compared to 0.3 h^{-1} under the other conditions (see Table4.4).The final biomass concentration and biomass yield (0.10 g biomass)*(g lactose)$^{-1}$ were the lowest with respect to the other two runs and the consequence is that more lactose is available for ethanol formation. Interestingly, the final glycerol

concentration and glycerol yield (0.029 g glycerol)*(g lactose)$^{-1}$ obtained in run 3, is about half of that in runs 1 and 2. Furthermore, the glycerol yield on biomass (0.29 g glycerol)*(g biomass)$^{-1}$ is similar to that at 37^{o}C (0.31 g*g^{-1}) and lower than at 34^{o}C (0.36 g*g^{-1}), suggesting that more lactose was indeed diverted to ethanol under the optimal conditions. A possible explanation can be provided by observing the proposed model. For the pathway leading to biomass precursors described in biochemical reaction I (Table4.1), it can be seen that this reaction involves, the formation of reducing equivalents (NADH was chosen in this work to represent any reducing equivalents form) which are consumed in reaction III, namely the one leading to glycerol formation (Verduyn et al, 1990a; 1990b). Therefore, the lower amount of biomass implies the formation of a less NADH, and thus, a lower amount of glycerol is produced.

4.4.2 Model Identification

The parameter estimation procedure (see *section 4.3.4*) provided the values reported in Table4.4. The value for t_d was obtained from inspecting a semi log plot of the data in Figure4.2 and was 1.9 h for both runs1 and 2, and at 1.7 h for run3.

Table 4.4. Estimated values of the parameters and the corresponding confidence intervals of the estimates. In the last column the standard deviation of the parameters in the three runs is reported with respect to the mean value obtained in the three runs.

Parameter	Units	Value (Run 1)	Value (Run 2)	Value (Run 3)	Standard deviation [%]
μ_m	h^{-1}	0.300 ± 0.018	0.300 ± 0.024	0.167 ± 0.019	7.7
K_{ss}	(C-mol)*L^{-1}	0.066 ± 0.036	0.059 ± 0.025	0.029 ± 0.004	3.0
K_e	(C-mol)*L^{-1}	0.382 ± 0.072	0.668 ± 0.009	0.701 ± 0.27	17.5
m	(mol ATP)*(C-mol biomass*h)$^{-1}$	0.022 ± 0.0078	0.036 ± 0.019	0.088 ± 0.02	3.5

In general, the results in Table 4.4 indicate values that are consistent with the literature. Values of μ_{max} have been reported to vary between 0.29 h^{-1} (Barba et al., 2001) and 0.55 h^{-1} (Longhi et al., 2004) for *K. marxianus* growth on cheese whey (Zafar, 2005), the value of K_{ss} is usually reported to be very low for yeast (Nielsen et al, 2003) and a mean value of *m* of ca 0.05 (mol ATP)*(C-mol biomass*h)$^{-1}$isconsistent with the literature (Flickinger and Drew, 1999; Roels and Kossen, 1978). Interestingly there is approximately a two-fold difference in the maximum specific growth rate, the maintenance coefficient and the limiting lactose uptake concentration in run 3 compared to run 1 and run 2. The inhibiting ethanol concentration in run 3 is similar to that of run 2 but twice as high as at run1. Differences in growth rate are to be expected and thus under less favorable growth conditions (e.g. low temperature) it may also be expected that maintenance would be higher, and ethanol inhibition more severe. Nevertheless when the standard deviations between the three fermentation types are determined, they are low except for the ethanol inhibition parameter (K_e = 17.5%), which, most likely is affected by a strong correlation with one or more parameters. The correlation matrixes for each run performed are reported in Table 4.5 and used to assess the effect of the numerical strategy, followed for the model identification, on the reliability of the model parameters. In other words, an evaluation about which parameters are dependant on each other, and which parameters vary independently and can thus be used as unique values for model development was performed and the results reported in Table 4.5.

Table 4.5. Correlation matrixes for runs 1, 2 and 3 (since the correlation matrix is symmetrical, the upper triangular matrix only is reported).

	Run 1				Run 2				Run 3			
θ	μ_m	K_{ss}	K_e	m	μ_m	K_{ss}	K_e	m	μ_m	K_{ss}	K_e	m
μ_m	1	-0.569	-0.908	-0.415	1	0.921	0.085	-0.492	1	-0.145	-0.825	-0.524
K_{ss}		1	0.837	0.251		1	0.164	-0.191		1	0.606	-0.254
K_e			1	0.257			1	0.170			1	0.067
m				1				1				1

A high correlation coefficient (which usually means values higher than 0.60) between two parameters means the values of these parameters are not suitable for use as unique values in modeling (since the parameter value itself is affected by the value of the correlated parameter). Inspection of Table 4.5 shows quite fair values of the correlation coefficients, mainly for runs 2 and 3. By looking at the matrix concerning run 1, it can be observed a strong correlation between μ_m and K_e (-0.908), and between K_{ss} and K_e (0.837). Such correlations indicate that the values of the involved parameters are not reliable. Rather, by looking at the other two correlation matrixes, better values of the correlation coefficients were achieved. About run 2, there is correlation betweenμ_m and K_{ss} (0.921), while, as far as run 3 is concerned, a certain correlation persists between $K_e$and μ_m. (0.825) About the maintenance parameter, m, the values of the correlation coefficients are acceptable in all runs suggesting that the estimated values are unique and can be relied on.

It can be concluded that the values of the kinetic parameter μ_m is always correlated with some other parameter, i.e. with $K_e$in runs 1 and 3 and with K_{ss} in run 2. With regard to K_{ss}, the correlation matrixes indicate the value obtained in run 3 is the most reliable; Same considerations can be made for $K_e$concluding that the values obtained in run 2 is more reliable because less correlated.

4.4.3 Evaluation of model descriptive performance

Although the model has already been demonstrated to give a good qualitative interpretation of the experimental data (see *section 4.1*) a deeper analysis of the model performance is needed and reported as follows. The continuous lines in Figure 4.2 represent the model simulations, while the dotted lines are the confidence intervals of the predictions. Qualitatively, the model fits the data well for all the runs. However, in run2 the final ethanol concentration predicted by the model is slightly higher than the experimental one after 10 h, when all of the lactose has been consumed. From the point

of view of process performance, it is interesting to compare the fitted process yields(i.e. Y_{sx}, Y_{se} and Y_{sg}, for yields of biomass, ethanol and glycerol on lactose, respectively) with those determined experimentally (Table4.6).The yields provided by the model are calculated as given in Equation 4.15.The rates q_j and q_i [(C-mol i,j)*(L*h)$^{-1}$] are determined from Equations 4.5, 4.6 and 4.7.

$$Y_{ij}(BSM) = \frac{q_j}{q_i} \tag{4.15}$$

Table 4.6[*]. Comparison of experimentally determined yields Y_{ij} (EXP) with the values provided by the model Y_{ij} (BSM). The percentage deviation of the model's yields from the experimental values is reported (calculated as $Ysi = (Y_{si}(EXP)-Y_{si}(BSM))/Y_{si}(EXP)$).

Run	Y_{sx} (EXP)	Y_{sx} (BSM)	Y_{se} (EXP)	Y_{se} (BSM)	Y_{sg} (EXP)	Y_{sg} (BSM)
1	0.164	0.155	0.598	0.592	0.047	0.045
2	0.148	0.148	0.565	0.599	0.039	0.041
3	0.116	0.119	0.636	0,639	0,027	0,030
Deviation of the fitted values from the experimental yield values [%]						
Run	**Y_{sx}**		**Y_{se}**		**Y_{sg}**	
1	5.65		1.08		5.32	
2	0.21		6.10		5.35	
3	2.75		0.50		12.47	

*Yields are measured in (C-mol *i*)*(C-mol *j*)$^{-1}$

The results in Table 4.6 show very good agreement between the model fittings and the experimental results, with all the differences less than 6.1%, except for Y_{sg} in run 3 (12.47%). Nevertheless, this is still acceptable given the very low absolute values of Y_{sg}.

The model developed so far, although still an unstructured one, possesses some important characteristics which give it "structure". The model is based on the stoichiometry of the main metabolic pathways through which the carbon is converted from lactose to the different products, which allows important qualitative conclusions to be made. The metabolic structure confirms, for instance, why glycerol cannot be

neglected in modelling the anaerobic fermentation in this microorganism. Glycerol is produced in reaction(III) in order to consume the reducing equivalents (NADH) produced in process (I) so as to maintain the balance (Table4.1) In the model developed, the rates of consumption or formation of the different intermediates and products have been calculated by applying simple but fundamental bioenergetics principles (ATP and reducing equivalents balances). Such a model allowed Equations 4.6 and 4.7 to be developed and that are meaningful unlike any equations coming from mere kinetic considerations. Equation 4.6 and 4.7, can be written in a general form (Equation 4.16 and 4.17) which is equivalent to the well-known linear product formation and linear substrate consumption equations involving the true yield coefficient (e.g. $Y_{i,j}^{true}$) and maintenance empirically introduced by Herbert and later on by Pirt (1965).

$$q_e = \frac{1}{Y_{ex}^t} q_x + m_e X \tag{4.16}$$

$$-q_s = \frac{1}{Y_{sx}^t} q_x + m_s X \tag{4.17}$$

By comparing these expressions with the ones obtained from the model (Equation 6 and 7), Equations 4.18 and 4.19 result.

$$\frac{1}{Y_{ex}^t} = 4.282 \quad and \quad m_e = 2m \tag{4.18}$$

$$\frac{1}{Y_{sx}^t} = 7.790 \quad and \quad m_s = 3m \tag{4.19}$$

In other words, the "true" yields, namely the true yield of ethanol on biomass, $\frac{1}{Y_{ex}^{t}}$, and the true yield of lactose on biomass, $\frac{1}{Y_{sx}^{t}}$, are fixed by metabolic constraints which are basically imposed by the thermodynamics of the bioprocess. The same holds for the maintenance coefficients. This is likely the most important benefit of the approach developed in the current work, i.e. the result is a system of equations where macroscopic quantities (rates of formation or consumption) are related to microscopic (mechanistic) quantities fixed by thermodynamics (ATP and reducing equivalent balances). In order to demonstrate this concept further, it is useful to report the general solution of the system constituted by Equations 4.2, 4.3 and 4.4 coupled with a substrate balance and this is given in Equations 4.20, 4.21 and 4.22.

$$q_g = 6(\delta_x - 0.05)q_x \tag{4.20}$$

$$q_e = 2[(K + \delta + 2\delta_x + 0.1)q_x + mX] \tag{4.21}$$

$$-q_s = [(3K + 3\delta + 13\delta_x + 0.4)q_x + 3mX] \tag{4.22}$$

The values of the true yields reported in Equations 4.18 and 4.19 were actually calculated from expressions 4.21 and 4.22 by fixing the values of the metabolic coefficients (see *section 4.2.2*). Now, in this form, the equations clearly show the relationship between the observable yields (macroscopic) and the true yields (functions of the metabolic coefficients). Thus, the following equations can be written:

$$\frac{1}{Y_{ex}} q_x = \frac{1}{Y_{ex}^{t}} q_x + m_e X = 2(K + \delta + 2\delta_x + 0.1)q_x + (2m)X \tag{4.23}$$

$$\frac{1}{Y_{sx}} q_x = \frac{1}{Y_{sx}^{t}} q_x + m_s X = (3K + 3\delta + 13\delta_x + 0.4)q_x + (3m)X \tag{4.24}$$

Here the yields on the left hand side are the observable yields (which can be calculated from the experiments). As a natural consequence, such model yields automatically consider the constraints imposed by thermodynamics, since bioenergetics principles have been used to derive them. This aspect gives the model an important physical meaning which is needed for a more rational approach to bioprocess development and scale-up.

4.5 Conclusions

A biochemically structured model has been developed which describes the anaerobic lactose fermentation process by *K. marxianus*. The structure of the model gives a very useful qualitative interpretation of experiments, providing a deep insight into the phenomena behind the fermentation process. Furthermore, the model also fits experimental batch fermentation data well and gives a good prediction of the experimentally derived yield coefficients. The model is based on metabolic constraints and provides values for the yields that satisfy both material and bioenergetic balances, thus such values do not violate the thermodynamics of the system. The model should now be further validated in new studies with different batches of cheese whey and at different volumes which are closer to industrial scale. Eventually, it must be considered that the model has been obtained under the assumption of pseudo-steady state conditions for biomass; this is a strong hypothesis for dynamic processes like a batch, indeed, it would be very interesting to assess the performances of the model if applied to continuous-mode lactose fermentation such as in chemostat configuration where the steady state conditions are trivially respected. This is what has been done in the next chapter.

Chapter V

Continuous lactose fermentation for ethanol production by *K. marxianus*: Development of a metabolically structured model

Anaerobic chemostat fermentations of lactose to ethanol by *K. marxianus* were performed in defined medium and the data used together with a new biochemically structured model to determine maintenance and true yield coefficients. Steady states at four dilution rates were achieved, namely 0.05, 0.10, 0.15, 0.20, however at 0.25 h^{-1} synchronous growth was seen. Remarkably high ethanol yields on substrate were observed, namely 0.653, 0.648, 0.633 and 0.623 (C-mol ethanol)*(C-mol lactose)$^{-1}$ at dilution rates of 0.05, 0.10, 0.15 and 0.20 h^{-1}, respectively, which were between 98 and 93% of the theoretical yield (0.666 (C-mol ethanol)*(C-mol lactose)$^{-1}$). A biochemically structured model was set up and the experimental data compared with it's predictions. There was very close agreement between the model and data, which permitted determination of the metabolic coefficients of the main pathways involved in the metabolic structure. Experimental data, coupled with the model, were used to determine the maintenance coefficients, i.e. m_s = 0.546 and m_e= 0.388 (C-mol i)*(C-mol x*h)$^{-1}$, and the true yields of Y^{true}_{sx} = 0.103, Y^{true}_{se} = 0.172 and Y_{sg} = 2.01 (C-mol)*(C-mol)$^{-1}$. The findings here can be used for designing bioethanol processes from waste whey feedstreams.

5.1 Introduction

Lactose conversion into ethanol can be achieved through anaerobic fermentation carried out by a suitable microorganism (Mawson, 1994; Berruga et al, 1997). This process has been considered for a long time (Whittier, 1944, Rogosa et al, 1947) as an alternative to dispose of (and valorize) the main waste from the dairy industry, namely cheese whey, which contains ca.5% (by weight) lactose. Several studies concerning lactose fermentation have been performed (Mawson, 1994; Ghaly and El Taweel, 1994; Zafar and Owais, 2005; Ozmihci and Kargi, 2007a; Sansonetti et al, 2009, Sansonetti et al, 2010). Most of the studies presented in the literature involve batch processes (Gawel and Kosikowski, 1978; Jenssens et al, 1984; Grubb and Mawson, 1993, Korkoutas et al, 2002; Longhi et al, 2004; Silveira et al, 2005). Nevertheless, the possibility to operate in continuous mode has been considered by several authors, using different reactor configurations (Linko et al, 1981; Cheryan and Mehaia, 1983; Kleine et al, 1995; Ozmihci and Kargi, 2007b, 2008).

Modelling of the fermentation process has been considered in several studies. They deal mainly with kinetic studies of the reactions involved (Lee and Rogers, 1983; Lee et al, 1983; Dourado et al, 1987). A collection of the most used kinetic equations for such fermentation processes was reported in a comparative study by Starzak et al. (1994). The main criticism of these models is the empirical nature of the equations involved. Indeed, no physical meaning can be attributed to the many terms constituting the model, and such a limitation results in poor descriptive and predictive performance. An interesting approach, that can be adopted to model the fermentation process, is the so called “biochemically structured approach” which was introduced for the first time by Roels and co-workers (1980; 1983). It is based on the consideration of the main metabolic pathways in the working microorganism, namely anabolic, catabolic, polymerization and maintenance reactions; its particular advantages will be illustrated later on. Several examples of this approach are found in the literature where the same principles have been applied to different processes (Krzystek and Ledakowicz, 1997, 2000; van Leeuwen et al, 1997; Heijnen et al, 1979). Although this approach has given fair results yielding deep

insights of the processes to which it has been applied, it has never been used for anaerobic fermentation of lactose. Furthermore, given there are few industrial applications of the lactose-to-ethanol process (all in batch configuration), namely *Carbery Group* (Ireland), *Anchor Ethanol* (New Zealand) and *Golden Cheese* (USA) it can be concluded that there is considerable scope for further efforts towards proper modeling aimed at a better characterization and optimization of the process.

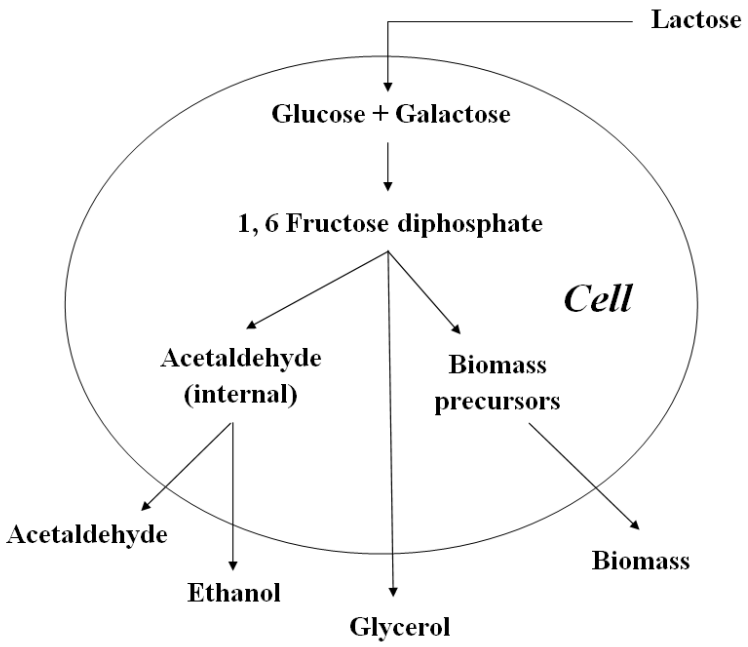
In the present work, chemostat fermentations of lactose to ethanol by the yeast *K. marxianus* have been studied at different dilution rates to generate a set of data which can be used to build a biochemically structured model.

5.2 Theoretical background and Modelling

5.2.1 Theoretical background

The biochemically structured approach of this work, is based on the main metabolic pathways involved in the carbon flux from the substrate to the products. Basically, these processes can be divided into: 1) anabolic reactions, in which the formation of biomass precursors is achieved, usually with the consumption of a certain amount of energy (ATP) and the production of reducing equivalents (indicated as NADH but representing all the forms of reducing equivalents). 2) Catabolic reactions, in which ATP is produced at the expense of a certain amount of substrate. 3) Polymerization of biomass precursors into active biomass with an extra consumption of ATP and 4) maintenance-associated ATP consumption. In order to identify the particular reactions involved in lactose fermentation, it is necessary to consider the metabolic structure involved. Lactose is mainly converted into biomass, ethanol, glycerol, carbon dioxide and acetaldehyde. The main metabolic pathways are depicted in Figure5.1. Besides it should be remarked that the excreted acetaldehyde is always present in negligible amounts (Nielsen et al, 2003) therefore it will not be considered in the model development.

Figure 5.1. Metabolic pathways considered in the model for the anaerobic bioconversion of lactose by *K. marxianus*.



Two other processes that play important roles in the fermentation reactions should also be considered in the model, namely polymerization of biomass precursors and biomass maintenance. With lactose as the only carbon source, the stoichiometry of each metabolic pathway is given in Table 5.1.

Table 5.1. Metabolic reactions involved in the model for anaerobic lactose fermentation by *K. marxianus*. Each reaction is written per unit of C-mol as recommended by Roels (1980). The rates of reaction, q_i, are expressed in [(C-mol i)*(L*h)-1].

Process	Stoichiometry
I. Formation of biomass* precursors	$(1+\delta_x)CH_{11/6}O_{11/12} + 0.2NH_3 + \delta ATP \xrightarrow{q_x'} CH_{1.8}O_{0.5}N_{0.2} + \delta_x CO_2 + 2(\delta_x - 0.05)NADH + \left(\frac{5-13\delta_x}{12}\right)H_2O$
II. Ethanol formation	$1.5CH_{11/6}O_{11/12} + 0.125H_2O \xrightarrow{q_e} CH_3O_{0.5} + 0.5CO_2 + 0.5ATP$
III. Glycerol formation	$CH_{11/6}O_{11/12} + \frac{1}{12}H_2O + \frac{1}{3}ATP + \frac{1}{3}NADH \xrightarrow{q_g} CH_{8/3}O$
IV. ATP consumption for polymerization of biomass	$CH_{1.8}O_{0.5}N_{0.2} + K \cdot ATP \xrightarrow{q_x} \frac{1}{n}(CH_{1.8}O_{0.5}N_{0.2})_n$
V. ATP consumption for maintenance	$ATP \xrightarrow{r_{ATP}} maintenancepuposes$

**K.marxianus* biomass is assumed to be$CH_{1.8}O_{0.5}N_{0.2}$ (Roels, 1983).

ATP consumption for both polymerization and maintenance results in a net ATP consumption rate, R_{ATP} [(mol ATP)*(L*h)$^{-1}$], which can be written as the sum of two terms, one growth-associated (IV), proportional to q_x, and one due to the maintenance (V). ATP consumption for maintenance is assumed to be directly related to the biomass concentration X [(C-mol)*L^{-1}] (Stouthamer and Bettenhaussen, 1973) as shown in Equation 5.1 where m [(mol ATP)*(C-mol biomass*h)$^{-1}$]represents the maintenance coefficient for biomass and K [(mol ATP)*(C-mol biomass)$^{-1}$] is the metabolic coefficient for biomass polymerization.

$$R_{ATP} = -Kq_x - mX \tag{5.1}$$

Process I contains two metabolic coefficients, δ_x [(C-mol lactose)*(C-mol biomass)$^{-1}$] that represents the amount of carbon lost as carbon dioxide and δ [(mol ATP)*(C-mol

biomass)$^{-1}$] which is the amount of ATP consumed in the anabolic formation of biomass precursors. In general this term depends on the culture conditions.

5.2.2 Model development

At steady-state conditions in a chemostat the conservation balances (referring to the stoichiometric scheme reported in Table 5.1) for ATP, reducing equivalents and biomass can be written as follows(Equations 5.2, 5.3 and 5.4).

$$-\delta q_x' + 0.5q_e - \frac{1}{3}q_g - R_{ATP} = 0 \quad (5.2)$$

$$(2\delta_x - 0.1)q_x' - \frac{1}{3}r_g = 0 \quad (5.3)$$

$$q_x{}' - q_x = 0 \quad (5.4)$$

The ranges of the values of the metabolic coefficients δ_x and δ are: δ_x [0.08, 0.14] (C-mol lactose)*(C-mol biomass)$^{-1}$ (Nielsen et al, 2003); it is often the value $\delta_x = 0.095$ (C-mol lactose)*(C-mol biomass)$^{-1}$ (Roels, 1983). The amount of ATP consumed for the formation of biomass precursors, δ, is more difficult to be specified since it depends on the culture conditions and on the carbon source; a value equal to 0.051 (mol ATP)*(C-mol biomass)$^{-1}$ can be assumed as reported in other cases for glucose fermentation by yeasts (Roels, 1980; 1983). The metabolic coefficient for biomass polymerization, K, is expected to be in the range 1.5 – 2.0 (mol ATP)*(C-mol biomass)$^{-1}$. Values of (mol ATP)*(C-mol biomass)$^{-1}$ 2 were experimentally found by Roels (1983) with glucose as the only carbon source for the yeast *S. cerevisiae*, the same value was also used by Starzak et al (1994) for ethanol fermentation on sucrose by *S. cerevisiae* and, 1.75 (mol ATP)*(C-mol biomass)$^{-1}$ was used by Krzystek and Ledakowicz (2000) for aerobic lactose conversion by *K. fragilis*. More recently, an empirical value of 1.8 (mol

ATP)*(C-mol biomass)$^{-1}$ was used for the total ATP consumption for biomass formation (precursor formation plus polymerization) for anaerobic fermentation of glucose by *S. cerevisiae* obtaining good agreement with experimental results (Nielsen et al, 2003).

By performing a substrate balance and solving the system constituted by Equations 5.2, 5.3 and 5.4, the expressions for substrate consumption, ethanol and glycerol formation can be obtained as given in Equations 5.5, 5.6 and 5.7.

$$q_g = 6(\delta_x - 0.05)q_x \tag{5.5}$$

$$q_e = 2[(K + \delta + 2\delta_x + 0.1)q_x + mX] \tag{5.6}$$

$$-q_s = [(3K + 3\delta + 13\delta_x + 0.4)q_x + 3mX] \tag{5.7}$$

In Equations 5.5, 5.6 and 5.7 the rates of consumption or production of the compounds are obtained as functions of the biomass growth rate and the maintenance coefficient (an exhaustive explanation will be given later on). Therefore, by dividing Equations 5.5, 5.6 and 5.7 by the biomass concentration X the expressions of the rates, r_i, [(C-mol i)*(C-mol biomass*h)$^{-1}$] are obtained (Equations 5.8, 5.9 and 5.10). These equations were written by considering sterile feed and steady state conditions, therefore $\mu = D$ (where D is the dilution rate).

$$r_g = 6(\delta_x - 0.05)D \tag{5.8}$$

$$r_e = 2[(K + \delta + 2\delta_x + 0.1)D + m] \tag{5.9}$$

$$-r_s = [(3K + 3\delta + 13\delta_x + 0.4)D + 3m] \tag{5.10}$$

In analogy with the linear relationships for substrate consumption and product formation introduced empirically by Herbert and Pirt (1965), the following equalities result (Equations 5.11-5.15).

$$\frac{1}{Y_{gx}} = 6(\delta_x - 0.05) \quad (5.11)$$

$$\frac{1}{Y_{ex}^{true}} = 2[(K + \delta + 2\delta_x + 0.1)] \quad (5.12)$$

$$\frac{1}{Y_{sx}^{true}} = [(3K + 3\delta + 13\delta_x + 0.4)] \quad (5.13)$$

$$m_e = 2m \quad (5.14)$$

$$m_s = 3m \quad (5.15)$$

5.3Materials and Methods

5.3.1 Yeast strain and inoculum preparation

The inoculum was prepared with a single colony withdrawn from the Petri dishes and incubated in a thermostated bath *Thermoscientific MaxQ 8000* (*USA*), maintained for 36 h at a temperature of 33°C with an orbital shaking velocity of 150 rpm. In all the experiments 150 mL of medium were poured in a 500 mL sterile flask. Each of the used materials, before performing this stage, was autoclaved at 121°C for 30 min. The inoculum medium had the composition as follows: lactose 40 g*L^{-1}, peptone 10 g*L^{-1} (from casein, *code 82303,Fluka, Denmark*), yeast extract 5 g*L^{-1} (*code 70161,Fluka, Denmark*) and ergosterol 10 mg*L^{-1}.

K. marxianus was maintained in a generic yeast medium having the following composition: agar, 10 g L^{-1} (*Fluka, Denmark*) lactose, 20 g L^{-1} (*Fluka, Denmark*),

peptone (from casein, *code 82303, Fluka, Denmark*), 10 g L^{-1}, and yeast extract, 5 g L^{-1} (*code 70161,Fluka, Denmark*). The culture was sterilized in autoclave at 121°C for 30 min, then it was poured on Petri dishes for solidification and, eventually, the yeast inoculum was spread on the surface and incubated at 20°C for 48 h. At completed growth, the dishes were kept at 4°C.

5.3.2 Fermentations in a controlled bio-reactor

Chemostats at five different dilution rates were conducted, namely 0.05, 0.10, 0.15, 0.20 and 0.25 h^{-1}. The other operating conditions were the same for all the runs, namely 33°C temperature, 195 rpm agitation rate, 5.4 pH and 0.2 $L*min^{-1}$ sparged nitrogen flow. The chemostats were started freshly for each steady state.

All the experiments were performed under anaerobic conditions using the procedure described in the following. A 1.5 liters fermentation medium was started with 75 mL inoculum (the 5% of the volume to be inoculated). Anaerobic conditions were ensured by a sparging with a continuous nitrogen flow (0.2 $L*min^{-1}$) pure at 99.9%. After a period of time of 12 hours in which the reactor had been operated in batch mode in order to allow the biomass to reach a certain concentration level, both the inflow (sterile feed) and the outflow were open at the dilution rate desired. Once steady state was reached, biomass and the other products were measured as described in *section 5.3.3*.

The lactose-based fermentation medium had the same composition as the medium used for the inoculum.

A controlled bioreactor, consisting of a 1.5 L autoclavable plexiglass cylinder (*Biostat B. Braun Int., Germany*), was used to perform the present experimental study. The main operating parameters (pH, dissolved O_2, temperature, agitation and foam level) were monitored by a set of sensors and controlled by means of a *Biostat B* controller (*Biostat B. Braun Int., Germany*).

5.3.3 Analytical methods

The cell dry weight was determined by filtering 10 mL of sample through a pre-dried and weighed 45 mm diameter 0.45 µm filter (*Millipore, USA*) which was subsequently dried in a *Samsung M1713* microwave oven at 200 W for 15 min, then weighed using a *Sartorius LE2445* analytical balance (*Germany*).

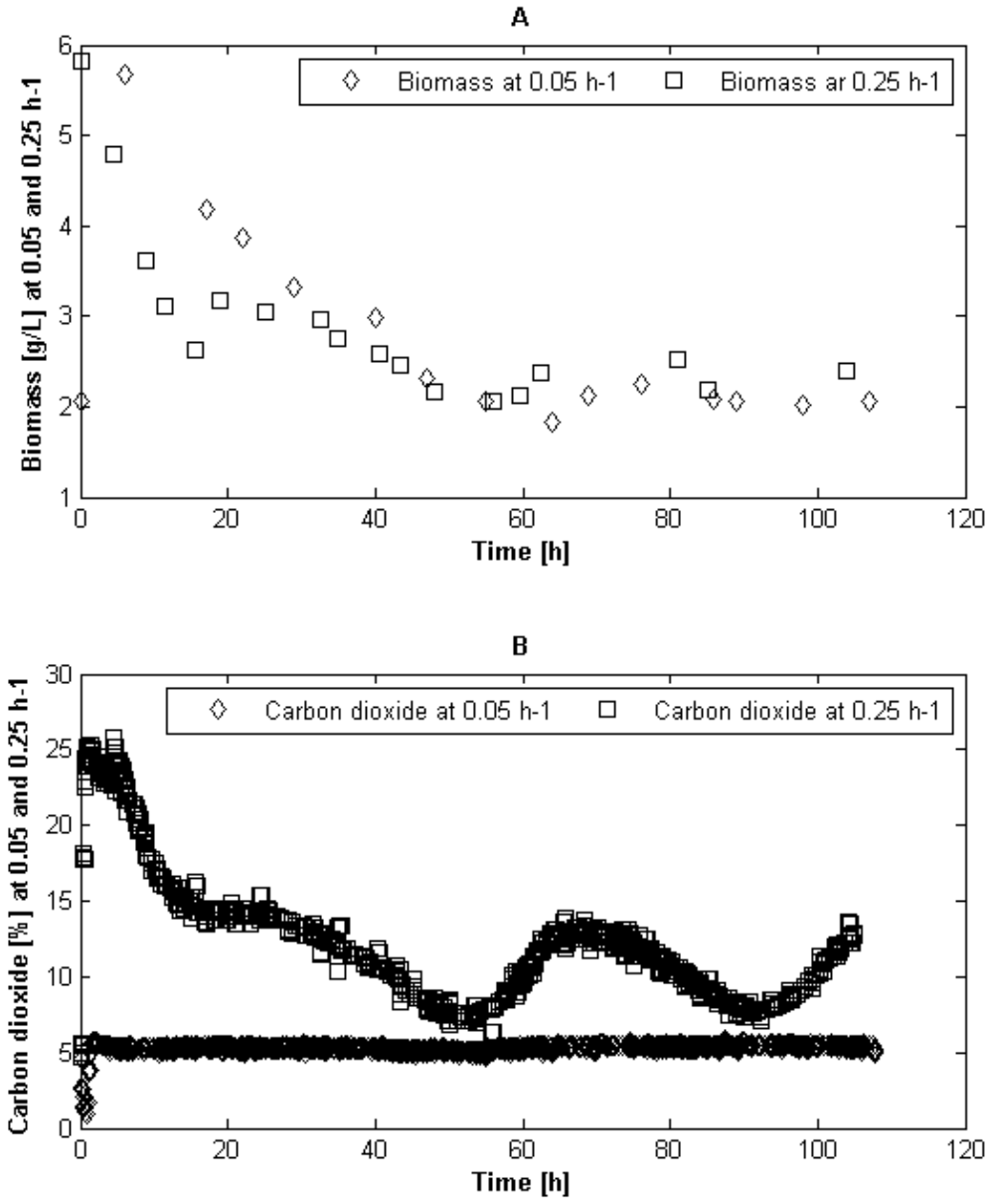
Lactose, glucose, galactose, ethanol, glycerol, pyruvate and acetate were measured using a HPLC method. A 1 mL sample was filtered to remove cells immediately after removal from the fermenter, frozen and analyzed later by HPLC. An *Agilent 1100* (*USA*) was used with an *Aminex HPX 87-H* 300x7.8 (*Bio-rad, USA*) column operated at 60°C. The amount of carbon dioxide produced in the off-gas was monitored by means of a *B. Braun MUX-11* gas analyzer (*Braun Int., Germany*).

5.4 Results and discussion

Chemostat cultivations were conducted at five different dilution rates and examples of their behaviour are given in Figure 5.2 where biomass concentrations (2A) and carbon dioxide (2B) for runs at 0.05 and 0.25 h^{-1} were reported.

In Figure 5.2A it can be seen that at a dilution rate of 0.05 h^{-1}, the cultivation achieved a steady state after about 70 h; the steady state is confirmed by the data concerning the carbon dioxide in the same run, indeed a constant value resulted during all the run (Figure 5.2B). The same behaviour was seen for all the cultivations (data not reported here), except at a dilution rate of 0.25 h^{-1} (Figures 5.2A and 5.2B). In Figure 5.2A is reported also the biomass trend at 0.25 h^{-1}, it is evident a synchronous growth started after approximately 20 h, and therefore that a steady state was not reached, which is consistent with the data regarding carbon dioxide shown in Figure 5.2B where it is clearly shown an established synchronous growth. Such a problem made the 0.25 h^{-1} run useless for the interpretation of the experimental data and thus was not considered in the rest of the study.

Figure 5.2. Dynamic experimental data regarding both biomass (A) and carbon dioxide (B) in chemostat fermentations at 0.05 and 0.25 h^{-1}.



The steady state values of the different species entering and leaving the chemostat at the five different dilution rates examined are reported in Table 5.2.

Table 5.2.Steady state values of the flow rates of the different species. The dilution rate D is expressed in [h^{-1}] and the flow rates in [C-mol*h^{-1}].

D	Lactose	Lactose	Ethanol	Biomass *	Glycerol	CO_2	Pyruvate	Acetate
	IN	OUT						
0.05	0.1128	0.0000	0.0737	0.0063	0.0042	0.0275	0.0004	0.0007
0.10	0.2254	0.0017	0.1449	0.0137	0.0086	0.0541	0.0007	0.0014
0.15	0.3483	0.0103	0.2141	0.0237	0.0155	0.0837	0.0013	0.0008
0.20	0.4917	0.0576	0.2705	0.0371	0.0194	0.1028	0.0006	0.0014

*Biomass formula was considered as follows: $CH_{1.8}O_{0.5}N_{0.2}$ (Roels, 1983)

The values in Table 5.2 can be used to calculate the yields on substrate of all the products using Equation 5.16 wherew_i and w_s are the C-molar flow rates of the compound *i* and the substrate (lactose), respectively, expressed in C-mol*h^{-1}. The results are presented in Table 5.3.

$$Y_{si} = \frac{w_i}{w_s} \tag{5.16}$$

Table 5.3. Experimental values of product yields (Cmol*$Cmol^{-1}$)on substrate. The following notation has been used: substrate (s), biomass (x), ethanol (e), glycerol (g), carbon dioxide (c), pyruvate (p), acetate (a). In the last column the sum of the yields for each chemostat is reported.

D [h^{-1}]	Ysx	Yse	Ysg	Ysc	Ysp	Ysa	Sum
0.05	0.0554	0.6535	0.0369	0.2435	0.0033	0.0063	0.9990
0.10	0.0613	0.6478	0.0386	0.2419	0.0031	0.0061	0.9987
0.15	0.0701	0.6334	0.0458	0.2477	0.0039	0.0025	1.0035
0.20	0.0854	0.6232	0.0446	0.2369	0.0014	0.0032	0.9946

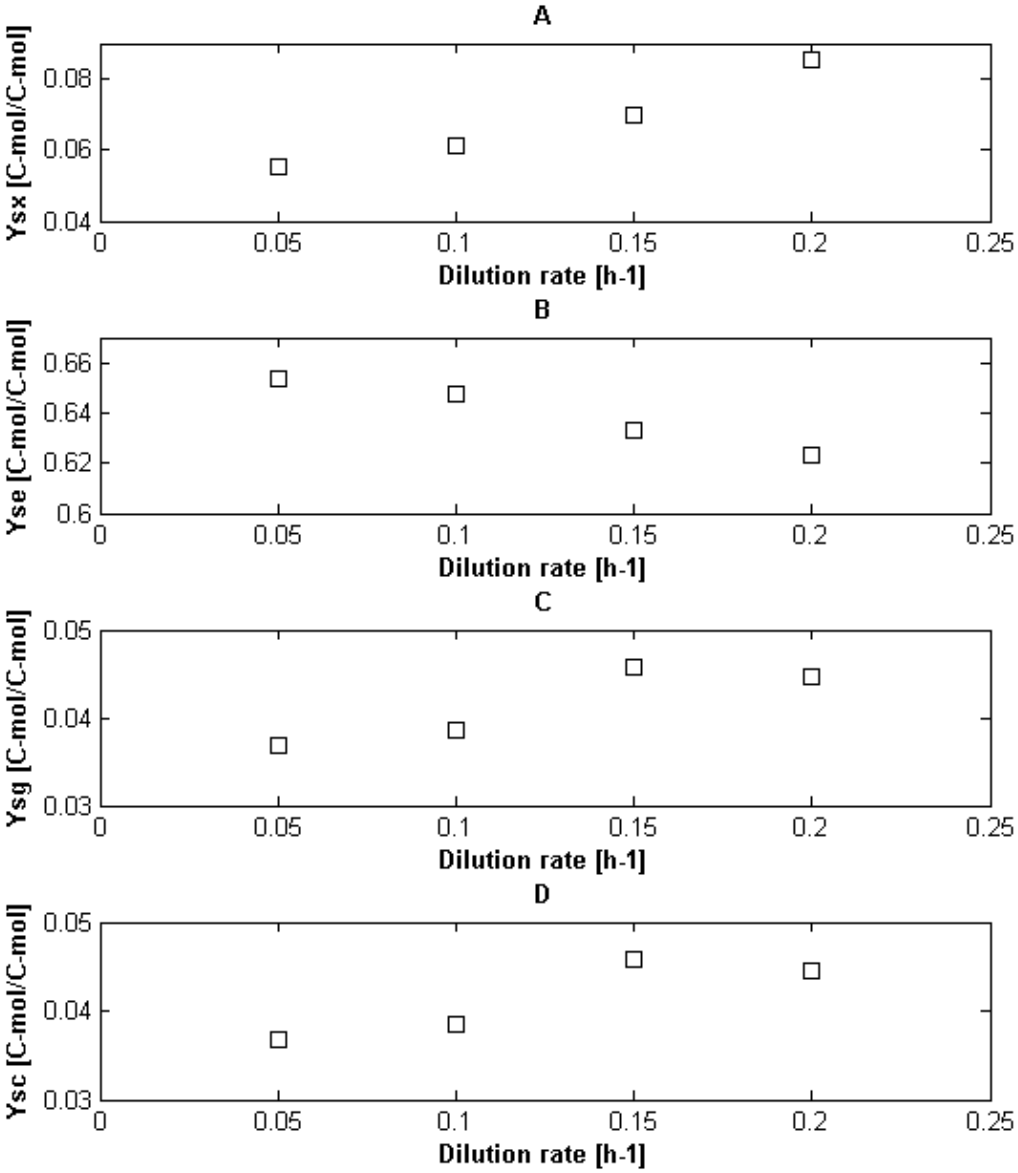
First observations of the data in Table 5.3 indicated that essentially all of the carbon could be accounted for. Given that the theoretical sum should add up to 1, differences of

only 0.11, 0.12, -0.34, and 0.47% for chemostats at D = 0.05, 0.1, 0.15 and 0.2 h^{-1}, respectively are seen. These differences can be considered as negligible.

The effect of dilution rate on the yields of greatest importance for the metabolic model (Figure 5.1) are plotted in figure 5.3. Figure 5.3A shows that Y_{sx} increases steadily with increases in dilution rate, from 0.055 to 0.085 C-mol x*C-mol s^{-1} at D = 0.05 h^{-1} to 0.20 h^{-1}, respectively. The opposite trend is seen for Y_{se} (Figure 5.3B),which decreases from 0.653 to 0.623 C-mol*C-mol h^{-1} from D = 0.05 h^{-1} to 0.20 h^{-1}. At low dilution rates the effects on Y_{sx} and Y_{se} can be postulated to result from maintenance, which most likely leads to ethanol production. Interestingly, the ethanol yields in Table 5.3 are very close to the theoretical value of 0.667 C-mol e*C-mol s^{-1} (calculated from process II in Table 5.1).

Another important observation is that the glycerol yield (Y_{sg}) increases from 0.037 to 0.045 C-mol g*C-mol s^{-1} as dilution rate is increased from 0.05 h^{-1} to 0.20 h^{-1} (figure 3C). This can be explained with the proposed model and the results for Y_{sx}. The biomass yield increased at higher dilution rates, leading to greater production of reducing equivalents (see NADH in process I, Table 5.1) and therefore greater production of glycerol is needed to consume these reducing equivalents. The carbon dioxide yield (Y_{sc}) does not vary greatly as a function of D (Figure 5.3D) and is approximately constant at an average value of 0.24 C-mol c*C-mol s^{-1}. This is consistent with the model, since CO_2 is a by-product of both process I and process II (Table5.1).

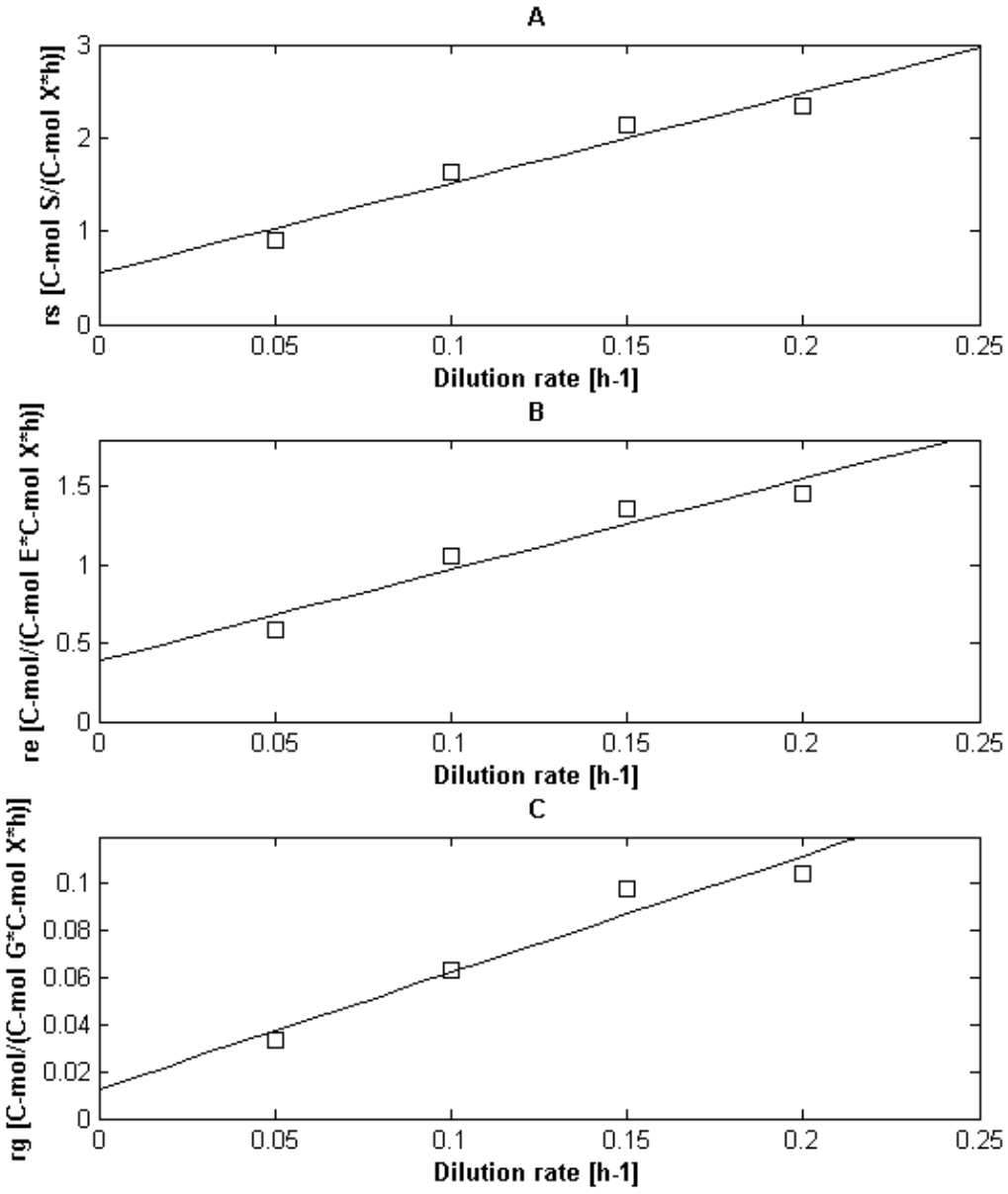
Figure 5.3.Yields of biomass (A), ethanol (B), glycerol (C)and carbon dioxide (D) relative to substrate consumed.



As was mentioned above, maintenance is expected to have an effect on the yields. This can be evaluated by fitting the experimental data to the model developed in *section 2.2*.

According to the model, Equations 5.11-5.13 describe a linear relationship between the rates of consumption or formation of glycerol, ethanol and lactose and the dilution rate D. Rearranging the experimental data to yield plots of the specific rates of ethanol (r_e) and glycerol formation (r_g), and the specific rate of substrate consumption (r_s) against D, the data can be fitted to evaluate the model. Plots of the data are shown in Figure 5.4, together with linear regressions through the data; regressions gave acceptable R^2 values, i.e. 0.94, 0.93 and 0.94 for r_s, r_e and r_g versus D, respectively. The following maintenance coefficients and true yield coefficients were obtained from the regressions: m_s = 0.546, m_e= 0.388 [Cmol i*(Cmol x*h)$^{-1}$]; Y^{true}_{sx} = 0.103, Y^{true}_{se} = 0.172, Y^{true}_{sg} = 2.01 (Cmol*Cmol^{-1}). These values can now be equated to Equations 5.11-5.15 thus obtaining Equations 5.17-5.21.

Figure 5.4. Rates of formation or consumption of main metabolic products and substrate against the dilution rate D. The straight line represents the linear regression of the data.



$$6(\delta_x - 0.05) = 0.497 \quad (5.17)$$

$$2[(K + \delta + 2\delta_x + 0.1)] = 5.818 \quad (5.18)$$

$$[(3K + 3\delta + 13\delta_x + 0.4)] = 9.658 \quad (5.19)$$

$$m_e = 0.388 \quad (5.20)$$

$$m_s = 0.546 \quad (5.21)$$

With the above equations from the model, the values of the metabolic coefficients can be stated. From Equation 5.17, a value of $\delta_x = 0.13$ (C-mol lactose)*(C-mol biomass)$^{-1}$results; the remaining Equations 5.18 and 5.19 are linearly dependent and cannot be solved together to give *K* and δ as explained in Nielsen et al. (2003). On the other hand, as was discussed in *section 2.2*a value of δ equal to 0.051 (mol ATP)*(C-mol biomass)$^{-1}$ can be chosen as first attempt. Therefore, one obtains a value of *K* equal to 2.62 (mol ATP)*(C-mol biomass)$^{-1}$ from Equations 5.18 and 2.459 (mol ATP)*(C-mol biomass)$^{-1}$ from Equation 5.19; from these, an average value of *K* equal to 2.54 (mol ATP)*(C-mol biomass)$^{-1}$ can be assumed.

The calculated coefficient representing the amount of carbon lost as carbon dioxide, δ_x, is within the range of values reported in the literature (see *section 5.2.2*). The coefficient K, which gives the amount of ATP consumed for the polymerization of biomass precursors, resulted in a value higher than expected, indeed it was (2.54 + 0.051) = 2.591 (mol ATP)*(C-mol biomass)$^{-1}$.On the other hand this solution is a function of the value chosen for δ. If, for instance, δ had a value of 0.090 (mol ATP)*(C-mol biomass)$^{-1}$, Equations 5.18 and 5.19 would give values of *K* equal to 2.46 and 2.43 (mol ATP)*(C-mol biomass)$^{-1}$, respectively, which are very close to the initial value used by Nielsen et al. (2003) for anaerobic growth of *S. cerevisiae* on glucose, namely 2.42 (mol ATP)*(C-mol biomass)$^{-1}$.

Eventually, a last interesting evaluation regards the value of ATP yield, Y_{ATP}. It can be calculated by Equation 5.22 (Verduyn at al., 1990).

$$Y_{ATP} = \frac{[biomass]}{[ethanol]-[glycerol]} \quad (5.22)$$

Where Y_{ATP} is expressed in g biomass formed per mol of ATP produced. By applying this equation to our data, the values of Y_{ATP} for each dilution rate can be calculated. These values were 4.33, 4.85, 5.72 and 7.08 (g biomass)*(mol ATP)$^{-1}$ for the runs at 0.05, 0.10, 0.15 and 0.20 h^{-1}, respectively. These values resulted to be lower than the values reported for anaerobic glucose-limited chemostat cultures (Verduyn et al., 1990), namely 16 (g biomass)*(mol ATP)$^{-1}$, but closer to the value of 8.6 (g biomass)*(mol ATP)$^{-1}$ reported by Dekkers et al. (1981) for both the same microorganism and substrate. The low value obtained in this paper is due to the particular low biomass growth which indicates that ATP consumption is mainly due to other phenomena rather than biomass growth, namely the maintenance (Equation 5.22 does not take into account such a phenomenon) that, indeed, resulted very high ($m_e = 0.388$ and $m_s = 0.546$).

5.4 Conclusions

K. marxianus is capable of fermenting lactose to ethanol at yields very close to those expected theoretically (e.g. up to 98%) at growth rates up to 0.2 h^{-1}. The reasons for this are linked to the particularly high maintenance coefficients of ca. 0.5 (C-mol)*(C-mol h)$^{-1}$ and the very low biomass yields (between 0.055 and 0.085 C-mol*Cmol^{-1}). This in turn leads to a quite low value of Y_{ATP} (in the range 4.33-7.08 g biomass*mol ATP^{-1} for D within 0.05-0.25 h^{-1}give value) compared to values seen by other workers for *S. cerevisae*. These results suggest that maintaining the growth rate of this microorganism below μ_{max}, e.g. in fed batch cultivations may be a successful strategy for optimising

ethanol yields and productivity in industrial processes. The biochemically structured model developed in this work was found to be a powerful tool for characterizing the metabolic structure of *K. marxianus* and could accurately predict experimental values from chemostat cultivations in defined medium. This model should be implemented for design and modeling of optimal fed batch processes for ethanol production from cheese whey.

General conclusions

The current thesis consisted of a comprehensive study aimed at characterizing the fermentation process whey lactose-to-ethanol. As it was pointed out at the beginning of the text, only a few industrial applications can be counted worldwide, which surely means that further efforts should be spent to make this process feasible and sustainable.

With this work a particular kind of cheese whey was studied even though the results are easily extended to embrace the whole category of dairy wastes.

First, the feasibility of ricotta cheese whey fermentation was demonstrated and its performances compared to the ones obtained with other similar substrates, namely cheese whey and cheese whey permeate. Ricotta cheese whey demonstrated to be an excellent alternative non vegetable source for bio-ethanol production.

Afterwards, the two developed models, one with a *data-driven* and the other with a *knowledge-driven* approach, demonstrated fairly good performances even if for different purposes. The empirical model demonstrated to be very useful to predict new experiment at different conditions, thus, it should be mainly used in industrial experimentations in order to save both time and money. On the other hand, such a model revealed next to nothing about the mechanisms behind the process therefore, from the point of view of a real breakthrough this model did not give so much (but still it must not be under-evaluated). For this reason a more appropriate and physically meaningful modeling was performed and this, in my opinion, is the very best achievement of this study. The biochemically structured model allowed the obtainment of an extremely meaningful system of equations represented by direct relationships between macroscopic design variables, namely the observable yields of the process, and microscopic (mechanistic) quantities, namely the metabolic coefficients.

Eventually, a continuous configuration of the process was experimented and excellent results were obtained thus laying the basis for a more comprehensive study in such a direction.

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