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Escola de Engenharia

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Implementation of the fermentation strategies for lactic acid bacteria

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e do
Doutor Nuno Costa Martins de Faria

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Declaração

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ABSTRACT

Implementation of the fermentation strategies for lactic acid bacteria

Lactococcus lactis is a Gram-positive bacterium with a generally regarded as safe (GRAS) status and a long standing history in industrial fermentations. It is often used as a starter culture in dairy products and contributes to the organoleptic characteristics of these same products. *L. lactis* is the most studied lactic acid bacteria and is nutritionally exigent. The present work aims to implement fermentation processes to optimize lactic acid bacteria growth, focusing in the identification of the best set of culture medium and environmental conditions. The strain used in this study was the *L. lactis* IL 1403, a organism derived from a dairy starter strain.

Biomass optimization was firstly examined by testing two carbon sources. In these experiments, the growth was evaluated using a bioreactor with control of temperature, pH and dissolved oxygen. The maximum biomass concentration (0.752 $g{\cdot}L^{-1}$) was obtained in complex medium supplemented with 5 $g{\cdot}L^{-1}$ lactose (CML). On the other side, growth in complex medium with 5 $g{\cdot}L^{-1}$ of glucose showed a growth rate approximately 32 % lower in comparison with CML. As opposed to what was observed in the complex medium, cells were not able to grow in a minimal defined medium supplemented with lactose. Additionally, the impact of pH control in IL 1403 growth was investigated in bioreactor *versus* non-pH control. As expected, the highest biomass concentration was obtained with pH control, corresponding to 0.696 $g{\cdot}L^{-1}$. A 38 % decrease in the biomass formation was observed for the non-controlled pH cultivation.

Afterwards, this work aimed at designing a minimal defined medium for *L. lactis* growth. For that purpose, the growth of *L. lactis* in the defined media SA, MS 10 and a modified SD3 was characterized. In the modified SD 3 medium, IL 1403 was able to grow until an optical density of 1, contrary to the other media where growth was not observed. Nevertheless, the modified SD3 medium does not satisfy the initial goal, since it is a complete medium. Therefore, other compositions were evaluated using as a base the modified SD3 medium. Under this scope, four main groups of nutrients were tested: mineral base, vitamins, nitrogen bases and amino acids compositions, which then were explored individually. From these experiments it was possible to conclude that an increase in K_2HPO_4 concentration from the mineral base favours growth; Vitamins tuning had no positive effect on IL 403 growth; and although not being essential to guarantee growth, nitrogen bases had a stimulatory effect. Furthermore, the set of essential amino acids for this strain were concluded to be methionine, valine, leucine, proline, serine, histidine,

glutamine, asparagine, cysteine and threonine. This achievement was of great importance due to the lack of consensus in the literature regarding the essential amino acids requirements for this strain.

RESUMO

Implementação de estratégias de fermentação para bactérias-lácticas

Lactococcus lactis é uma bactéria Gram-positiva com estatuto GRAS e com um longo historial em processos fermentativos. Trata-se da bactéria do ácido láctico (BAL) mais estudada e é nutricionalmente exigente. É frequentemente usada como uma cultura de arranque em produtos lácteos e contribui para as características organoléticas dos mesmos. O presente trabalho visava a implementação de estratégias de fermentação com o objetivo de otimizar o crescimento desta BAL, focando-se na identificação do melhor meio de cultura, assim como das melhores condições ambientais. A estirpe usada neste trabalho foi a *L. lactis* IL 1403 que não possui plasmídeo e deriva de culturas de arranque de produtos lácteos. Primeiramente foram avaliadas duas fontes de carbono na otimização da produção de biomassa. Nestes ensaios, o crescimento bacteriano foi avaliado num bioreator com controlo da temperatura, do pH e do oxigénio dissolvido. A concentração máxima de biomassa, 0,752 $g{\cdot}L^{-1}$, foi obtida em meio complexo suplementado com 5 $g{\cdot}L^{-1}$ de lactose (MCL). Por sua vez, no meio complexo com 5 $g{\cdot}L^{-1}$ de glucose, a taxa de crescimento obtida foi 32 % inferior à registada no MCL. Adicionalmente foi avaliado, em bioreator, o impacto do controlo do pH no crescimento da IL 1403 comparando-o com a ausência de controlo do mesmo. Como esperado, a maior concentração de biomassa, 0,696 $g{\cdot}L^{-1}$, foi obtida no ensaio com controlo de pH, tendo-se obtido menos 38 % no ensaio sem controlo. Ao contrário do que se observou em meio complexo, *L. lactis* IL 1403 não foi capaz de crescer em meio mínimo definido com lactose.

O crescimento da *L. lactis* em diferentes meios definidos (SA, MS10 e meio SD 3 modificado) foi, também, caracterizado. Em meio SD 3 modificado, a estirpe IL 1403 cresceu até uma densidade ótica máxima de 1, enquanto nos restantes meios não foi observado crescimento. No entanto, o meio SD 3 modificado não satisfaz um dos objetivos estabelecidos, por ser um meio definido completo. Desta forma, este meio foi usado como base para definir um meio mínimo para a *L. lactis* IL1403. Neste âmbito, a composição dos 4 principais grupos de nutrientes (base mineral, vitaminas, bases azotadas e aminoácidos), foi testada individualmente. Destes ensaios pode-se concluir que o aumento da concentração do K_2HPO_4 influencia fortemente o crescimento; a redução das vitaminas não afeta o crescimento da IL 1403 e as bases azotadas estimulam o crescimento, não sendo por isso consideradas essenciais. Por fim, os aminoácidos essenciais determinados para esta estirpe são a metionina, valina, leucina, prolina, serina, histidina,

glutamina, asparagina, cisteína e treonina. Esta última informação foi de maior importância devido à falta de consenso na literatura sobre os requisitos mínimos para a IL1403 em relação aos aminoácidos. Em suma, neste trabalho foi otimizada a produção de biomassa e determinado o meio mínimo para a *L. lactis* IL 1403.

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LIST OF ABBREVIATIONS

μ_{max}	Maximum specific growth rate
1,3-BPGA	1,3-diphosphoglyceric acid
2-PGA	2-phosphoglyceric acid
3-PGA	3-phosphoglyceric acid
Ala	Alanine
ALS	α-acetolactate synthase
Arg	Arginine
Asn	Asparagine
ATP	Adenosine triphosphate
CDW	Cells dry weight
CMG	Corynebacterium medium with glucose
CML	Corynebacterium medium with lactose
Cys	Cysteine
DHAP	Dihydroxyacetone phosphate
EMP	Embden Meyerhoff Parnas
F6P	Fructose-6-phosphate
FBP	Fructose-1,6- phosphate
FDA	Food and Drug Administration
G1P	Glucose-1-phosphate
G6P	Glucose-6-phosphate
Ga3P	Glyceraldehyde-3-phosphate
Gal6P	Galactose-6-phosphate
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
GRAS	Generally Regarded As Safe
His	Histidine
HPLC	High Performance Liquid Chromatography
Ile	Isoleucine
LAB	Lactic Acid Bacteria
LDH	Lactate dehydrogenase
Leu	Leucine

Lys	Lysine
Met	Methionine
NAD	Nicotinamide adenine dinucleotide
OD	Optical density
P	Cell productivity
PDHc	Pyruvate dehydrogenase complex
PEP	Phosphoenolpyruvate
PEP:PTS	Phosphoenolpyruvate- sugar phosphotransferase system
PFL	Pyruvate formate-lyase
Phe	Phenilalanine
Pro	Proline
q_S	Specific substrate consumption rate
ROS	Reactive Oxygen Species
rpm	Revolution per minute
Ser	Serine
TCA	Tricarboxylic Acid Cycle
t_d	Duplication or double time (h)
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine
$Y_{P/S}$	Lactic acid yield per substrate consumed
$Y_{X/S}$	Cell mass yield per substrate consumed

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1. CONTEXTUALIZATION

In this chapter a framing of the work and are their main objectives are described

In recent years, the human diet has suffered some modification due to an increase of the demand of products with bioactive or physiotherapeutic properties (Fumagali *et al.*, 2008; Gobbo-Neto and Lopes, 2007). In consequence, the economic interest in these compounds has increased and, subsequently, the search of compounds with these proprieties have been intensified (Gobbo-Neto and Lopes, 2007).

Plants are important sources of bioactive phytochemicals (Martins, 2012) because they are able to synthetize a high variety of secondary metabolites such as essential oils (Gobbo-Neto and Lopes, 2007), terpenoids and sterols (Fumagali *et al.*, 2008), pigments and phenolic compounds (BacHBerry, 2013). From this category, the most common and important from a biotechnological point of view are the phenolic compounds. The beneficial effects of these compounds are supported by several studies that demonstrate that consumption of food rich in phenolics help to prevent chronic diseases, like diabetes and cardiovascular diseases. For this reason, phenolic compounds are in the spotlight and an urge to produce them at a competitive price has arisen.

The European project BacHBerry aims to develop innovative and sustainable methodologies to optimize the potential of phenolic compounds from berry fruits.

Most high-value phenolic compound found in the market are obtained from natural sources via biomass extraction. This procedure presents several drawbacks since it depends on several factors as seasonality, environmental and geographic conditions. In addition, this process needs large amounts of plant raw materials to meet the market requirements. All these factors contribute for the choice of a chemical route of production. In turn, this alternative is comprised of several steps that facilitate the synthesis and extraction of the product of interest, but has the concomitant production of toxic compounds harmful to the environment. For this reason, an alternative way of producing these compounds is needed. Even more, in order to meet consumer demands, a "natural" way to obtain these phenolics is mandatory. Therefore, the BacHBerry project intends to apply metabolic engineering tools to genetically modify organisms to produce high-value compounds. This proposal is based in the production of phenolic compounds, in large scale, from lactic acid bacteria (LAB).

This work was performed under the scope of this large project and its main goal is to implement fermentation strategies to optimize the growth of *Lactococcus lactis*. This microorganism is a Gram-positive bacterium with a generally regarded as safe (GRAS) status and a long standing history in industrial fermentations. *L. lactis* is widely used for the production of dairy products and antimicrobial substances (bacteriocins).

The optimization of the fermentation process will be focused on the identification of the best sets of environmental conditions (controlled and uncontrolled pH), such as composition of culture medium (complex and defined media, carbon source and minimal nutritional requirements).

2. LITERATURE REVIEW

This chapter includes the state of the art regarding the lactic acid bacteria, as well as their industrial application. Subsequently, the LAB used in this work, *Lactoccocus lactis*, is described, as well as its carbon and pyruvate metabolism.

Finally, a description is made on the nutritional requirements of *L. lactis* growth.

2.1 LACTIC ACID BACTERIA

Since the ancient times that microorganisms have been used in several technological purposes, such as human and animal food and health (Axelsson, 2004).

The food-processing fermentation with lactic acid bacteria (LAB) is a form of bioconservation that has been used for centuries, because it inhibits growth of microorganisms that can deteriorate food (Nagalakshmi *et al.*, 2013). The LAB were initially isolated from milk but it is possible to detect these organisms in other products, such as meat, dairy products (cheese, butter) and fermented vegetables (Aleksandrzak-Piekarczyk, 2013). Due to a long history of safeness in fermentation processes, LAB have a generally regarded as safe (GRAS) status given by the Food and Drug Administration (FDA) agency (Aleksandrzak-Piekarczyk, 2013; Berlec and Strukelj, 2009) and the use of these bacteria in these processes allow to increase the shelf-life of the products (Neves, 2001).

Lactic acid bacteria are gram-positive bacilli or cocci, non-movable or rarely movable, non-sporulating, and most of this group is catalase-negative (Axelsson, 2004). Moreover, these bacteria are unable to synthetize porphyrinic groups, such as the heme group (Lan *et al.*, 2006; Lechardeur *et al.*, 2011) and are very demanding in terms nutrients due to the fact that these organisms are found in a very nutrient rich habitat (milk, meat and decomposing plant material) and some cavities of human and animals (mouth, intestine) (Konig and Frohlich, 2009; Konings *et al.*, 1999).

The morphology of cells, like form, Gram reaction and endospores; of the colonies as colour, dimension and form; its biochemistry as fermentation type and lactic acid configuration; and its physiology as growth in different environmental conditions, are the main characteristics for classification as a lactic acid bacteria (Axelsson, 2004; Konig and Frohlich, 2009). The LAB group includes 11 genera, as *Lactococcus*, *Pediococcus*, *Lactobacillus*, *Oenococcus*, *Leuconostoc* and others (Aleksandrzak-Piekarczyk, 2013). Some pathogenic microorganisms (*Streptococcus pneumoniae*) are included in the LAB group, but only non-pathogenic microrganisms, like *Lactococcus lactis*, are used in food-fermentation processes (Axelsson, 2004).

LAB have an important role in some fermentative processes (fermented products and beverages). Namely, they are used as starter cultures (Aleksandrzak-Piekarczyk, 2013; Neves, 2001), and they contribute to flavour (diacetyl, acetaldeyde) and texture (exopolysacharides) of the fermented foods (Neves, 2001). The enhancement of the taste and aroma of the products also results from the hydrolysis of the milk proteins, as casein, and the action of some enzymes (Neves,

2001). LAB have the ability to produce antimicrobial compounds (Gobbo-Neto and Lopes, 2007) such as organic acids, bactericins and hydrogen peroxide (Nagalakshmi *et al.*, 2013).

2.2 *Lactococcus lactis*

The genus *Lactococcus* can be found in two main environments, plants and milk (Aleksandrzak-Piekarczyk, 2013). In 1919, *Lactococcus lactis* was described by Orla-Jensen (Aleksandrzak-Piekarczyk, 2013) and was initially denominated as *Streptococcus lactis.* In 1985, it was renamed as *L. lactis.*

L. lactis is a gram-positive bacterium which presents a typically spherical or ovoid form (Figure 1), with 0.5-1 μm of diameter. In liquid culture, cells can appear in pairs or in short chains (Allegretti, 2009; Nagalakshmi *et al.*, 2013; Neves, 2001; Schleifer *et al.*, 1985; Tamime, 2002). This LAB does not form spores, is non-motile (Nagalakshmi *et al.*, 2013) and catalase-negative (Allegretti, 2009). *L. lactis* is often used as a starter culture in dairy products (Neves, 2001).

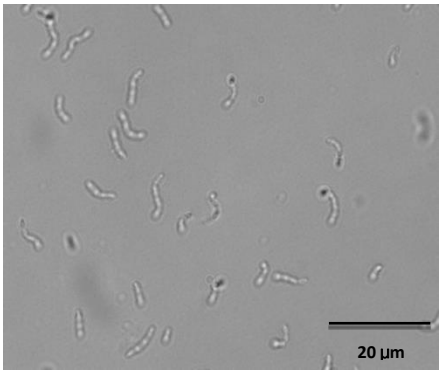

Figure 1 - *Lactococcus lactis* IL 1403 (photograph taken by an OLYMPUS BX51 microscope, Germany) (1000 x).

The genus *Lactococcus* is mesophilic, meaning that it grows optimally between 15 and 40 ºC. Studies performed by Holt *et al.* (1994) and Ruoff (1994) demonstrated that the optimum temperature of *L. lactis* subps. *lactis* is 30 ºC. In addition, *L. lactis* is a facultative anaerobe that tolerates high concentrations of organic acids, alcohol, and can resist to stress conditions (BacHBerry, 2013).

During milk fermentation, *L. lactis* converts the milk sugar (namely lactose) in lactic acid (Nagalakshmi *et al.*, 2013). The accumulation of lactic acid in the extracellular space decreases pH to a point that makes the propagation of other organisms difficult, contributing this way to the preservation of the fermented products (Aleksandrzak-Piekarczyk, 2013; Neves, 2001; Parente *et al.*, 1994). In accordance with Parente *et al.* (1994), the optimal pH for the growth of *L. lactis* is

6.5, but cells can grow as high as pH 9.2 (Aleksandrzak-Piekarczyk, 2013). However, at pH below 4.4 this bacterium cannot grow.

The species *L. lactis* may be divided into four subspecies (*lactis*, *cremosis*, *hordniae* and *tructae*) (Aleksandrzak-Piekarczyk, 2013). Nomura *et al.* (2002) and Schleifer *et al.* (1985) refer that the subspecies *lactis* can grow up to 40 ºC in environments with 4 % of NaCl. It can also use arginine and maltose as carbon source, whereas the subspecies *cremoris* do not (Aleksandrzak-Piekarczyk, 2013).

Lactococcus lactis has a homofermentative metabolism as converts about 95 % of sugar milk in lactic acid, the major end-product of its fermentation (Neves, 2001). However, this LAB is able to produce other products (acetate, formate, acetoin, ethanol) depending on the environmental conditions, see section 2.3.1 (Akerberg *et al.*, 1998; Aller *et al.*, 2014; Cocaign-Bousquet *et al.*, 1996). This characteristic increments its potential applications in food industry, because *L. lactis* can also produce bacteriocins (for example nisin) (Nagalakshmi *et al.*, 2013), prebiotics and exopolysaccharides (Lan *et al.*, 2006; Neves, 2001).

Lactococcus lactis is the most studied lactic acid bacteria due to its importance in the food industry. Its simple carbon metabolism and small genome facilitate the studies about metabolism, physiology and molecular biology and allow the genetic manipulation and expression of some the proteins from eukaryotic plasma membrane (Jorge, 2012). These characteristics makes the *L. lactis* an ideal model of LAB group for metabolic engineering (BacHBerry, 2013; Neves, 2001).

Lactococcus lactis was the first LAB with complete sequenced genome, including the strains IL 1403 and MG 1363 (Aleksandrzak-Piekarczyk, 2013). Additionally, Bolotin *et al.* (2001) sequenced the genome of *Lactococcus lactis* strain IL 1403 that comprises 2 365 589 base pairs in a circular chromosome and codifies for approximately 2300 proteins.

In accordance with the criteria stablished by the BacHBerry project, the choice of microorganism was based in the fact that this bacterium is commonly utilised in industrial fermentation processes (long history of application in this area) and to have the GRAS status. (BacHBerry, 2013).

2.3 SUGAR UPTAKE AND METABOLISM IN *L. lactis*

Glucose, xylose, lactose, maltose and sucrose are the mains carbon sources used by *Lactococcus lactis* (Loubière *et al.*, 1997; Martinez *et al.*, 2013), and it cannot metabolize rhamnose and sorbitol.

Most of the sugars enter the cells in two distinct forms: the phosphoenolpyruvate- sugar phosphotransferase system (PEP:PTS) or via permease transport. Glucose, lactose and galactose can be transported by these two (Figure 2) (Cocaign-Bousquet *et al.*, 1996; Thompson, 1979; Thompson *et al.*, 1985). Other sugars like fructose and sucrose are transported by a specific PEP:PTS, contrary to maltose which uses exclusively the permease transport (Benthin *et al.*, 1993; Cocaign-Bousquet *et al.*, 1996; Neves, 2001).

The transport of fructose can occur via fructose PTS or mannose PTS (Benthin *et al.*, 1993). However, the fructose metabolism in *L. lactis* has received little attention (Benthin *et al.*, 1993) because this LAB is mostly used in dairy products in which the main sugars are glucose, lactose and galactose (Crow and Thomas, 1984; Neves, 2001).

In PEP-PTS system, the phosphorylation of the sugar via enzymatic reactions occurs simultaneously with the transport through plasma membrane (Aleksandrzak-Piekarczyk, 2013; Benthin *et al.*, 1993; Thompson, 1979). The carbohydrate is phosphorylated and accumulated in cells (Cocaign-Bousquet *et al.*, 1996; Neves, 2001). The sugar uptake by PTS systems depends of the availability and phosphoenolpyruvate production rate (Thompson, 1978).

In *L. lactis*, the glucose uptake is made via the mannose-PTS system (man-PTS), which is the main sugar transporter in this LAB (Benthin *et al.*, 1993; Jorge, 2012; Neves, 2001). In some cases, glucose is uptaken via a permease system, Figure 2 (Neves, 2001). In man-PTS, glucose is transported and phosphorylated in glucose-6-phosphate and enters the glycolysis pathway where is promptly metabolized in pyruvic acid (Neves, 2001). In the case of glucose being transported by the permease system, the phosphorylation occurs once the sugar is inside cells, following glycolysis afterwards.

In the case of galactose, this monosaccharide can enter cells in the three distinct forms: permease transport (Cocaign-Bousquet *et al.*, 1996; Neves, 2001); through lactose hydrolysis in the reaction catalysed by β-galactosidase (Cocaign-Bousquet *et al.*, 1996) or by galactose-PTS (Neves *et al.*, 2005). When the specific galactose-PTS is used, the monosaccharide is metabolized in trioses (Ga3P and DHAP) via the Tagatose pathway (Neves *et al.*, 2005), while when it is transported by permease, galactose is metabolized by the Leloir pathway (Cocaign-Bousquet *et al.*, 1996), Figure 2.

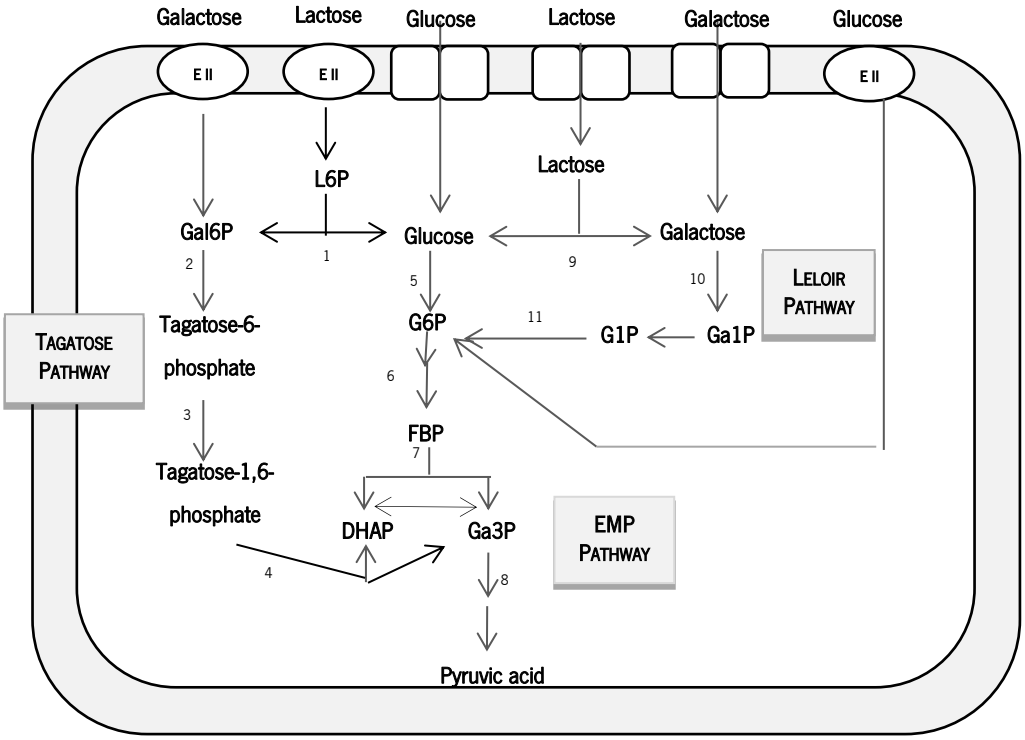

Figure 2 - Schematic representation of glucose, lactose and galactose uptake by *Lactococcus lactis*. The enzymes involved in the reactions indicated are: 1. phospho-*β*- galactosidase; 2. galactose-6-phosphate; 3. tagatose-6-phosphate; 4. tagatose- 1, 6-bisphosphate aldolase; 5. glucokinase; 6, 7 and 8. EMP enzymes; 9. *β*- galactosidase; 10. galactokinase; 11. phosphoglucomutase. The compounds involved in pathways are: L6P (lactose-6-phosphate), G6P (glucose-6-phosphate), FBP (fructose1,6-bisphosphate), DHAP (dihydroxyacetone phosphate) and Ga3P (glyceraldehyde-3-phosphate). Circules corresponds to PTS systems while the double squares consist in permease transport (Garrigues *et al.*, 1997; Neves, 2001; Thompson *et al.*, 1985).

Lactose uptake in lactic acid bacteria can start by the use of two pathways, namely a lactose-specific phosphotransferase system (lactose-PTS) and the permease system (Crow and Thomas, 1984; Neves, 2001; Thompson, 1979). When lactose-PTS is used, a specific enzyme which phosphorylates the lactose to lactose-6-phosphate (L6P) is necessary (Neves, 2001). This compound is then hydrolysed to glucose and galactose-6-phosphate (Gal6P), in a reaction catalysed by phospho-*β*-galactosidase (Crow and Thomas, 1984; Thompson *et al.*, 1985). Glucose is then phosphorylated via glucokinase originating G6P and this way enters in the Embden Meyerhoff pathway (EMP)(Thompson *et al.*, 1985). In turn, Gal6P is metabolized via the Tagatose pathway (Crow and Thomas, 1984; Thompson *et al.*, 1985). Tagatose pathway uses three enzymes, galactose-6-phosphate, tagatose-6-phosphate kinase and tagatose-1,6-diphosphate aldolase which allow to metabolize Gal6P to triose phosphates (glycolytic intermediates) (Crow and Thomas, 1984; Neves *et al.*, 2005). The last enzyme converts tagatose-1,6- diphosphate in Ga3P

and DHAP which are further metabolized in glycolysis (Aleksandrzak-Piekarczyk, 2013; Neves, 2001).

The permease system requires the presence of an electrochemical gradient through cytoplasmatic membrane (ion translocation) which allows the entry of lactose and other sugars (Aleksandrzak-Piekarczyk, 2013; Jorge, 2012; Neves, 2001). Within cells, lactose is then hydrolysed in galactose and glucose by *β*-galactosidase (Thompson *et al.*, 1985). After that, glucose follows glycolysis, and galactose is metabolized by the Leloir pathway. In this pathway, three enzymes are involved in the transformation the galactose into glucose-1- phosphate (G1P) which is then converted to glucose-6-phosphate via phosphoglucomutase. From this step, it can enter glycolysis (Aleksandrzak-Piekarczyk, 2013; Neves, 2001).

According to Bolotin *et al.* (2001), the homolactic fermentation is associated to PTS sugars, while the mixed fermentation has been reported for sugars that are transported by permeases and ABC transport. This way it has been suggested that there is a relationship between the type of transport used by the bacterium and the fermentation profile (homolactic or mixed fermentation).

2.3.1 Carbohydrate metabolism

Fermented products contain many LAB involved in the process and the use of these microorganisms allows the increase the product shelf-time allowing their conservation (Axelsson, 2004; Hartke *et al.*, 1996; Pampulha, 1998).

Lactic fermentation can be made by bacteria (*Lactobacillus* sp., *Lactococcus* sp., *Streptococcus* sp.), micro-algae (*Chlorella*), protozoa and animals (Madigan *et al.*, 2003).

In the case of lactic fermentation performed by bacteria, the fermentation of sugars to lactic acid is accomplished by two distinct ways: homofermentative or heterofermentative pathways (Neves, 2001). The main difference between these two pathways is the way of cleavage of the six-carbon molecule and, consequently the products formed (Dragone *et al.*, 2007).

The homofermentative pathway produces lactic acid as the main or only product of fermentation from the conversion of 95 % of the sugar (Konig and Frohlich, 2009; Madigan *et al.*, 2003). Glycolysis, also called the Embden Meyerhoff pathway, consists of a set of 10 sequential biochemical reactions in which one molecule of hexose is broken down into two molecules of pyruvic acid, Figure 3 (Campos, 1999; Faia and Castro, 1998; Madigan *et al.*, 2003; Neves, 2001; Neves *et al.*, 2005).

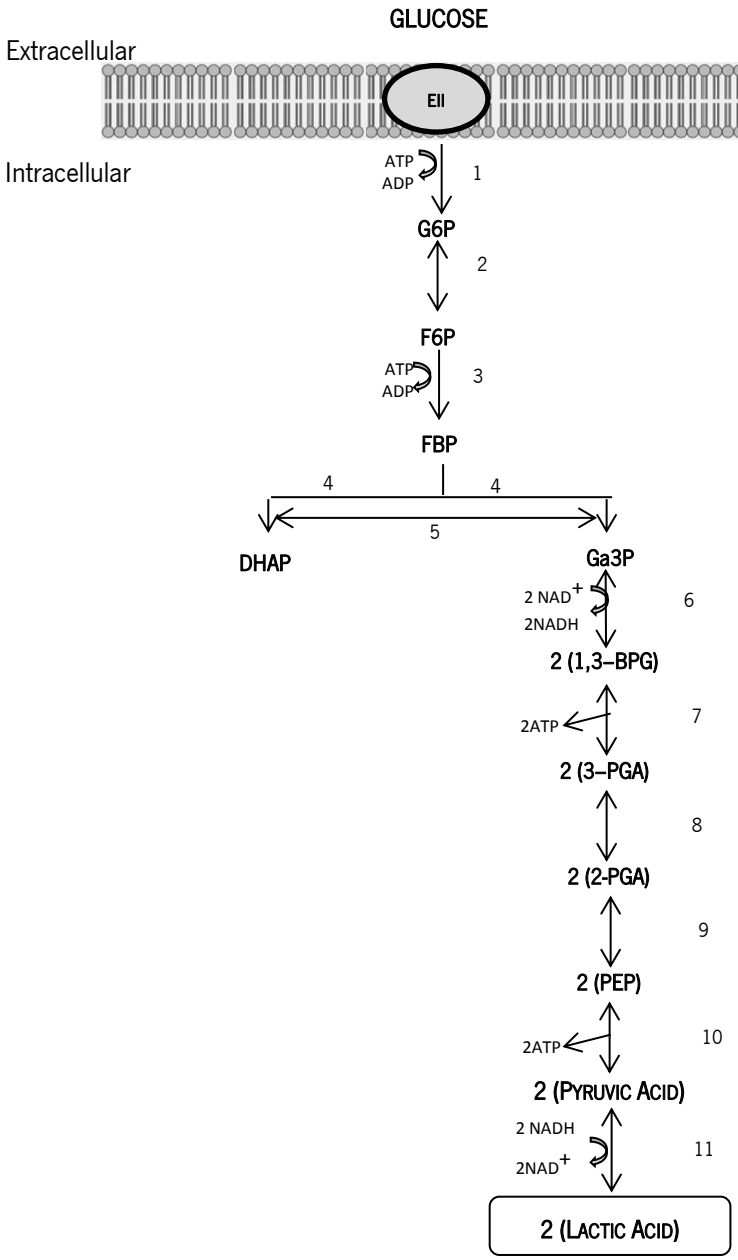

Figure 3 - Schematic representation of glycolysis and lactic acid production by homolactic fermentation The enzymes involved in the glycolysis are: 1. hexokinase, 2. phosphoglucose isomerase; 3. 6-phosphofructo-1-kinase, 4. fructose-1,6-biphosphate aldolase, 5. triosephosphate isomerase; 6. glyceraldeyde-3- phosphate dehydrogenase; 7. phosphoglycerate kinase; 8. phosphoglyceromutase; 9. enolase; 10. pyruvate kinase and 11. lactate dehydrogenase (Campos, 1999; Madigan *et al.*, 2003; Neves, 2001).

In the first step of glycolysis (which is absent when the PTS systems is used), glucose is phosphorylated, at the cost of an ATP molecule, into glucose 6-phosphate (G6P). This reaction is catalysed by the hexokinase (glucokinase) (Madigan *et al.*, 2003). Then, the isomerization of glucose 6-phosphate into fructose 6-phosphate (F6P) takes place, where the last is subsequently phosphorylated into fructose 1,6-bisphosphate (FBP). In this reaction, the enzyme phosphofruto-1-kinase catalyses the phosphorylation by using a second ATP molecule (Campos, 1999; Neves,

2001). FBP is broken down, by the action of the FBP aldolase, into two interconvertible three-carbon molecule, dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3- phosphate (Ga3P). DHAP and Ga3P are isomers and inter-converted through the action of the triose phosphate isomerase (Campos, 1999; Neves, 2001). Ga3P suffers oxidation producing 1,3–diphosphoglyceric acid (1,3-BPGA) (Campos, 1999). During this step, two hydrogen atoms are removed from the substrate and NAD^+ is reduced to NADH (Madigan *et al.*, 2003). The next reactions involve the action of kinases, mutases and enolases which enable the formation of pyruvic acid.

During glycolysis 4 ATP molecules are produced, but two of them are used along the process (first and third stage) and this way the final energy balance is 2 ATP molecules per glucose. The ATP is produced during the conversion of 1,3-BPGA in 3- phosphoglyceric acid (3-PGA) by phosphoglycerate kinase and, t.hen the phosphoenolpyruvate is converted in pyruvic acid by pyruvate kinase (Madigan *et al.*, 2003) that is, the energy is obtained by substrate-level phosphorylation (Madigan *et al.*, 2003; Neves, 2001; Neves *et al.*, 2005). 2 NADH molecules are also produced.

In an anaerobic environment, the electron transport chain is not able to function because it dependents on availability of oxygen, as the final acceptor of electrons (Campos, 1999). Thus, NADH is recycled through the reduction of metabolites, allowing the oxidation of glucose into fermentative end-products (Cocaign-Bousquet *et al.*, 1996). In the homolactic fermentation, the pyruvic acid is degraded in lactic acid by the oxidation of a NADH molecule, in a reaction catalysed by lactate dehydrogenase (LDH) (Campos, 1999; Madigan *et al.*, 2003). Most *Lactoccus lactis* strains are classified as homolactic under anaerobic metabolism (Cocaign-Bousquet *et al.*, 2002; Lan *et al.*, 2006; Mercade *et al.*, 2000).

However, lactic acid bacteria carbohydrate metabolisms are not always homofermentative (Neves, 2001). In response to stress conditions, some homofermentative LAB, as *L. lactis,* shift from a homolactic fermentation to a mixed acid fermentation (Cocaign-Bousquet *et al.*, 1996; Felipe and Hugenholtz, 1999; Lan *et al.*, 2006; Neves *et al.*, 2005). The presence of oxygen, high pH, low temperature, carbon source limitation in chemostat cultures in anaerobisis and galactose, lactose and maltose metabolism may increase the metabolic flexibility and induce the shift towards a mixed acid fermentation (Akerberg *et al.*, 1998; Felipe and Hugenholtz, 1999; Lan *et al.*, 2006; Neves *et al.*, 2005). In situations with carbon limitation, PEP and 3-PGA accumulate in cells, and inhibit the pyruvate kinase resulting in a decrease of FBP and triose pools. As consequence of this,

the lactate dehydrogenase is inactivated (Neves *et al.*, 2005) and, consequently occurs a increase of the formate, ethanol and acetate, fermentation products obtained from the action of pyruvate formate lyase (Cocaign-Bousquet *et al.*, 1996).

In the mixed acid fermentation, the pyruvic acid can be metabolised by different enzymes, namely via lactate dehydrogenase, pyruvate formate-lyase (PFL), pyruvate dehydrogenase complex (PDHc) or α- acetolactate synthase (ALS), Figure 4 (Cocaign-Bousquet *et al.*, 1996; Garrigues *et al.*, 1997; Neves, 2001; Thompson, 1979). The genes that codify for these enzymes are present in the *L. lactis* genome (Bolotin *et al.*, 2001). During mixed fermentation there is an accumulation of ethanol, acetate, formate, diacetyl and acetoin in addition to the main end-product which is the lactic acid (Akerberg *et al.*, 1998; Garrigues *et al.*, 1997; Neves, 2001).

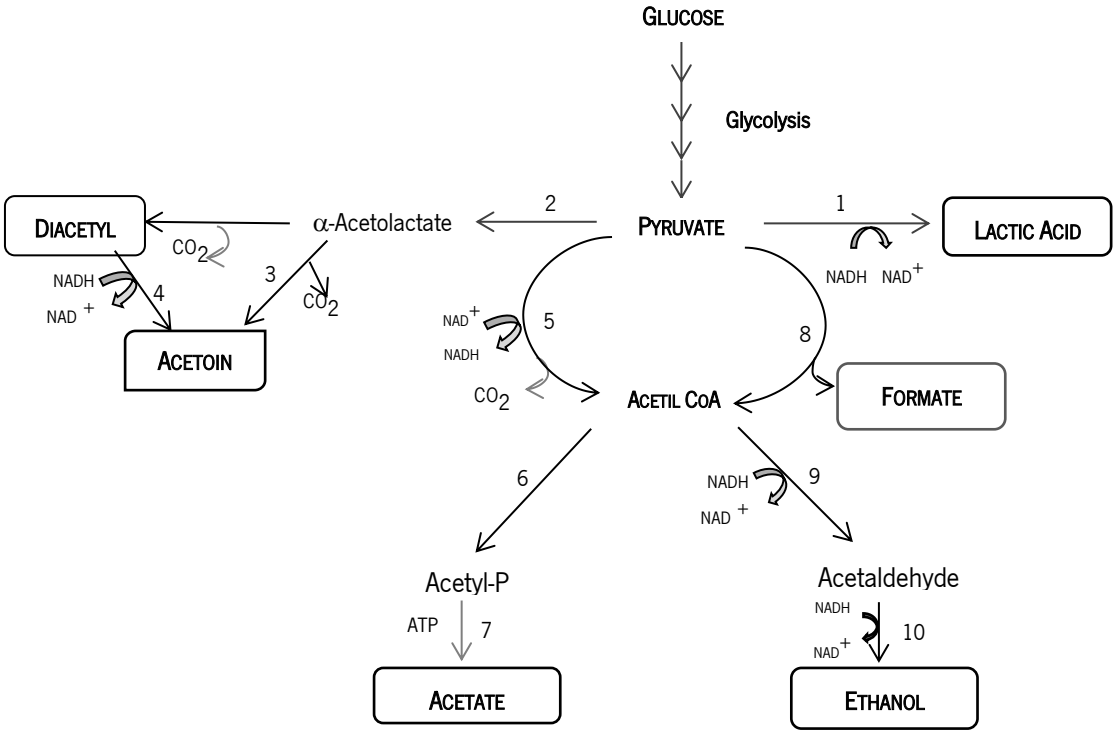

Figure 4 - Pyruvate metabolism in *Lactococcus lactis*. The several reactions are catalysed by:1. lactate dehydrogenase; 2. α-acetolactate synthase; 3. α- acetolactate decarboxylase; 4. 2,3-butanediol dehydrogenase; 5. pyruvate dehydrogenase complex; 6. acetate kinase; 7. phosphotransacetylase; 8. pyruvate formate-lyase; 9. acetaldehyde dehydrogenase; 10. alcohol dehydrogenase (Cocaign-Bousquet *et al.*, 1996; Garrigues *et al.*, 1997; Neves, 2001).

The enzyme pyruvate formate-lyase transforms pyruvic acid into formate and acetyl-coenzymeA (acetyl-CoA). Pyruvate can be also converted into acetyl-CoA through a decarboxylation by the pyruvate dehydrogenase complex (Cocaign-Bousquet *et al.*, 1996; Lan *et al.*, 2006; Neves, 2001). The PDHc is present in facultative anaerobic bacteria like *L. lactis*, and is constituted by three

enzymes that catalyse the reactions (reductive decarboxylation) from pyruvate to acetyl-CoA with the release of a CO_2 molecule and the reduction of one NAD^+ (Neves, 2001).

Acetyl-CoA production results of PLF or PDHc activities, being metabolized to ethanol or acetate, respectively. Acetate production requires the action of phosphotransacetylase and acetate kinase and yields one ATP molecule. Ethanol production, with the regeneration of two NAD^+ molecules involves the action of the aldeyde dehydrogenase and the alcohol deydrogenase (Cocaign-Bousquet *et al.*, 1996; Garrigues *et al.*, 1997; Neves, 2001).

NADH oxidase is an enzyme that catalyses the formation of NAD^+ from NADH with the spend of an O_2 molecule. Thus, the activity of this enzyme is regulated by the presence of O_2, and increases its catalytic activity when this co-factor is abundant in the medium (Duwat *et al.*, 2001; Felipe and Hugenholtz, 1999; Neves *et al.*, 2005).

Under such conditions, the enzymes lactate dehydrogenase, acetaldehyde dehydrogenase, ethanol dehydrogenase and NADH oxidase compete for NADH pool (Neves, 2001). Moreover, in aerobiosis, the production of important flavour compounds used in the food industry is stimulated. Example of these molecules are the diacetyl and acetoin (Neves, 2001). The production of these two metabolites is also stimulated when cells are grown in complex medium with lactose (Cocaign-Bousquet *et al.*, 1996). In these cases, pyruvate is metabolized by the α-acetolactate synthase from two molecules of pyruvic acid to yield acetolactate. This compound can be accumulated but is unstable in the presence of oxygen from which originates diacetyl (Cocaign-Bousquet *et al.*, 1996).

The decarboxylation of the acetolactate originates acetoin by the action of acetolactate decarboxylase (Cocaign-Bousquet *et al.*, 1996; Lan *et al.*, 2006). Acetoin can also be produced by an alternative pathway, through diacetyl reduction performed by the diacetyl reductase.

In the presence of oxygen, the levels of pyruvate dehydrogenase and the acetolactate synthase increase, contrary to lactate dehydrogenase (Cocaign-Bousquet *et al.*, 1996; Neves *et al.*, 2005).

The manipulation of *L. lactis* metabolism, namely the pyruvate metabolism, allows the formation of the products that contribute to its important role in food industry. Diacetyl and acetaldehyde have an important role in buttermilk and yoghurt flavours, respectively (Jorge, 2012).

2.3.2 Aerobic Respiration Metabolism

L. lactis is considered a LAB facultative anaerobe (Duwat *et al.*, 2001; Neves, 2001). Although, its genome contains the genes to perform the aerobic respiration metabolism with exception of the

genes required for heme group production (Lan *et al.*, 2006). In LAB, the shift from fermentation metabolism to aerobic respiration occurs when provided with heme and/or menaquinone for some lactic acid bacteria (Lechardeur *et al.*, 2011; Pedersen *et al.*, 2012)

In the case of *L. lactis,* there is no pathway to synthesise the heme group and it needs to be added to the medium in order to allow an aerobic metabolism (Duwat *et al.*, 2001; Lan *et al.*, 2006; Neves *et al.*, 2005; Pedersen *et al.*, 2012). Heme consists of a ferrous iron present in a protoporphyrin ring (Lechardeur *et al.*, 2011; Madigan *et al.*, 2003) and is an indispensable cofactor in the cytochrome oxidase system (Bolotin *et al.*, 2001; Duwat *et al.*, 2001; Lan *et al.*, 2006).

The aerobic respiration is divided in four stages: glycolysis, acetil-CoA formation, tricarboxylic acid cycle (TCA) and electron transport chain (Madigan *et al.*, 2003). In accordance to genome analysis, *L. lactis* have a partial tricarboxylic acid cycle (TCA), meaning that, the pyruvic acid obtained from the glycolysis cannot be totally oxidized into CO_2, and H_20 (Bolotin *et al.*, 2001; Duwat *et al.*, 2001; Lan *et al.*, 2006).

The benefits of the hemin-stimulated aerobic respiration has been reported by several authors that state that respiration metabolism is more advantageous than fermentation because it allows to obtain a higher biomass concentration and a lower production of by-products. Moreover, the stress due to the presence of oxygen decreases over time, once cells became resistant to oxygen, there is an increase in the long-term survival (Duwat *et al.*, 2001; Lan *et al.*, 2006; Lechardeur *et al.*, 2011; Pedersen *et al.*, 2012). Furthermore, the presence of oxygen and the heme group influences the growth kinetic parameters (higher specific growth rate and biomass accumulation) and the products formed (Lan *et al.*, 2006).

2.4 NUTRITIONAL REQUIREMENTS

The growth of any microorganism is a dynamic process that requires energy and nutrients for the synthesis of the cellular components and cell maintenance. The microorganisms are versatile and diversified in of terms nutrients. The chemical composition of the cell indicates the principal requirements for growth. The water represents 80-90 % of total cell weight. Other nutrients like carbon, oxygen, hydrogen, nitrogen, magnesium, phosphorus and potassium are considered macronutrients, meaning that they are necessary in large quantities and play an important role in the structure and metabolism of cells (Pampulha, 1998). The micronutrients (trace elements) are necessary in minor quantities but are equally important. Cobalt, copper, manganese, zinc, iron and

among others are examples of micronutrients that have an important function to some enzymes (Madigan *et al.*, 2003).

The knowledge of the nutritional requirements from a microorganism allows us to growth these microbes in the laboratory. In the composition of the media, essential nutrients must be included at non-toxic concentrations.

The genus *Lactococcus* is very demanding in terms of nutrients because its biosynthetic capacity is limited due to the lack of some biosynthetic pathways. Carbon and nitrogen sources, vitamins, minerals, purines and pyrimidines have to be added to the growth medium (Cocaign-Bousquet *et al.*, 1995; Oliveira *et al.*, 2005). As a consequence, complex media, namely M17, are used to grow *L. lactis*. However, with rich media the determination of the consumption of amino acids, for example, is impossible, and the characterization of metabolic and energetic behaviour is difficult (Aller *et al.*, 2014; Jensen and Hammer, 1993; Novak *et al.*, 1997; Oliveira *et al.*, 2005).

Complex media have all the components necessary for microbial growth and enable cells to grow at its maximal. However, these media are a major drawback because they are constituted made of complex nutrients, such as peptone, tryptone, yeast extract, meaning that the composition is undetermined (Madigan *et al.*, 2003; Pampulha, 1998).

Thus, metabolic experiments and *in silico* growth predictions are made in defined medium (Jensen and Hammer, 1993). The most commonly used defined or synthetic medium in *L. lactis* is the Chemically Defined Medium (CDM) described by Otto *et al.*, (1983) and later modified by Poolman and Konings (1988). CDM contains 47 compounds, including 18 amino acids, 4 nitrogen bases, 5 trace metals and 14 vitamins (Cocaign-Bousquet *et al.*, 1995; Novak *et al.*, 1997). Defined media allows the conduction of studies about physiology (metabolic pathways), genetics (gene regulation) and protein expression (Neves, 2001; Novak *et al.*, 1997). However, the bacterial growth in define medium is lower comparatively to complex medium (Jensen and Hammer, 1993; Novak *et al.*, 1997).

The microorganisms isolated from rich environments, as from dairy products, have several nutritional requirements which makes the growth in simple defined medium, difficult. The shift from rich media to defined media leads to the loss of some functions, as amino acids or vitamins synthesis (Godon *et al.*, 1993). Godon el al. (1993) give some examples of genetic defects: the genes necessary for amino acids biosynthesis are present and synthesize functional proteins, but their expression is low; genes are present in the genome but encode non-functional proteins; or genes that are not present in the genome.

Lactococcus lactis is able to metabolize various sugars such as, glucose, maltose, lactose, sucrose, fructose, galactose, ribose and trehalose (Loubière *et al.*, 1997; Martinez *et al.*, 2013; Oliveira *et al.*, 2005). Approximately 95 % of these sugars are converted into lactic acid. The remaining 5 % are used in biomass production (Oliveira *et al.*, 2005).

Nitrogen is the second most abundant compound after carbon, and it can be made available in organic and inorganic forms. Ammonium chloride, ammonium sulphate and others correspond to the inorganic forms, while amino acids (monomers of proteins) and nucleotides (monomers of nucleic acids) correspond to organic forms. The mineral nitrogen is not sufficient to grow LAB when exogenous sources of amino acids are absent (Cocaign-Bousquet *et al.*, 1995).

Several research groups have identified the essential amino acids for different strains of *L. lactis* through the single omission technique (Cocaign-Bousquet *et al.*, 1995; Jensen and Hammer, 1993; Oliveira *et al.*, 2005). These research groups have concluded that nearly all strains of *L. lactis* have the core of essential amino acids composition: isoleucine, leucine, glutamic acid, methionine, valine and histidine (Bolotin *et al.*, 2001; van-Niel and Hahn-Hagerdal, 1999). However, some strains need others amino acids to grow. For example, the *L. lactis* strains from vegetable (NCDO 2118) and dairy origin (IL 1403) have different amino acids requirements. The first strain is prototrophic for all amino acids with exception of 5 amino acids mentioned above, plus histidine which is replaced by serine. Relatively to the second strain, it needs the above 6 amino acids plus serine, arginine and threonine (Cocaign-Bousquet *et al.*, 1995). Godon el al. (1993) states that *L. lactis* obtained from milk is auxotrophic for branched-chain amino acids (isoleucine, leucine and valine), contrary to the strains from vegetable origin, which are prototrophic. As mentioned, the shift from complex to defined medium can put into evidence some genetic defects, like the loss of capacity to synthetize some amino acids. *L. lactis* has all necessary genes for the synthesis of the 20 amino acids, but some of them may be inactivated (Bolotin *et al.*, 2001; Caspi *et al.*, 2010; Delorme *et al.*, 1993) explaining why some of these amino acids are required for growth.

L. lactis is catalase-negative and under aerobic conditions, the absence of this enzyme promotes the accumulation hydrogen peroxide (H_2O_2) in cells and, consequently the destruction of cells components. The addition of exogenous of catalase, yeast extract, reducing agents or asparagine avoids H_2O_2 accumulation and inhibition of bacterial growth. Van Niel, *et al.* (2002) studied the effect of oxygen on the growth of *L. lactis* ATCC 19435, testing different conditions (aerobiosis, O_2 limitation and anaerobiosis) with and without asparagine in the culture medium.

The results indicate that under aerobiosis without asparagine, the specific growth rate is low (0.015 h^{-1}) and only 0.09 $g.L^{-1}$ biomass was formed. Contrary, in aerobiosis with asparagine, the specific growth rate is higher (0.58 h^{-1}) and the biomass formed was 8.6 $g.L^{-1}$. According to this, the presence of asparagine in the culture medium helps cell to cope with the stress associated with O_2 metabolism.

Amino acids, vitamins, purines and pyrimidines are considered growth factors, i. e., they are organic substances required in small amounts (Madigan *et al.*, 2003; Pampulha, 1998).

Relatively to nitrogen bases, several authors sustain that these have a stimulatory effects in *L. lactis,* promoting an increase of approximately 35 % of growth when added to complex or synthetic medium (Anderson and Elliker, 1953; Cocaign-Bousquet *et al.*, 1995; van-Niel and Hahn-Hagerdal, 1999).

The defined medium for *L. lactis* must contain at least seven vitamins, namely the B group vitamins (nicotinic acid, calcium pantothenate, biotin, folic acid, pyridoxamine, riboflavin and thiamine) to allow a sustainable growth. The genes necessary for synthesis of the cofactors, folic acid, menaquinone, riboflavin and thioredoxin are present in *L. lactis* genome, however they are inactive or produce non-functional proteins (Bolotin *et al.*, 2001). Table 1 shows some functions of the vitamins of B group (Bolotin *et al.*, 2001; Cocaign-Bousquet *et al.*, 1995; Neves, 2001; Razvi *et al.*, 2008; van-Niel and Hahn-Hagerdal, 1999).

Table 1 - Some vitamins of the B group and respective functions (Madigan *et al.*, 2003; Pampulha, 1998; Razvi *et al.*, 2008; van-Niel and Hahn-Hagerdal, 1999)

VITAMINS	FUNCTIONS
Nicotinic acid (B3)	Production of NAD(P)
Calcium pantothenate (B5)	Precursor of coenzyme A
Biotin (B7)	Fatty acids and aspartic acid synthesis; CO_2 fixation
Pyridoxamine (B6)	Amino acids synthesis
Riboflavin (B2)	Precursor of FMN and FAD

In accordance to Cocaign-Bousquet *et al.* (1995) growth is possible only by adding nicotinic acid, calcium pantothenate and biotin. However, this fact depends of the culture media composition. Several authors mention that pyridoxamine is not essential for growth, but when nitrogen bases are not present, vitamin B6 becomes essential (Cocaign-Bousquet *et al.*, 1995; van-

Niel and Hahn-Hagerdal, 1999). The joint action of alanine and pyridoxamine stimulates growth but when both are absent cells cannot duplicate (Niven, 1994; van-Niel and Hahn-Hagerdal, 1999).

Riboflavin, vitamin B2, consists in the parent compound of the flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). The enzymes responsible for redox reactions, such as NADH oxidase, need the presence of these flavin coenzymes (Madigan *et al.*, 2003). Van-Niel and Hahn-Hagerdal (1999) affirms that NADH oxidase activity is influenced by riboflavin concentration, namely the enzymatic activity increases with the increase of riboflavin concentration. NADH oxidase catalyses the NADH oxidation with consumption of oxygen and production of hydrogen peroxide and carbon dioxide. Studies developed by Cocaign-Bousquet *et al.*, (1995) demonstrated that riboflavin has a different purpose in vegetable and dairy *L. lactis* strains. This author compared the growth of *L. lactis* IL 1403 in MS 10 medium (see Table 3 in section 3.2) with and without riboflavin and found that in the medium supplemented with vitamin B2 allowed growth, contrary to the other scenario. Then, in dairy strains, riboflavin is essential for growth.

Thus, to obtain high productivities, cells have to be supported by a culture medium that guarantees a sustainable growth and viability. For this, the essential nutrients, as carbon and nitrogen sources, vitamins and minerals must be added to the medium (Martinez *et al.*, 2013).

3. MATERIAL AND METHODS

In this chapter the microorganism and its conservation will be described, as well as the culture media, growth conditions, methodologies and the equipment used in all experiments performed in shake flasks and bioreactor.

The analytical methods applied in the laboratorial execution in this work are also mentioned.

3.1. MICROORGANISM

The microorganism used in this thesis is the *Lactococcus lactis* subsp. *lactis* from dairy origin, IL 1403 (wild type), and was obtained from DSMZ, Germany.

The stock cultures contained cells in exponential phase allowing microorganisms to remain viable, genetically homogeneous and protected from contaminations (Pampulha, 1998). So the strain was maintained in Corynebacterium medium (5 $g \cdot L^{-1}$ yeast extract, 5 $g \cdot L^{-1}$ NaCl and 10 $g \cdot L^{-1}$ casein peptone) supplemented with glycerol (30 %) and stored at – 80 ^{o}C.

3.2. CULTURE MEDIA

The growth of the *L. lactis* was studied in different culture media, namely the Corynebacterium medium (complex medium), Table 2, and four defined synthetic media,Table 3.

Table 2 - Composition of the Corynebacterium medium used in *Lactococcus lactis* IL 1403 growth

Component	Concentration ($g \cdot L^{-1}$)
Yeast extract	5
Glucose	5
NaCl	5
Casein peptone	10

The solution containing yeast extract, sodium chloride and casein peptone was prepared with distilled water and sterilized by autoclave (20 min at 121 ^{o}C). The glucose and lactose were sterilized, separately by autoclave using a cycle of 30 minutes at 110 ^{o}C to prevent caramelization and were added, individually, to the remainder medium after sterilization.

The four defined synthetic media used in the growth of *L. lactis* IL 1403 were SA medium, MS 10, modified SD3 and MS 15 (Table 3) and were all supplemented with 10 $g \cdot L^{-1}$ of glucose. The original version of SD3 medium contains casamino acids (acid hydrolysed of casein), making it a semi-defined medium; in the modified SD3 medium this mixture of amino acids obtained through the acid hydrolysis of casein was replaced by a mixture of the 19 amino acids present in the SA medium. The defined synthetic media were prepared from concentrated stock solutions (vitamins, amino acids, trace metals) which were prepared individually and stored at 4 ^{o}C, after sterilization by filtration. These solutions cannot be autoclaved because high temperatures can cause

degradation of the vitamins and of the amino acids. The final pH of the vitamins solution was adjusted to 6.5. The stock solutions were added aseptically to the culture medium.

The mineral base of the defined media was sterilized by filtration with cellulose acetate membrane (0.22 µm; Sartorius). After autoclaving the carbon source, mineral base, vitamins, trace metals, amino acids and nitrogen bases were added.

Table 3 - Composition of each defined media ($g \cdot L^{-1}$) used for *L. lactis* growth (Cocaign-Bousquet *et al.*, 1995; Jensen and Hammer, 1993; van-Niel and Hahn-Hagerdal, 1999)

Groups	Compounds	MS 15	MS 10	SA	Modified SD 3
AMINO ACIDS	Alanine	–	0.24	0.3	0.3
	Arginine	0.12	0.12	0.18	0.18
	Aspartic acid	–	–	–	–
	Cysteine	–	0.17	0.1	0.1
	Glutamic acid	0.10	–	0.31	0.31
	Glycine	–	0.17	0.2	0.2
	Histidine	0.11	0.11	0.05	0.05
	Isoleucine	0.20	0.2	0.1	0.1
	Lysine HCl	–	0.35	0.2	0.2
	Methionine	0.12	0.12	0.1	0.1
	Phenylalanine	–	0.28	0.2	0.2
	Proline	–	0.68	0.3	0.3
	Serine	0.34	0.34	0.3	0.3
	Threonine	0.23	0.23	0.2	0.2
	Tryptophan	–	0.05	0.1	0.1
	Tyrosine	–	0.29	0.05	0.05
	Valine	0.33	0.33	0.11	0.11
	Leucine	0.47	0.47	0.1	0.1
	Asparagine	–	0.34	0.11	0.11
	Glutamine	–	0.51	0.1	0.1
VITAMINS	Thiamine HCl	–	–	0.0001	0.001
	Riboflavin	0.001	–	0.001	0.001
	Nicotinic acid	0.001	0.001	0.001	0.0013

(Continued)

Table 3 - Composition of each defined media ($g \cdot L^{-1}$) used for *L. lactis* growth (Cocaign-Bousquet *et al.*, 1995; Jensen and Hammer, 1993; van-Niel and Hahn-Hagerdal, 1999) (continued)

Groups	Constituents	MS 15	MS 10	SA	Modified SD 3
VITAMINS	*p*-aminobenzoic acid	–	–	–	0.0001
	Calcium pantothenate	0.001	0.001	0.001	0.0012
	Folic acid	–	–	0.001	0.001
	Pyridoxamine	0.005	0.005	0.002	0.0023
	Biotin	0.01	0.01	0.0001	0.0001
	myo-Inositol	–	–	–	0.0015
NITROGEN BASES	Adenine	–	–	–	0.03
	Guanine	–	–	–	0.03
	Uracil	–	–	–	0.06
MINERAL BASE	Acetate	–	–	1.2	–
	$(NH_4)_2SO_4$	–	0.18	0.48	3.74
	NaCl	–	–	2.9	0.525
	KH_2PO_4	9	9	–	2.5
	K_2HPO_4	7.5	7.5	0.18	3.25
	$MgSO_4 \cdot 7H_2O$	0.2	0.2	0.106	0.87
TRACE METALS	$(NH_4)_6Mo_7O_{24}$	–	–	0.0000037	0.0004
	$MnSO_4 \cdot 4H_2O$	–	–	0.000013	0.001
	$CaCl_2 \cdot 2H_2O$	–	–	0.055	0.08
	$ZnSO_4 \cdot 7H_2O$	–	–	0.000003	0.0048
	$CoCl_2 \cdot 6H_2O$	–	–	0.000007	0.0003
	$CuSO_4 \cdot 5H_2O$	–	–	0.00002	0.0003
	$FeSO_4 \cdot 7H_2O$	–	–	0.0015	0.003
	H_3BO_3	–	–	0.000025	0.001
OTHERS	MOPS	–	–	8.36	–
	EDTA	–	–	–	0.015

The SA medium described by Jensen and Hammer (1993) has 42 components, among which 19 are amino acids and 9 are vitamins. Modified SD 3 medium contains the same amino acids composition as the SA medium, but is enriched in nitrogen bases, namely adenine, guanine and uracil. The MS 10 medium is constituted by 18 amino acids, but is simpler than the SA medium because it only contains 4 vitamins and does not have any trace metals. The MS 15 corresponds to the minimal medium specific for the strain used in this work, IL 1403, and contains 17 components, including 9 amino acids and 5 vitamins (Cocaign-Bousquet *et al.*, 1995).

3.3 SHAKE FLASKS EXPERIMENTS

3.3.1 Cultivation

Initially, the stock cultures were thawed at room temperature and inoculated in 250 mL sterile shake flasks (first pre-culture) containing 50 mL of Corynebacterium medium. The cultures grew on a rotary shaker at 30 ºC and 200 rpm, during seven hours. Then, cells were separated from the medium by centrifugation (8000 rpm, 10 minutes at 4 ºC) and the pellets were washed twice with 0.9 % of a NaCl solution. Next, cells were resuspended in 250 mL shake flasks (second pre-culture) with 100 mL of the modified SD 3 medium and let grew overnight under the same conditions. Thereafter cells were centrifuged and washed twice with NaCl (0.9 %) and resuspended in 5 mL of NaCl solution. These cells were used as inoculum of a 50 mL shake flask. The initial optical density ($OD_{600\ nm}$) of corrected to approximately 0.1. The growth conditions of the main culture were the same used in the second pre-culture.

When the composition of the main culture was the complex medium, the second pre-culture was omitted, as well as the centrifugations.

3.3.2 Influence of the carbon source in growth

In the experiments about the influence of the carbon source in growth, glucose or lactose were added to the Corynebacterium medium. The experiments were made in a bioreactor and the initial optical density ($OD_{600\ nm}$) was corrected to approximately 0.1. The initial sugar concentrations were 5 $g{\cdot}L^{-1}$. The first pre-culture grew at 30 ºC and 200 rpm, overnight.

3.3.3 Definition of the minimal medium

A main objective of this work consisted in the definition of an appropriate minimal medium for *L. lactis* subsp *lactis*. This way the influence of vitamins, mineral base and amino acids compositions were evaluated, individually, in the growth of *L. lactis* IL 1403.

To test the vitamins group, *L. lactis* IL 1403 was cultivated in the modified SD3 medium; however, the vitamin composition corresponded to the SA medium (see Table 3). The modified SD3 medium supplemented with 10 $g{\cdot}L^{-1}$of glucose was used, as a control.

The mineral base study was carried out as stated above but using the mineral base composition from the SA medium. The composition of the main culture corresponded to the modified SD3 medium, but using the vitamins from the SA medium.

Table 4 shows the composition of the main cultures used in the experiments on the vitamins and mineral base.

Table 4 - Main cultures compositions used in the study about the influence of vitamins and mineral base in the *L. lactis* IL 1403 growth

	Influence of vitamins	Influence of mineral base
Base mineral	SD3 medium	SA medium
Amino acids	SD3 medium	SD3 medium
Trace metals	SD3 medium	SD3 medium
Vitamins	SA medium	SD3 medium
Glucose	10 $g{\cdot}L^{-1}$	10 $g{\cdot}L^{-1}$

Additionally, the effect on *L. lactis* growth of each compounds of the mineral base was explored. For this, the mineral base of SA medium was enriched, individually for each compound of mineral base. The enrichment consists in the utilization of the concentrations present in modified SD 3 medium.

Relatively to amino acids, 6 combinations were tested, as described in Table 5. The amino acids isoleucine, valine, leucine, histidine and methionine were present in all combinations.

Table 5 - Composition of the various amino acid combinations (Bolotin *et al.*, 2001; Cocaign-Bousquet *et al.*, 1995; Michelsen *et al.*, 2010; van Niel *et al.*, 1999;)

COMBINATION 1 (C1)		COMBINATION 2 (C2)		COMBINATION 3 (C3)	
Component	$g{\cdot}L^{-1}$	Component	$g{\cdot}L^{-1}$	Component	$g{\cdot}L^{-1}$
Isoleucine	0.2	Isoleucine	0.2	Isoleucine	0.2
Valine	0.33	Valine	0.33	Valine	0.33
Leucine	0.47	Leucine	0.47	Leucine	0.47
Histidine	0.11	Histidine	0.11	Histidine	0.11
Methionine	0.12	Methionine	0.12	Methionine	0.12
Glutamic acid	0.1	Glutamic acid	0.1	Glutamic acid	0.1
		Arginine	0.12	Arginine	0.12
				Serine	0.34
				Threonine	0.23
COMBINATION 4 (C4)		**COMBINATION 5 (C5)**		**COMBINATION 6 (C6)**	
Component	$g{\cdot}L^{-1}$	Component	$g{\cdot}L^{-1}$	Component	$g{\cdot}L^{-1}$
Isoleucine	0.1	Isoleucine	0.2	Isoleucine	0.2
Valine	0.1	Valine	0.33	Valine	0.33
Leucine	0.1	Leucine	0.47	Leucine	0.47
Histidine	0.05	Histidine	0.11	Histidine	0.11
Methionine	0.1	Methionine	0.12	Methionine	0.12
Glutamic acid	0.3	Arginine	0.12	Arginine	0.12
Arginine	0.2	Serine	0.34	Asparagine	0.1
Asparagine	0.1	Threonine	0.23	Glutamic acid	0.3
				Lysine	0.35

All experiments were made in duplicate and the modified SD3 medium was used as control.

3.4 BIOREACTOR EXPERIMENTS

All fermentations were carried in a 5-L stirred-tank bioreactor BIOSTAT®B-DCU II (Sartorius Stedim, Germany) with 3-L working volume. This bioreactor is designed for microbial cultures, in discontinuous and continuous process (Sartorius, 2009). The bioreactor was sterilised in an autoclave at 121 °C, 40 minutes. This temperature must be maintained during at least 30 minutes to guarantee a good sterilization. The culture media used in bioreactor experiments was the Corynebacterium medium, described in 3.2.

L. lactis strain was precultured in complex medium (as described in section 3.3.1) and the first pre-culture was used in the inoculation of the bioreactor to an optical density of 0.1.

In all experiments temperature, pH and dissolved oxygen (DO) were controlled automatically. The temperature was maintained at 30 ºC using a temperature sensor (SONTEC). The pH was

measured with a pH probe (325/12-VP-HM, Hamilton, USA) and kept constant at 6.5. In the experiments with pH control 15 % orthophosphoric acid or 3 M NaOH were added automatically. In the experiments about the influence of the non-control of pH in *L. Lactis* growth, the pumps of the base and acid were disconnected. Relatively to dissolved oxygen (DO), this parameter was maintained at 30 % using an oxygen electrode (325/12-VP-HM-optical, Hamilton, USA) and to maintain the desired percentage, the sparging air was kept at a rate of 1 vvm. When necessary, antifoam was added to the bioreactor.

The fermentations were coupled with a BioPAT_DCU which allows the control of the process and data acquisition during the fermentations.

In the bioreactor, the influence of the carbon source (glucose and lactose) and the control of pH in the growth of *L. lactis* IL 1403 were analysed. All experiments were carried out in duplicate.

3.5 ANALYTICAL METHODS

In all experiments, samples were taken to quantify the bacterial growth. In bioreactor experiments, the samples were also used to determinate substrate consumption and products concentrations. The bacterial growth was quantified by optical density (*OD*) using the microplate reader (Sunrise, TECAN), at a wavelength of 600 nm. The *OD* values obtained were converted to dry cell weight (CDW), using a calibration curve (Annex A.1).

To determine the concentrations of the substrate and fermentation products, 3 mL samples were centrifuged at 8000 rpm during 5 minutes. Then, the supernatant was filtered using 0,2 μm filters, for complete removal of the cells and others residues, and, stored in vials at -20 ^{o}C for later analysis.

Lactose concentrations were determined by high performance liquid chromatography (HLPC) with a MetaCarb 87H Varian column (300 x 7.8 mm) coupled to a RI detector (model RI 1530). The analysis of this compound was made at 60 ^{o}C, flow rate of 0.5 mL·min^{-1} and the eluent used was H_2SO_4 at 5 mM concentration. The eluent was filtered with a membrane (0.2 μm porosity) and degassed in an ultrasonic bath for 30 minutes, before utilisation.

The remaining compounds were determined using enzymatic kits from Boehringer Mannheim/R-Biopharm, Germany: glucose (Cat. No. 10 716 251 035) and lactic acid (Cat. No. 10 139 084 035). The samples used in the experiments were thawed and boiled at, approximately, 80 ^{o}C during 15 minutes to stop the enzymatic reactions and, subsequently centrifuged (8000 rpm, 5 minutes). Then, the supernatant was utilized in the determination of the referred

compounds. The absorbance was measured at a wavelength of 340 nm and converted in concentrations by standard curves determined previously for each compound (Annex C1). The supplier instructions for each kit are described in Annex C2.

Cell dry weight (*CDW*) was determined at the end of the exponential phase by filtration of the sample through a mixed cellulose ester filters (0.2 μm porosity). The filters were previously dried in a microwave at 300 W for 10 minutes, placed in a desiccator for 15 minutes to avoid the water vapour adsorption and weighed in an analytical balance. Next, 40 mL of culture were filtered and the filters were washed once with 15 mL of distilled water. The filters were, again, dried in a microwave in the same conditions, placed in desiccator and weighted. The difference between both weights corresponds to the cell dry weight.

3.6 CALCULATIONS OF FERMENTATION PARAMETERS

Applying the logarithm to the biomass concentration, the exponential phase is then linearized and the slope of this linearization corresponds to the maximum specific growth rate (μ_{max}). The fermentation parameters were determined as described in table 6.

Table 6 - Equation to the several fermentation parameters calculated

Parameter	Equation		Units
t_d	$t_d = \frac{ln2}{\mu_{max}}$	1	h
$Y_{X/S}$	$Y_{X/S} = \frac{X_f - X_i}{S_f - S_i}$	2	$g \cdot g^{-1}$
q_s	$q_s = \frac{\mu_{max}}{Y_{X/S}}$	3	$g \cdot g^{-1} \cdot h^{-1}$
P	$P = \frac{X_f - X_i}{t_{incubation}}$	4	$g \cdot L^{-1} \cdot h^{-1}$

Where:

X – Biomass concentration ($g \cdot L^{-1}$) in the end (X_f) and begin (X_i) of exponential phase;

S – Substrate concentration ($g \cdot L^{-1}$) in the end (X_f) and begin (X_i) of exponential phase;

q_s – Specific substrate consumption rate;

$Y_{X/S}$ – Biomass yield per substrate consumed;

P – Biomass productivity.

3.7 STATISTICAL ANALYSIS

Average and standard deviations, of each variable evaluated, were calculated from two independent fermentation experiments.

Statistical analysis of similarity between biomass concentrations, using different carbon sources, was performed using a t-test with the software R (RStudio Desktop, version 0.95.262).

4. RESULTS AND DISCUSSION

This chapter covers the results obtained concerning the influence of the carbon source and control of pH in *Lactococcus Lactis* IL 1403 growth.

Also, the optimization of defined culture media and the minimal amino acids composition were investigated for *L. lactis* and, the results obtained are presented in this chapter.

4.1 INFLUENCE OF THE CARBON SOURCE

The culture medium should contain all nutrients, including carbon and nitrogen sources that allow the growth of microorganisms (Anderson and Jayaraman, 2003).

In order to understand the effect of the carbon source in *L. lactis* batch growth, two different sugars were used in the bioreactor experiments. To do so, the Corynebacterium medium (CM) was individually supplemented with glucose (CMG) and lactose (CML).

The fluctuations in the price of the media components used in fermentations (*e.g.*: sugars, amino acids, minerals) have contributed to the necessity of finding economic alternatives. This category includes by-products from the food industry, such as the cheese whey, coffee grinds, edible oils, among others. For instance, cheese whey is a by-product of the dairy industry and has approximately 5 % of lactose, in addition to proteins (β-lactoglobine, lactoferrin), minerals and lactic acid (Madrona *et al.*, 2009). Worldwide, cheese whey production reaches 120 million tons every year and is often discarded in lands and rivers without treatment. For this reason, cheese whey wastes are considered one of the most polluting food wastes (Madrona *et al.*, 2009) and the increase of awareness for environmental protection contribute for the use of this stream as culture medium and motivates the study about the influence of lactose in *L. lactis* growth (BacHBerry, 2013). Glucose was chosen also as a carbon source because it is the preferential sugar for bacterial growth and is present in nature either as a monosaccharide or as part of polysaccharides (Madigan *et al.*, 2003).

To see the effect of lactose and glucose on the growth of *L. lactis*, cells were cultured in Corynebacterium medium with the corresponding carbon source (see details in "Material and Methods"). A typical growth curve for the two carbon sources is shown in Figure 5.

The lag phase of the cells was very short for both carbon sources, and growth was observed upon one hour after the inoculation and lasted for 10 hours, Figure 5. The similarity between the cultivation conditions in shake flasks and the bioreactor has a great impact in these reduced lag phase since cells were already adapted to the culture medium and environmental conditions (Madigan *et al.*, 2003).

The exponential phase had different durations in CMG and CML. In the experiment with lactose, the exponential phase lasted 3 hours, while with glucose it lasted 4 hours.

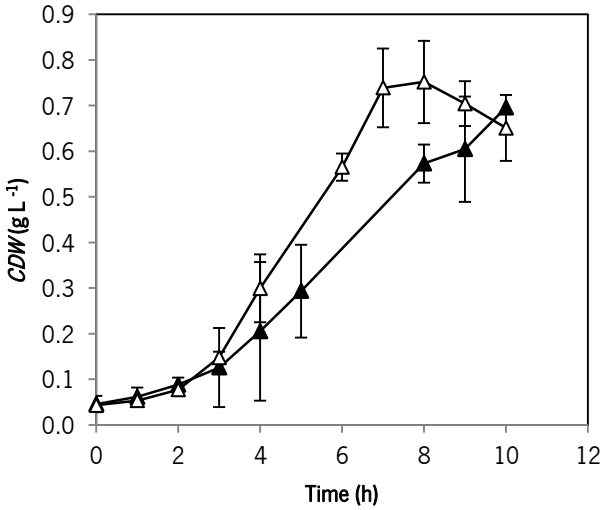

Figure 5 - *L. lactis* IL 1403 growth curve in a 5-L bioreactor. Cells were cultivated in the Corynebacterium medium with 0.5 % lactose (△) and glucose (▲) at 30 ^{o}C with pH at 6.5 and 30 % DO. Average and standard deviation were calculated from two independent fermentation experiments.

After exponential growth, the cells reached the late exponential phase and the cells growth starts to decelerate because one or more substrates achieved limiting concentrations or are depleted from the medium. In addition, growth of cells can be arrested due to the accumulation of inhibitory or toxic metabolites (Madigan *et al.*, 2003; Pampulha, 1998). The maximum biomass concentration was reached along this phase. *Lactococcus lactis* IL 1403 grew better in medium supplement with lactose than in glucose medium, since the maximum biomass concentration was higher for CML, 0.752 g·L^{-1}, contrary to 0.696 g·L^{-1} obtained in CMG. This is probably due to the fact that the strain *L. lactis* IL 1403 has dairy origin (Cocaign-Bousquet *et al.*, 1995). The analysis of similarity between biomass concentrations was performed using t-test. In accordance with the results obtained (p value = 0.672), is possible to affirm with 95 % confidence that the biomass concentrations are not statistically equivalent.

In the medium supplement with lactose, cells reached stationary phase seven hours after the inoculation, Figure 5.

Substrate consumption and lactic acid production profiles for the experiments are shown in

Figure 6. When cells reach the stationary phase, different profiles of carbon source consumption are observed. For CML, lactose is completely depleted from the medium suggesting that growth is arrested due to the limitation of this substrate, while in CMG growth stops before glucose is depleted, indicating that this substrate is not the limiting nutrient and some essential growth factor,

as amino acids or vitamins could be exhausted and, consequently cause the growth cessation (Razvi *et al.*, 2008).

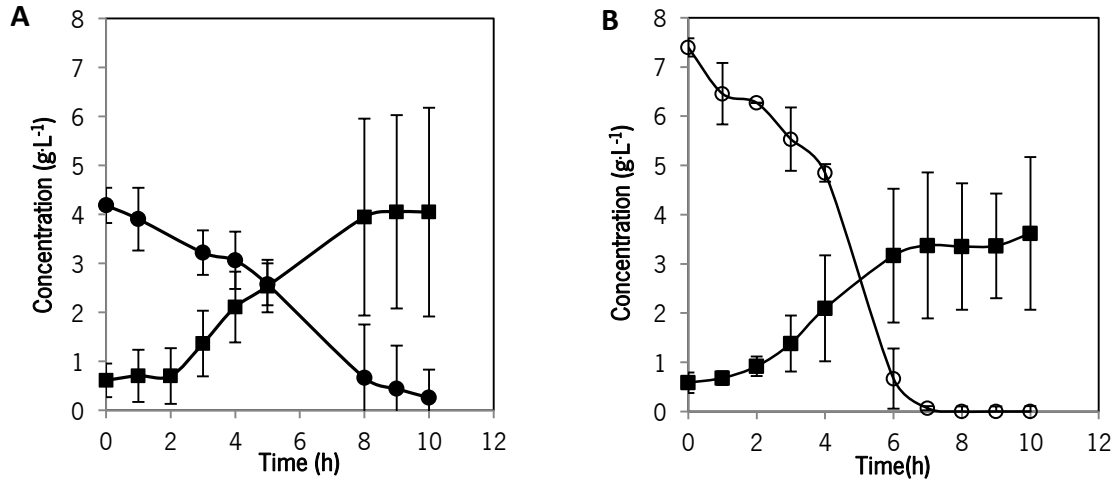

Figure 6 - Substrate consumption and end-product production in *L. lactis* (A) Glucose consumption (●) and lactic acid production (■) and (B) lactose consumption (○) and lactic acid production (■) during of the fermentation process. Data are presented as the mean and the standard deviation calculated from two independent fermentation experiments in the bioreactor

The biomass yield ($Y_{X/S}$) was calculated by the ratio between cells mass yield per carbon source mass consumed (Fonseca and Teixeira, 2006) at end of the exponential phase. As shown in Table 7, the highest biomass yield was obtained in the medium supplemented with glucose. The lowest value was obtained with lactose. As mentioned above, the maximum of biomass concentration in CML and CMG are 0.752 and 0.696 $g \cdot L^{-1}$, respectively. In addition we have observed that during the exponential phase, lactose was more promptly consumed than glucose, which contributed to a biomass yield was lower. Although a similar biomass concentration was obtained for the two substrates tested, the cell productivity (P) is higher in the MCL condition, because the lactose consumption in this case is faster.

The exponential phase is linearized applying the logarithm to the biomass concentration and slope corresponds to the maximum specific growth rate (μ_{max}). The parameters obtained for *Lactococcus lactis* IL 1403 for the different carbon sources tested are shown in Table 7.

The maximum specific growth rate of *L. lactis* IL 1403 is enhanced by the use of lactose as carbon source (Table 7). Consequently, cells cultivated in CML have a faster duplication time than cells cultivated in CMG. The time needed for the formation of two cells from one is called the doubling or generation time (t_d) (Madigan *et al.*, 2003). The doubling time and the specific growth rate depend of the intrinsic characteristics of each species, of the extrinsic environmental

conditions (pH, temperature, water availability and aeration) and composition of the growth medium (Fonseca and Teixeira, 2006; Madigan *et al.*, 2003; Pampulha, 1998).

Table 7 - Maximum specific growth rate (μ_{max}), duplication time (t_d), biomass yield ($Y_{X/S}$) at end of the exponential phase, specific substrate consumption rate (q_S) and maximum productivity in biomass (P) for *Lactococcus lactis* IL1403 grown in glucose and lactose Data about $Y_{X/S}$, q_S and P are presented as the mean and the standard deviation of two independent fermentations in the bioreactor

Parameters	Glucose	Lactose
μ_{max} (h^{-1})	0.398 ± 0.041	0.582 ± 0.237
t_d (h)	1.743 ± 0.181	1.191 ± 0.484
$Y_{x/s}$ ($g \cdot g^{-1}$)	0.174 ± 0.107	0.156 ± 0.036
q_S ($g \cdot g^{-1} \cdot h^{-1}$)	2.282 ± 0.676	3.810 ± 0.989
P ($g \cdot L^{-1} \cdot h^{-1}$)	0.065 ± 0.009	0.095 ± 0.020

Lan *et al.*, (2006) compared *L. lactis* growth under anaerobic and aerobic conditions, with and without heme in complex medium, M17, supplemented with 65 $g \cdot L^{-1}$ glucose. The operational conditions used were similar to those used in the carbon source experiments above. However, in the experiment under aerobic condition without heme dissolved oxygen was kept at 2 % saturation, while in the present work 30 % was the condition used (see in section 3.4) and the initial concentration on CMG was 5 $g \cdot L^{-1}$. In the aerobic fermentation without heme, Lan *et al.* (2006) obtained a maximum biomass concentration of 1.801 $g \cdot L^{-1}$, while a value of 0.696 $g \cdot L^{-1}$ was obtained for MCG, although the initial substrate concentrations are not comparable. Additionally, the specific growth rate was 0.4 h^{-1}, a value similar to the one obtained in MCG. The difference observed in the biomass concentration can be based on the growth medium composition, since the presence of other limiting compounds improve the growth rate and, subsequently decrease the doubling time, as well as due to the *L. lactis* strain used.

The ratio between the maximum specific growth rate and the biomass yield allowed the determination of the specific substrate consumption rate (q_S) (Fonseca and Teixeira, 2006; Lopes, 2013). In accordance to the results obtained (Table 7), this parameter is affected by the sugar used as carbon source, obtaining a higher value for the CML. These results are in conformity with

several authors that refer that strains from dairy origin metabolize lactose more quickly (Aleksandrzak-Pierkarczyk *et al.*, 2005; Axelsson, 2004).

4.2 OPTIMIZATION OF DEFINED CULTURE MEDIA

Once *L. lactis* growth in complex medium was characterized, the characterization of the growth in different defined media was studied. In this study, four different media were used, namely minimal medium (MS 15), MS 10 medium, SA medium and modified SD 3 medium.

Cocaign-Bousquet and co-workers (1995) compared the growth of two strains of *L. lactis* (NCDO 2118 and IL 1403) and defined the minimal nutritional requirements for each strain. In accordance to this paper, the minimal medium for strain IL 1403, called MS 15 (see composition in Table 3), contains only the indispensable compounds that allow cell growth (Pampulha, 1998). The MS 15 medium was tested with a minor modification in which glucose was replaced by lactose, Figure 7. The experiment was conducted at 30 ^{o}C and 200 rpm in a rotary shaker.

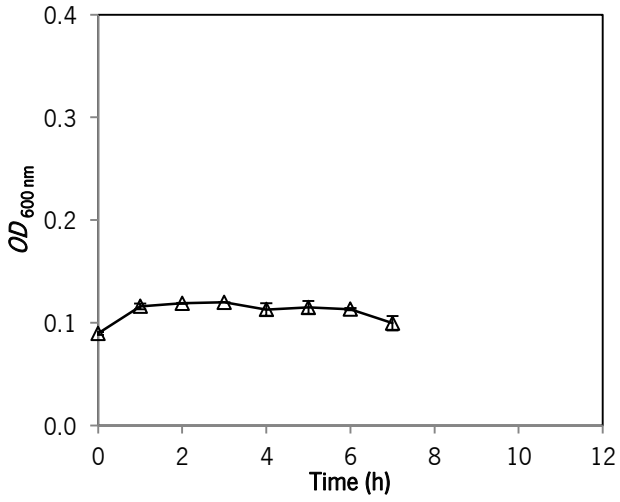

Figure 7 - *Lactococcus lactis* IL 1403 growth curve in shake flasks. Cells were cultivated in minimal medium (MS15) with 5 g·L^{-1} lactose at 30 ^{o}C and 200 rpm. Average and standard deviation were calculated from two independent fermentation experiments.

In these conditions, *L. lactis* IL 1403 was not able to grow as shown in Figure 7. As previously mentioned, *Lactococcus lactis* is used as a start culture in many food production processes, namely in the dairy industry. This LAB is able to metabolize lactose and the catabolism of this sugar is encoded by genes present in the lactose operon (lac operon) (Kowalczyk *et al.*, 2000). However, various studies have shown that the strain used in this work, IL 1403, is lactose negative meaning that it is unable to transport and metabolize lactose (Aleksandrzak-Pierkarczyk *et al.*, 2005; Kowalczyk *et al.*, 2000).

The IL 1403 strain of *L. lactis* is a derivative of the wild-type IL 594 strain, with no plasmids that was isolated from dairy products (Aleksandrzak-Piekarczyk, 2013; Bolotin *et al.*, 2001). Through the analysis of the genome sequence of IL 1403, it is possible to sustain that the lactose-PTS transporter is not present. For the case of the strain IL 1403, the genes that encode for the PTS system, as the phospho- β–galactosidase, are not present, including the genes that degrade lactose via the Tagatose pathway. The lack of the enzymatic activity involved in lac-PTS and the tagatose pathway was previously reported by Crow and Thomas (1984).

Additionally, lactose can be transporter by lactose permease (lacS) and the cellobiose specific permease (celB). The expression of these transporters is regulated by the sugar present in the medium. For instance, in medium with glucose and lactose both activities are absent. Furthermore, in medium with galactose, the activity of these enzymes is close to zero. Nevertheless, if the medium is supplemented with cellobiose, an activator of these proteins, the activity of these enzymes reaches its maximum value (Aleksandrzak-Pierkarczyk *et al.*, 2005)

Relatively to lactose permease system, *L. lactis* IL 1403 contains the galactose genes that allow the catabolism via the Leloir pathway and the enzymes required for lactose assimilation, namely the β-galactosidase (lac Z) and thiogalactosidase acetyltransferase (lac A) (Aleksandrzak-Pierkarczyk *et al.*, 2005). Although the IL 1403 strain contains all the genetic elements, it is not able to metabolize lactose in defined media (Figure 7). Nevertheless, this phenomenon can be explained by the low affinity of the lactose permease (lacS) towards lactose and the absence of β-galactosidase expression (Aleksandrzak-Piekarczyk, 2013; Aleksandrzak-Pierkarczyk *et al.*, 2005). Indeed, Aleksandrzak-Piekarczyk and co-workers (2005) state that in the strain IL 1403, the lac Z in is not being expressed in defined media supplemented with lactose. Additionally, these authors sustain that the lactose permease, has a higher affinity to galactose than to lactose. These results corroborate our own data and explain why there is no lactose being transported into cells.

Bolotin *et al.* (2001) was able to sequence the genome of *L. lactis* IL 1403, which led to the identification of the genes associated to cellobiose-specific PTS system, namely the bglS (β-glucosidase A) and EII components, as celB (cellobiose-specific PTS EII C), ptcA (cellobiose-specific PTS EII A) and ptcB (cellobiose-specific PTS EII B) (Aleksandrzak-Piekarczyk, 2013). These last two EII components are responsible for phosphorylating cellobiose, while the first helps sugar translocation via the membrane. Relatively to bglS, this enzyme cleaves lactose phosphate in glucose and galactose-6-phosphate, that is, the bglS activity is similar to phospho-β-glucosidase

and phospho-β-galactosidase. Glucose towards the EMP pathway and galactose-6-phosphate after dephosphorylation follows the Leloir pathway (Aleksandrzak-Piekarczyk, 2013).

Then it can be concluded that if the culture medium is supplemented with this disaccharide, the cellobiose-PTS is activated and lactose can be metabolized because celB is the only permease used in lactose uptake in *L. lactis* IL 1403 (Aleksandrzak-Piekarczyk, 2013).

As mentioned in section 4.1, cells were able to grow in a CML, Figure 5. However, the same behaviour was not observed in minimal defined medium supplemented with lactose, Figure 7. Aleksandrzak-Piekarczyk, *et al.*, (2005) compared the *Lactococcus lactis* growth in two scenarios: *L. lactis* strains growing in a chemically defined medium (CDM) with 10 $g{\cdot}L^{-1}$ of lactose and supplemented with a low cellobiose concentration (0.1 $g{\cdot}L^{-1}$) and, the same medium but without cellobiose. In the scenario without cellobiose, *L. lactis* did not grow. In contrary, when cells were grown with cellobiose, growth was immediately observed. These results allow the authors to speculate that cellobiose induces the permease transport and the phosphorylation of lactose. Given this fact, we can speculate that growth observed in CML results from the presence of some compound that activate lactose metabolism. Indeed, the strain IL1403 is only able metabolize lactose under two conditions: a) cellobiose, which is the inductor, is in the medium and b) the control catabolite protein A (*ccp*A$^-$) is not repressing the lactose-associated genes. The *ccpA* is a protein specific of gram positive bacteria, responsible to regulate the β-glucosides and lactose metabolism. In the presence of glucose, this protein represses the enzymes involved in these carbohydrate metabolisms in a phenomenon called carbon catabolite repression. In accordance to the literature, the absence of glucose leads to inactivation of *ccpA* which allows cells to degrade other sugars that are on its surrounding. Concluding, the absence of glucose (inactivation of *ccpA* gene) and the presence of cellobiose in culture medium leads to an efficient lactose uptake and subsequently degradation.

4.2.1 Development of a Defined Medium

Several media were tested: MS 10, SA and a modified SD3 medium (Figure 8). SA medium described by Jensen and Hammer (1993) has 42 components, among which 19 amino acids and 9 vitamins. The modified SD 3 medium contains the same amino acids composition that SA medium but is enriched in nitrogen bases, namely adenine, guanine and uracil. The MS 10 medium is constituted by 18 amino acids, but is simpler than the SA medium because it only contains 4 vitamins and does not have trace metals (Cocaign-Bousquet *et al.*, 1995).

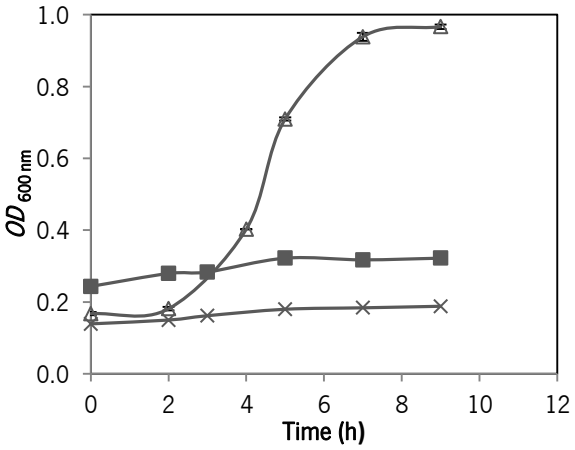

Figure 8 - Growth curve of *L. lactis* IL1403 in different culture media. Symbols stand for: △ modified SD3; ■ MS10 medium and × SA medium) with 10 $g \cdot L^{-1}$ glucose, at 30 ^{o}C and 200 rpm.

Figure 8 shows that no growth is detected for the media MS 10 and SA, 9 hours after inoculation. However, in the modified SD 3 medium *L. lactis* IL 1403 was able to grow until an *OD* of approximately 1.This medium differs from the other two media by having the inclusion or higher concentrations of several groups of nutrients. With the view of finding a minimal medium that sustains the growth of *L. lactis,* several groups of nutrients were tested. One group of nutrients which is only present in the SD3 medium is a combination of nitrogen bases, namely adenine, guanine and uracil. Therefore, we aimed to find the essentiality of these compounds by growing cells with and without them, keeping the remaining composition of the SD3 medium. To guarantee a pool of viable cells upon inoculation, cells were grown in SD3 medium overnight and washed twice in an isotonic solution to avoid the contamination of the different media. Results are shown in Figure 9 and indicate that while not being essential, nitrogen bases mixture promotes growth. The presence of nitrogen bases allow reaching an optical density around 1, while without them maximum *OD* is only 0.5.

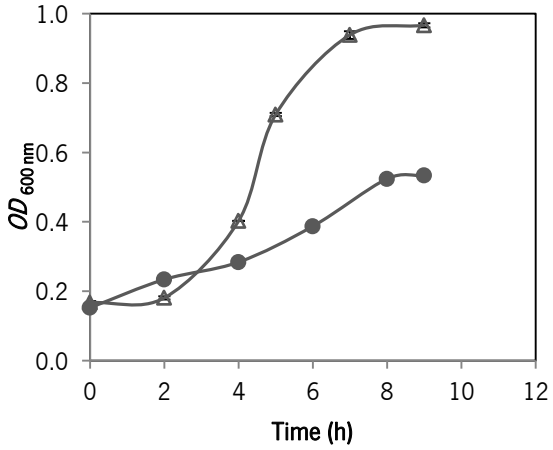

Figure 9 - Growth curve of *L. lactis* IL1403 in modified SD3 with (△) and without (●) nitrogen bases. The experiments were made at 30 ^{o}C, 200 rpm.

In this situation the specific growth rate was 0.289 h^{-1} while without nitrogen bases it was 0.126 h^{-1}. According to van-Niel and Hahn-Hagerdal (1999) these compounds are not essential for *L. lactis* growth, but have a stimulatory effect. The Figure 9 is consistent with the results obtained by these authors. Anderson and Elliker (1953) affirm that when all purines and pyrimidines are added to the culture medium with 20 amino acids, they sharply increased growth.

However, *L. lactis* could grow in the SD3 medium where nitrogen bases have been eliminated, since pyridoxamine is present in the vitamins composition of the medium used. This vitamin is not essential for growth, but when nitrogen bases are not present in the culture medium this becomes essential (Cocaign-Bousquet *et al.*, 1995; van-Niel and Hahn-Hagerdal, 1999). Then, in minimal medium the nitrogen bases can be removed provided that pyridoxamine is present in the composition of the culture medium.

4.2.1.1 Influence of the mineral base and vitamins in growth

As mentioned above, a main goal of this work is to find out a minimal medium that sustains the growth of *L. lactis* IL1403. Besides the nitrogen bases, the main difference between SD 3 medium and MS10 and SA media are the vitamins and mineral base composition. Consequently, the influence of the composition of these two groups in *Lactococcus lactis* IL 1403 growth was studied and results are shown in Figure 10. Cells were grown in modified SD3 medium overnight, and subsequently transferred to media with different vitamins and mineral base composition (Table 4, see 3.3.3 of "Materials and Methods"). Cells cultivated in SD3 were used as the control of the experiment.

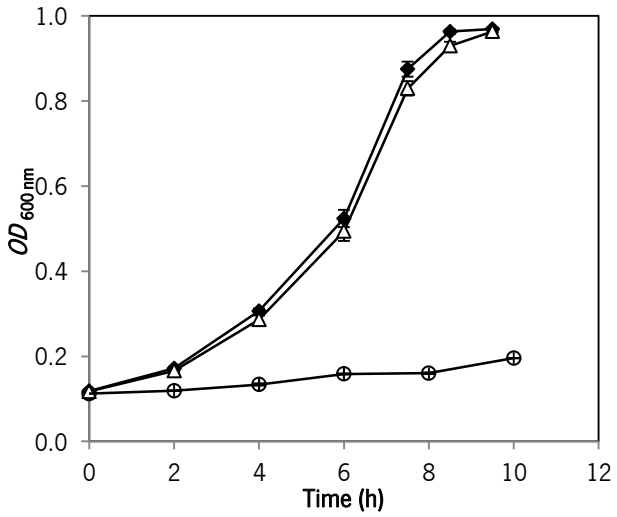

Figure 10 - Growth curve of *L. lactis* IL1403 in media with different vitamins (△) and mineral base (○) compositions. The modified SD 3 medium (◆) was used as control. The experiments were made at 30 ^{o}C and 200 rpm. Average and standard deviation were calculated from two independent fermentation experiments.

The composition of the mineral base has a strong influence in the growth of *L. lactis* IL 1403, Figure 10. The mineral base of the SA medium is not sufficient to meet the needs of the *L. lactis* strain used in this thesis. As shown in Figure 10, growth is monitored along 10 hours, in which the optical density remains approximately the same. The main difference between the compositions of this group of nutrients in the SA medium *versus* modified SD 3 medium is the concentration of the compounds and the absence of acetate. Due to this fact, each compound (K_2HPO_4, KH_2PO_4; NaCl, $(NH_4)_2SO_4$ and $MgSO_4{\cdot}7H_2O$) were evaluated, Figure 11.

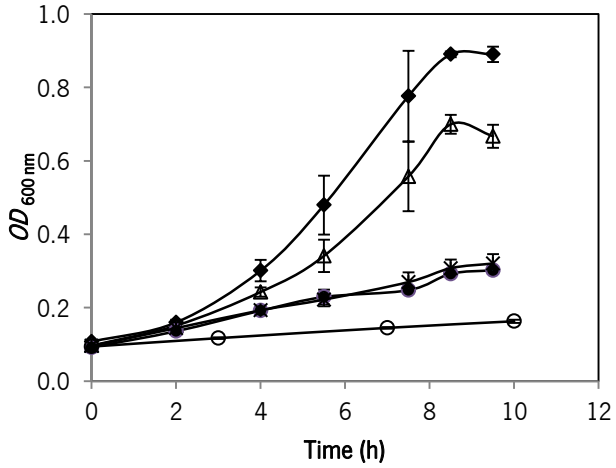

Figure 11 - Growth curve of *L. lactis* IL 1403 in modified SD 3 media enriched, individually for each compound of the mineral base. The experiments were made at 30 ^{o}C and 200 rpm. Symbols stand for: ◆ control (modified SD3 medium), △ K_2HPO_4, ● $(NH_4)_2SO_4$, ∗KH_2PO_4 and ○ $MgSO_4$. Average and standard deviation were calculated from two independent fermentation experiments.

The nitrogen is an essential compound for bacterial growth. This is found in nature in inorganic and organic forms, and is crucial to ensure growth. Nitrogen is present in the structure of proteins, vitamins and nucleotides (Madigan *et al.*, 2003). In the majority of the culture media, nitrogen is added as salts (inorganic forms) and/or as amino acids (organic forms) (Pampulha, 1998). Relatively to salts, the most commonly used is the ammonium sulphate, $(NH_4)_2SO_4$. In accordance to Figure 11, this compound does not influences *L. lactis* IL 1403 growth because the nitrogen requirements are, probably, filled from the 19 amino acids present in the growth medium. The results in Figure 11 are identical to the ones achieved by Cocaign-Bousquet *et al.*, (1995).

K_2HPO_4 and KH_2PO_4 are normally used as buffers in the culture medium (Coelho *et al.*, 2011; Honorato *et al.*, 2007). Additionally, phosphorus is used in the synthesis of nucleic acids, while the potassium is necessary as a cofactor for several enzymes (Madigan *et al.*, 2003). In the SA medium, potassium phosphate monobasic (KH_2PO_4) is absent, suggesting that absence of growth in this medium may be also due to its absence. However, in the experiments with the medium enriched with KH_2PO_4, growth is not observed, meaning that the presence of KH_2PO_4 does not influence the growth of the *L. lactis* strain used in this study. An increase of K_2HPO_4 in the medium, from 0.18 to 3.25 $g{\cdot}L^{-1}$ has a positive effect in the growth of *L. lactis* IL 1403. Coelho *et al.*, (2011) and Honorato *et al.*, (2007) studied the influence of K_2HPO_4 concentration in cell growth, with the same environmental conditions applied in complex medium and obtained the same behaviour found in the experiments described in this thesis. These authors state that by adding this natural buffer, the medium maintains nearly the same pH, which allows cells to grow longer. To test if K_2HPO_4 acts like a natural buffer we set to monitor pH values throughout the fermentation. Indeed, the pH varied from 6.99 to 5.64. In contrast, when medium is not enriched with K_2HPO_4, the pH varied much more from 6.5 to 4.2.

Another important nutrient for *L. lactis* is magnesium, which is necessary as a cofactor in several enzymatic reactions, as well as a stabilization factor in cell membrane and ribosomes (Campos, 1999; Madigan *et al.*, 2003; van-Niel and Hahn-Hagerdal, 1999). In accordance to Loubière *et al.*, (1997), the growth rate is affected by magnesium when the concentration is lower than 0.36 $mg{\cdot}L^{-1}$ and it becomes limiting at the concentration of 2.4 $mg_{magnesium}{\cdot}g_{biomass}^{-1}$. In SA medium, the concentration of $MgSO_4{\cdot}7H_2O$ is 0.2 $g{\cdot}L^{-1}$ meaning that this nutrient is not affecting growth rate or final biomass concentration.

The next group of nutrients tested was vitamins. The comparison performed is between the composition and concentration of vitamins in the SA medium *versus* the SD3 medium. Growth with both compositions was tested and showed that the composition used in the SA medium is already optimized to allow *L. lactis* growth (Figure 10) and the addition of aminobenzoic acid and *myo*-Inositol are not necessary. Niven (1994) and van Niel *et al.*, (1999) affirm that various vitamins of the B group are important for LAB growth and all *L. lactis* strains required obligatorily three vitamins, namely calcium pantothenate, nicotinic acid and biotin. Other B group vitamins stimulate bacterial growth (Cocaign-Bousquet *et al.*, 1995; van-Niel and Hahn-Hagerdal, 1999). This fact explains the occurrence of growth with fewer vitamins. The main difference between the vitamins composition tested is the number. Modified SD3 medium contains two more vitamins, *myo*-Inositol and *p*-aminobenzoic acid comparatively to SA medium. Both media contain the three essential vitamins required for all *L. lactis*.

In accordance to Cocaign-Bousquet *et al.*, (1995), the strain with dairy origin, as IL 1403, needs riboflavin in the culture medium to allow growth. This author compared the growth of this strain in the MS 10 medium (see Table 3 in section 3.2) with and without riboflavin and found that in the medium supplemented with vitamin B2 growth rate is 0.50 h^{-1}, contrary to other scenario. The omission of riboflavin in vitamins composition of MS 10 medium may be one of the reason for the lack of *L. lactis* growth. As demonstrated above in Figure 9, the nitrogen bases had a stimulatory effect in the *L. lactis* growth. However, when these are absence in the medium it is necessary to add pyridoxamine into the culture medium (Cocaign-Bousquet *et al.*, 1995).

4.2.1.2 Optimization of amino acids composition

With the objective of finding a minimal medium that sustains the growth of *L. lactis*, the minimal requirements in terms of amino acids were evaluated. The genus Lactococcus is nutritionally demanding, and it is not able to grow in medium that contains only nitrogen of mineral origin (Cocaign-Bousquet *et al.*, 1995). Oliveira *et al.*, (2005) affirms that the amino acids composition for *L. lactis* is not simple to define. To determine which were the essential amino acids for a sustainable growth, different combinations of these compounds were tested (Table 5, see "Material and Methods"), having as control cells growing in modified SD3 medium (amino acid composition of 19 amino acids). Cells were firstly grown in an inoculum with complex medium, and then transferred to the SD3 medium, for an overnight growth. To guarantee the mineral nitrogen

requirements in the experiments, the mineral base of SD 3 medium was used in which the concentration of $(NH_4)_2SO_4$ was 3.74 g·L^{-1}.

The starting point for the combinations tested was the amino acids composition present in combination 1, established by Cocaign-Bousquet and co-workers (1995) for the strain used in this work, IL 1403. Other amino acids were added to the combination 1 in accordance with what was reported by several authors (Bolotin *et al.*, 2001; Michelsen *et al.*, 2010; van-Niel and Hahn-Hagerdal, 1999; Zhang *et al.*, 2009). The composition of these combinations is discriminated in the "Materials and Methods" section 3.3.3, Table 5. The results obtained are shown in Figure 12 and demonstrate that all combinations of amino acids fail to allow cell growth.

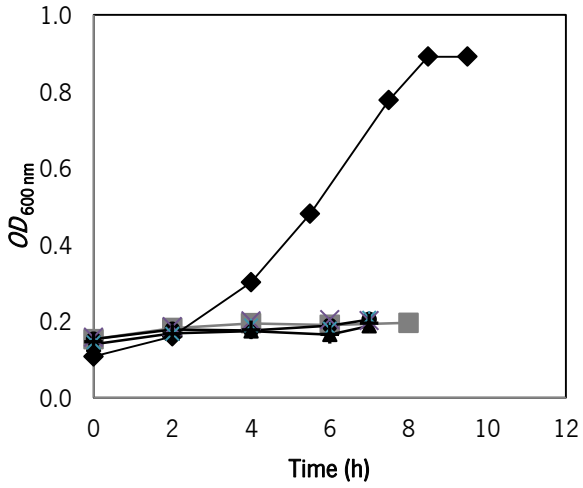

Figure 12 - Growth curve of *L. lactis* IL1403 on several amino acids combinations, at 30 ºC and 200 rpm. Symbols stand for: ◆ control; ■ C1; × C2; ✱ C3; ● C4; + C5 and ▲ C6) and the amino acids composition of the control medium (modified SD3 medium).

Firstly, IL 1403 was cultivated in a synthetic medium with 6 amino acids essential for nearly all *L. lactis* strains (combination 1) without success. In addition, other amino acids (see Table 5, section 3.3.3) were added to combination 1, but nonetheless cells did not grow, as it can be observed in Figure 12. Due these results was applied the single omission technique. This technique was used by Cocaign-Bousquet *et al.* (1995) to establish a minimal synthetic medium for two strains of *L. lactis,* NCDO 2118 and IL 1403. This technique is constituted by two processes: elimination of each metabolic family of amino acids (histidine, serine, branched chain, aspartate, glutamate and aromatic family) and elimination of an individual amino acid. Then, the effect of these processes was evaluated in terms of maximum specific growth rate (μ_{max}). In accordance with the results obtained in this study for strain IL 1403, the removal of phenylalanine, tyrosine and

tryptophan (elements of the aromatic family), alanine and cysteine from the medium did not affect cellular growth. Similar results were obtained in the experiments in which each amino acid was removed individually, as can be seen in Table 8.

Table 8 - Maximum growth rate (μ_{max}) obtained for each combination tested by the single omission technique The control was constituted by all amino acids except aspartic acid

Amino acids	μ_{max} (h^{-1})	Amino acids	μ_{max} (h^{-1})
Alanine (Ala)	0.217	Lysine (Lys)	0.179
Arginine (Arg)	0.197	Methionine (Met)	0.003
Asparagine (Asn)	0.121	Phenylalanine (Phe)	0.181
Cysteine (Cys)	0.287	Proline (Pro)	0.008
Glycine (Gly)	0.275	Serine (Ser)	0.034
Glutamic acid (Glu)	0.228	Tyrosine (Tyr)	0.246
Glutamine (Gln)	0.279	Threonine (Thr)	0.199
Histidine (His)	0.005	Tryptophan (Trp)	0.239
Isoleucine (Ile)	0.161	Valine (Val)	0.004
Leucine (Leu)	0.003	Control	0.287

When cysteine, glycine and tryptophan are individually absent from the medium, the growth rate is not affected, Figure 13. The presence of serine in the amino acid composition contributes for this behaviour because it is used as a precursor in the synthesis of these amino acids (Azizan *et al.*, 2013; Caspi *et al.*, 2010).

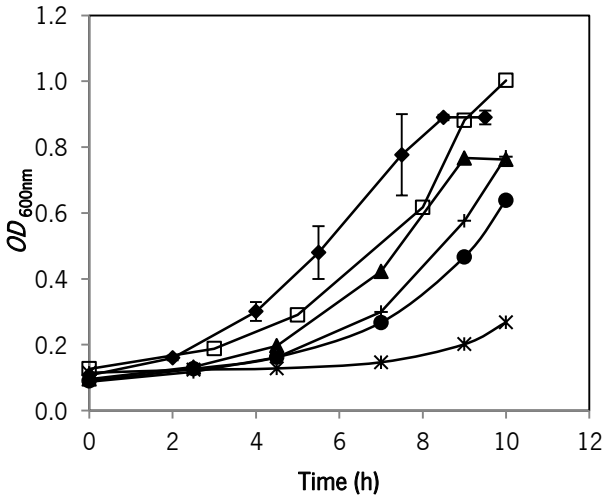

Figure 13 - *Lactococcus lactis* IL 1403 growth curve in shake flasks. Cells were cultivated in modified SD 3 media without some amino acids, as □ tryptophan, ▲ glycine, ┼ cysteine, ● threonine and ✳ serine and 10 $g \cdot L^{-1}$ of glucose at 30 ^{o}C and 200 rpm. The modified medium (◆) with 19 amino acid was used as control.

However, in the present work, the depletion of serine caused a decrease of 88 % in growth, contrary to 48 % obtained by (Cocaign-Bousquet *et al.*, 1995). Besides having a crucial role in the production of some amino acids, serine is used directly or indirectly in the synthesis of other compounds, as phospholipids (Caspi *et al.*, 2010).

In the case of branched chain amino acids, the absence of leucine and valine affects significantly, 99 %, the growth rate, Figure 14.

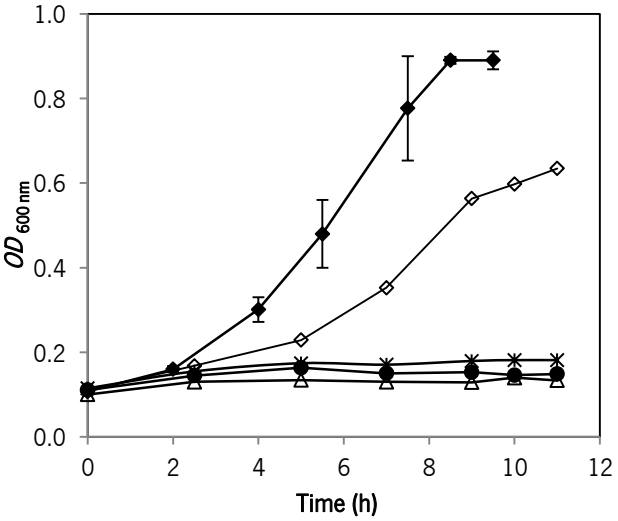

Figure 14 - *Lactococcus lactis* IL 1403 growth curve in shake flasks. Cells were cultivated in modified SD 3 medium without some amino acids, as ◇ isoleucine, △ leucine ● valine and ✳ histidine and 10 $g \cdot L^{-1}$ of glucose at 30 ^{o}C and 200 rpm. The modified medium (◆) with 19 amino acid was used as control.

In turn, the removal of isoleucine, another branched chain amino acid, leads to a reduction of 44 % of the evaluated parameter. In accordance with Cocaign-Bousquet *et al.* (1995), the omission of isoleucine affects in 100 % the μ_{max}. However, by analysing the isoleucine biosynthesis pathway, this amino acid is produced through threonine or aspartate. This way, the isoleucine synthesis is ensured, since when it is absent it can be synthesised through threonine and aspartate (Azizan *et al.*, 2013; Caspi *et al.*, 2010).

The loss of some metabolic functions, for instance, the ability to synthetize or degrade a substance, constitutes some of the genetic defects that result from the shift of a natural rich environment to simple defined media (Delorme *et al.*, 1993; Godon *et al.*, 1993). In accordance to Godon *et al.*, (1993), the auxotrophy reported by several authors for branched chain amino acids (BCAA) can be explained by a non-functionality of the genes required for their synthesis, since the required genes are present in the IL1403 genome (Cocaign-Bousquet *et al.*, 1995; Delorme *et al.*, 1993; Godon *et al.*, 1993). In the study developed by Godon *et al.*, (1993) about the inactivation of the genes involved in the synthesis of BCAA amino acids, it was proven that *leuC* and *ilvD* are active while *leuA* and *leuB* are inactive. In the same study two nonsense mutations and two deletions were detected in *leu* genes, which make *L. lactis* unable to synthetic these amino acids. Two base substitutions were found in *leuA* and *leuB* genes that added two stop codons and probably led to the formation of two non-functional proteins (Godon *et al.*, 1993; Grilo and Videira, 2001). Relatively to the two deletions, it was found that they were localized in the *leuA* gene and before of the *leuC* gene. The extension of the genetic defects determined by Godon *et al.*, (1993) is not revertible to prototrophy for the mutated genes. In some microorganisms, these lesions can be reverted via single mutations.

The omission of methionine and proline severely affects the growth rate, with a reduction of 99 and 97 %, respectively (Figure 15). Methionine is an important amino acid for microorganisms since it is involved in several cellular processes, for example, is the initiation of protein synthesis (Grilo and Videira, 2001). Sperandio *et al.*, (2005) affirms that strains obtained from dairy products are not able to growth when methionine is removed from the medium, and therefore it is considered a key amino acid in *L. lactis* growth. The synthesis of methionine is dependent on two amino acids, namely cysteine and aspartic acid and its synthesis involves six more reactions (Caspi *et al.*, 2010; Dale *et al.*, 2010; Sperandio *et al.*, 2005). Although the genes needed for this synthesis are present in the genome, the reason why there is not activity may be due to a lack of expression or insufficient gene expression (Bolotin *et al.*, 2001; Godon *et al.*, 1993).

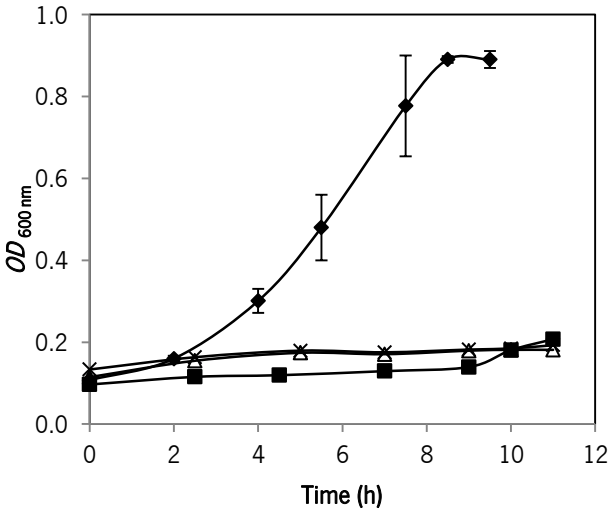

Figure 15 - *Lactococcus lactis* IL 1403 growth curve in shake flasks. Cells were cultivated in modified SD 3 medium without some amino acids, as ■ proline, △ histidine and × methionine and 10 $g \cdot L^{-1}$ of glucose at 30 ^{o}C and 200 rpm. The modified medium (◆) with 19 amino acid was used as control.

As expected, when histidine was removed of the medium composition, the growth rate was affect in 98 % (Figure 15). The dairy strains have lost the ability to synthetize histidine (Delorme *et al.*, 1993; Godon *et al.*, 1993). Delorme *et al.*, (1993) analysed the inactivation of histidine biosynthesis genes in strains isolated by dairy and non-dairy products. In order to grow, most of dairy strains need histidine in the culture medium. In resemblance to what happens with branched chain amino acids, this author and co-workers conclude which strains have genes required for the histidine synthesis. In addition to not growing in the absence of histidine, the IL 1403 strain does not grow in the presence of histidinol (intermediate in histidine biosynthesis) suggesting that the *his*D gene is inactive (Delorme *et al.*, 1993). The histidine operon of auxotrophic strains (*his*CGDBHA) was cloned and compared with prototrophic strains. Five deletions and one insertion were found, resulting in six frameshift mutations in the coding region (Delorme *et al.*, 1993). These mutations lead to the inactivation of all enzymes that constitute the histidine operon genes. Thus, IL 1403 is auxotrophic in relation to histidine and, consequently the presence of this amino acid in the culture medium is mandatory.

The absence of threonine provokes a reduction of 30 % in the growth rate, similarly to what was obtained by Cocaign-Bousquet *et al.* (1995). However, the omission of lysine by this author does not affect the μ_{max}. This result is contradictory to the ones described in this thesis. The absence of lysine reduces growth in 38 % (Table 8) in comparison with the control.

In accordance with the results obtained, the elements of the aromatic family, alanine, cysteine, glutamic acid and aspartic acid do not affect significantly the bacterial growth, a behaviour similar to what was found by Cocaign-Bousquet *et al.* (1995) and Zhang *et al.* (2009). The elements of the aromatic family and alanine were removed together and the results are shown in Table 9. In this case, the growth rate was reduced in 22 %, probably due to phenylalanine, because this is the amino acid whose individual omission had a greater impact in the parameter evaluated.

Table 9 - Maximum growth rate (μ_{max}) obtained in experiments where several amino acids were removed simultaneously Without Extras (WE) correspond to absence of Phe+Ala+Trp+Tyr

Amino acids	μ_{max} (h^{-1})	Amino acids	μ_{max} (h^{-1})	Amino acids	μ_{max} (h^{-1})
WE	0.224	WE + Gly	0.189	WE + Asn	0.143
WE + Arg	0.196	WE + Ile	0.162	WE + Gln	0.177
WE + Cys	0.188	WE + Lys	0.168	Control	0.287
WE + Glu	0.182	WE + Thr	0.173		

The amino acids that had a low impact in *L. lactis* growth were firstly removed individually and, then, removed together with aromatic amino acids and alanine (Table 9). In accordance to the results obtained, the growth rate in most of the experiments is affected by approximately 40 %. In the experiments where asparagine is removed, μ_{max} decreased 51 % comparatively to the experiments with 19 amino acids (control). This amino acid is involved in the production of nucleotides and proteins, but in most cases is promptly directed to the aspartic acid synthesis (Lahtvee *et al.*, 2011). Aspartic acid can also be produced from the TCA cycle intermediate oxaloacetate and, is required for the synthesis of isoleucine, methionine and threonine. Nevertheless, this amino acid was not added into the medium since asparagine is able to fulfil the need of aspartic acid (Azizan *et al.*, 2013; Caspi *et al.*, 2010; Dale *et al.*, 2010; Lahtvee *et al.*, 2011).

As demonstrated in Table 8, methionine, histidine, leucine, valine, proline and serine are the amino acids that have a higher impact in the maximum specific growth rate, and for this reason are considered essential amino acids for *Lactococcus lactis* metabolism. However, growing cells with only these amino acids, a sustainable growth is not guaranteed, as seen in Figure 16.

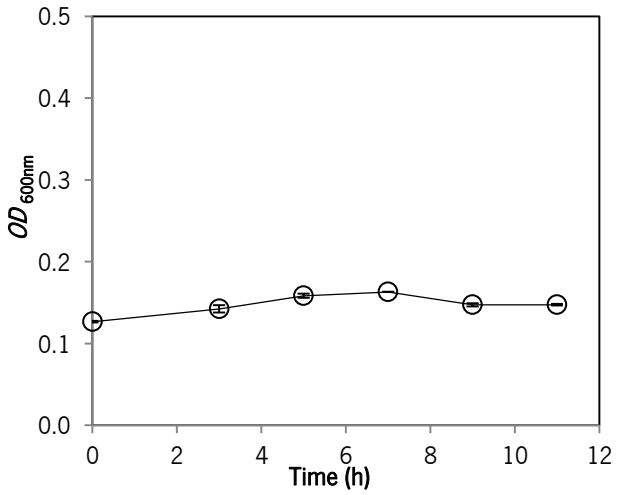

Figure 16 - *Lactococcus lactis* IL 1403 growth curve in shake flasks. Cells were cultivated in a modified SD 3 medium supplemented only by the amino acids methionine, histidine, leucine, valine, proline and serine and 10 $g \cdot L^{-1}$ of glucose at 30 ^{o}C and 200 rpm. Average and standard deviation were calculated from two independent fermentation experiments.

One of the most widely used and trusted database for the metabolism of small molecules is MetaCyc. This database contains information about reactions, genes, enzymes and metabolites present in the different microorganisms that were inferred or tested experimentally and reported in the scientific literature. The current version v.18.0, contains information of more 2000 pathways from 2000 microorganisms, as *L. lactis* (Caspi *et al.*, 2010; Caspi *et al.*, 2006; Dale *et al.*, 2010). In accordance with what is indicated in MetaCyc, for *L. lactis* IL1403, additional amino acids must be added to culture medium in order to guarantee a good cell growth. That is the case of glutamine, glutamic acid, cysteine, asparagine and threonine. Glutamine and glutamic acid can interconvert into each other, meaning that only one is needed to be added into the medium. These two amino acids are required to synthesise arginine, proline and tryptophan (Azizan *et al.*, 2013; Caspi *et al.*, 2010). As a neutral amino acid, glutamine is more stable than glutamic acid, and for this reason was preferably added to the medium composition (Campos, 1999). Cysteine is an important source of sulphur, a compound indispensable to some vitamins (lipoic acid, biotin and thiamine), along with coenzyme A and methionine (Sperandio *et al.*, 2005). In accordance with these data it was stablished four new amino acid combinations, Table 10.

Table 10 - Amino acid combinations tested based in the data present in the Metacyc database The abbreviation AA corresponds to the methionine, histidine, leucine, valine, proline and serine

Combination 7 (C7)	Combination 8 (C8)	Combination 9 (C9)	Combination 10 (C10)
AA + Cys + Gln + Thr + Asn	AA + Cys + Gln + Thr	AA + Cys + Gln + Asn	AA + Cys + Gln

Cells were cultivated overnight in the modified SD3 medium and then were transferred to defined media with different amino acid combinations. In Figure 17, the growth of *L. lactis* for these amino acid compositions is represented.

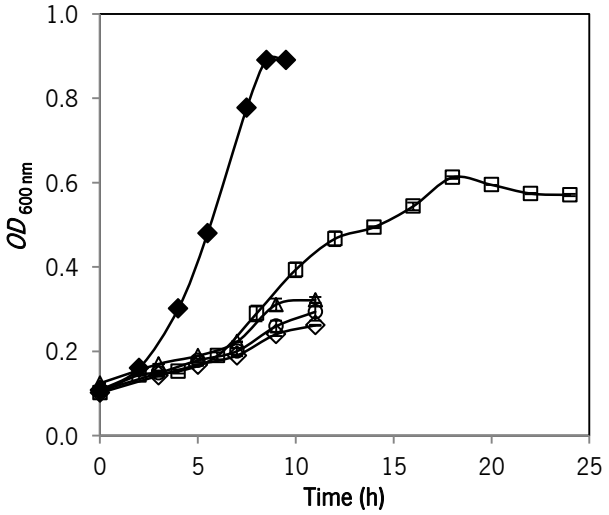

Figure 17 - *L. lactis* IL 1403 growth curve in shake flasks. Cells were cultivated in media with different amino acid combinations and 10 $g \cdot L^{-1}$ glucose, at 30 ^{o}C and 200 rpm. Symbols stands for: ◆ control, □ combination 7, ○ combination 8, △ combination 9 and ◇ combination 10 (see Table 10). Average and standard deviation were calculated from two independent fermentation experiments.

In combination 7, cells have grown until an optical density of 0.6, while nearly no growth is observed for combination 10.

Van Niel *et al.*, (2002) studied the effect of oxygen in the growth of *L. lactis* ATCC 19435, and for this tested different conditions (aerobiosis, O_2 limitation and anaerobiosis) with and without asparagine in the culture medium. The results show that in anaerobiosis and without asparagine the specific growth rate is very low (0.66 h^{-1}) and only 1.02 g L^{-1} of biomass was formed. Contrary, in anaerobiosis with asparagine, the specific growth rate is higher (0.86 h^{-1}) and the biomass formed was 1.21 $g \cdot L^{-1}$. This behaviour was similar to the one obtained in combinations 7 and 8, Figure 17. In this last combination, the asparagine is absent and the biomass produced was lower than in combination 7 that contains this amino acid. In the tested conditions (shake flask) it is

possible to affirm that the presence of asparagine in the culture medium is not essential, as it can be seen in Table 9, because growth was possible in the absence of this amino acid. In conclusion, we can state that the presence of asparagine in anaerobic conditions improves cellular growth (van-Niel *et al.*, 2002).

Concluding, the sustainable growth of *Lactococcus lactis* IL 1403 is possible in a medium with minimal amino acids requirement that contains methionine, histidine, serine, valine, proline, leucine, glutamine, cysteine, asparagine and threonine.

In accordance with the results obtained in the other experiments, the minimal medium for *L. lactis* IL 1403 has 29 components, among which 10 are amino acids and 7 are vitamins, table 11.

Table 11 – Composition of minimal medium for *L. lactis* IL 1403

Minimal Medium for *L. lactis* IL 1403			
Vitamins	**Amino acids**	**Mineral Base**	**Trace Elements**
Biotin	Cysteine	Acetate	$(NH_4)_6Mo_7O_{24}$
Calcium pantothenate	Histidine	$(NH_4)_2SO_4$	$MnSO_4 \cdot 4H_2O$
Folic acid	Methionine	NaCl	$CaCl_2 \cdot 2H_2O$
Pyridoxamine	Proline	KH_2PO_4	$ZnSO_4 \cdot 7H_2O$
Thiamine HCl	Serine	K_2HPO_4	$CoCl_2 \cdot 6H_2O$
Riboflavin	Threonine	$MgSO_4 \cdot 7H_2O$	$CuSO_4 \cdot 5H_2O$
Nicotinic acid	Valine		
	Leucine		
	Asparagine		
	Glutamine		

4.3 INFLUENCE OF THE pH IN *L. lactis* GROWTH

Each microorganism has an optimal pH where the growth rate reaches a maximal value. Notwithstanding, cells can grow in a pH range without provoking a significant variation in the μ_{max} (Fonseca and Teixeira, 2006; Pampulha, 1998). Most environments in which the microorganisms inhabit have a pH between 5 to 9 (Madigan *et al.*, 2003). Most of the lactic acid bacteria are neutralophiles, meaning that they grow best at a neutral pHs (Fonseca and Teixeira, 2006; Pampulha, 1998).

To see if pH control has a meaningful contribution in *L. lactis* growth, we performed cell cultivations with and without pH control. Growth was performed in a bioreactor with aeration and agitation. For the experiment without pH control, pH was initially set to 6.5 (Figure 18), and growth was followed, in addition to glucose consumption and lactic acid production.

As mentioned, *L. lactis* is able to convert glucose in organic acids, as lactic acid, which leads to an acidification of the medium and consequent pH decrease. Cells are inoculated in the medium with pH of 6.5 which decreases until the end of growth until 4. The pH decrease is more accentuated during the exponential phase, the stage in which cells are metabolically more active. The lactic acid production leads to a decrease of the extracellular pH and prevents the growth of other microorganisms, such as, spoilage and pathogenic bacteria (Andersen *et al.*, 2009; Parente *et al.*, 1994). Apart from the acidification of the culture medium, the lactic acid production ultimately leads to a growth arrest and even cell death (Rallu *et al.*, 1996).

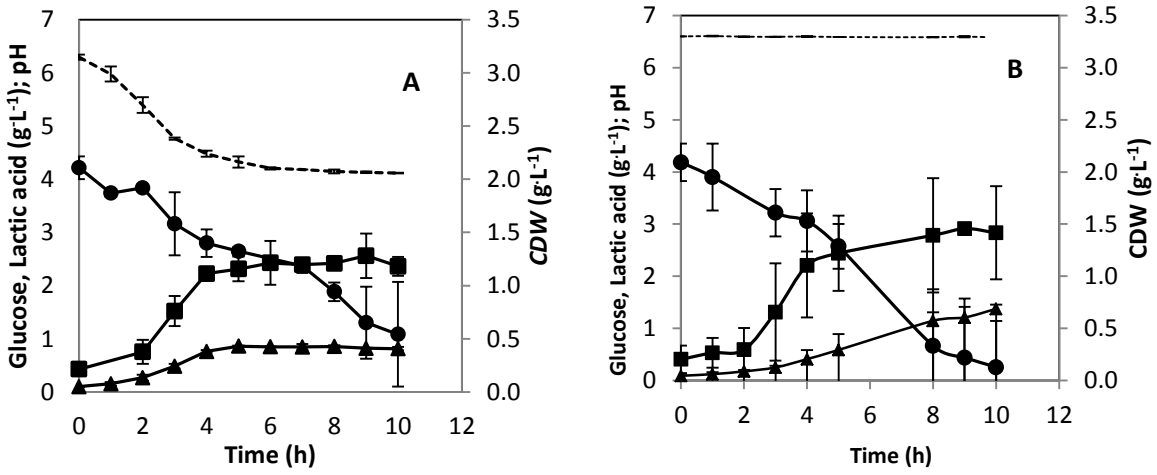

Figure 18 - Typical batch cultivation of *L. lactis* in the Corynebacterium medium at 30 ^{o}C with an agitation of 250 rpm and 30 % DO. (A) Without pH control. pH (- - -), glucose concentration (●), *CDW* (▲), lactic acid production (■) and (B) with pH control, pH (- - -), glucose concentration (●), *CDW* (▲), lactic acid production (■). Data are presented as the mean and the standard deviation calculated from two independent fermentation experiments in bioreactor.

When the pH is around 4 the bacterial growth ceases (Andersen *et al.*, 2009) and, enters in the stationary phase five-hours after inoculation, as shown in Figure 18 - A. From this moment, lactic acid production slows down. The maximum lactic acid concentration obtained without pH control was 2.425 g·L^{-1}, six hours after inoculation, and it was maintained along the stationary phase. During this period, the value of pH was constant.

Although *Lactococcus lactis* can grow in an acid medium, the production of organic acids contributes to a natural stress (Fonseca and Teixeira, 2006; Rallu *et al.*, 1996). In this experiment,

the maximum biomass concentration obtained was 0.430 $g{\cdot}L^{-1}$, Table 12. Although the organism can grow in an acidified media, the glycolysis rate is largely affected when the extracellular pH is lower than 5. Consequently, the glucose consumption rate is lower than in the controlled pH condition, meaning that cells are not able to import and consume glucose completely (Andersen *et al.*, 2009; Elmarzugi *et al.*, 2010).

When the pH is not controlled, the stationary phase has a duration of 5 hours (see Figure 18), and during this period the biomass concentration remains constant. According to Cock and Stouvenel (2006), when the pH is not controlled and the medium has a low glucose concentration (< 20 $g{\cdot}L^{-1}$), only approximately 75 % of the glucose is consumed. These results are corroborated by the ones obtained in our conditions, where there is a consumption of 76 % of the glucose. Andersen *et al.*, (2009) shows that a low extracellular pH decreases the phophoenolpyruvate concentration and subsequently the uptake of glucose, ultimately stopping glycolysis.

The optimum pH for *L. lactis* subsp *lactis* growth is 6.5 in accordance to studies developed by Parente *et al.*, (1994). This author evaluated *L. lactis* growth during batch fermentations with different pH values (5-6.5) obtaining the best biomass concentration, 3.067 $g\ L^{-1}$, with a pH of 6.5. This way, in the experiment with pH control, this parameter was controlled at 6.5.

Table 12 - Comparison of the kinetic parameters with and without pH control in *L. lactis* Maximum biomass (X_{max}) concentration and specific grow rate (μ_{max}) on complex medium, with or without pH control at the end of the exponential phase

Kinetic parameters	Non-controlled pH	Controlled pH
X_{max} ($g{\cdot}L^{-1}$)	0.430 ± 0.004	0.696 ± 0.115
μ_{max} (h^{-1})	0.548 ± 0.090	0.398 ± 0.041

As expected, when we compare cell growth in the two conditions (with and without pH control) the maximum biomass concentration is obtained when the pH is kept at 6.5, Table 12. Similar results were obtained by Cock and Stouvenel, (2006) affirming that by keeping constant the optimal pH for *L. lactis*, cell growth was at its maximum and, consequently the biomass produced was higher in comparison with cultures without pH control.

During bacterial growth and fermentation experiments, the pH of the cytoplasm decreases with the acidification of the medium due to the production and excretion of fermentation end-products, namely lactic acid, promoting the acid stress (Hartke *et al.*, 1996). This stress is provoked by the

entrance of organic acids into cells by passive diffusion; after this diffusion, the acids dissociate realising a proton (Fonseca and Teixeira, 2006). With the increase of organic acids concentrations, the maintenance energy used to excrete protons is too high and the action of the H^+ efflux pump is not sufficient to maintain the optimal cytoplasm pH (Kashket, 1987). When the intracellular pH reaches a threshold, the enzymatic functions are inhibited and cells enter in death phase (Kashket, 1987).

Since one of the objectives of this work is to maximize the biomass concentration, the *L. lactis* growth under controlled pH revealed to be the best option. To support this, Rallu *et al.* (1996) also recognizes that the cell growth in uncontrolled (natural) conditions is suboptimal comparatively to controlled conditions.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

In this chapter are referred the main conclusions of this work and presented future perspectives.

The present work had as main objectives the optimization of minimal medium composition for *L. lactis* IL 1403 and pH control in the bioreactor experiments.

In a first stage, the influence of two different carbon sources, glucose and lactose, was evaluated in the growth of *Lactococcus Lactis* IL1403, using a 5-L bioreactor with pH, temperature and dissolved oxygen control. As expected, the biomass concentration was higher in the complex medium supplemented with 5 $g{\cdot}L^{-1}$ lactose, totalizing 0.752 $g{\cdot}L^{-1}$of biomass. This result is supported by the fact that this strain was isolated from dairy products, whose main sugar is lactose.

As opposed to what was observed in the complex medium, cells were not able to grow in a minimal defined medium supplemented with lactose. IL 1403 strain had lost the ability to metabolize lactose in defined medium. Indeed, if we look into the *L. Lactis* IL 1403 genome we can observe that some genes required for the transport/degradation of lactose are not present. However, the addition of cellobiose in the culture medium succumbs this difficulty.

In the three complete defined media tested, *L. lactis* only grew in the modified SD 3 medium. Thus, this medium was used as a reference to determine a minimal medium for *L. lactis* strain IL1403. From all the groups of nutrients evaluated, the one that produced a higher impact on growth in shake flasks was the mineral base, specifically the increment of K_2HPO_4. Since this compound is a natural buffer, it can be speculated that K_2HPO_4 maintains the pH for a longer period of time, favouring this way the bacterial growth. Relatively to other nutrient groups tested, IL 1403 is able to grow with seven vitamins and the nitrogen bases have a stimulatory effect, not being essential for growth. The essential amino acids determined for *L. lactis* IL 1403 are methionine, histidine, serine, valine, proline, leucine, glutamine, cysteine, asparagine and threonine.

Lastly, the influence of the pH in growth was also evaluated in a bioreactor. During the fermentation process, *L. lactis* converted glucose into organic acids, mostly lactic acid, leading to an acidification of the medium and a consequent decrease of the extracellular pH to 4.2 at the end of the fermentation when the pH is not under control, which leads to an arrest of growth. As expected, the maximum biomass concentration (0.696 $g{\cdot}L^{-1}$) was obtained when the pH is controlled.

The European project BacHBerry aims to evaluate the use of alternative media, such as cheese whey, whose main sugar is lactose. To do so, there is probably the need to replace the strain IL 1403 for its parental strain, the IL 594, which is commonly used as a dairy starter culture. This

choice is based on the fact that the current strain is not able to degrade lactose in a minimal medium, so the use of another strain is mandatory.

Lactococcus lactis is traditionally a fermentative organism but also possesses the genes to accommodate the aerobic respiration metabolism. In accordance to several authors, this situation is energetically more favourable to the cell and results in a higher biomass concentration. Thus, it would be interesting to add the heme group, in minimal medium and compare the biomass, end-products production and substrate consumption in fermentation experiments with the minimal medium but without the heme group.

There are different ways to cultivate bacterial cells in bioreactors. The fed batch mode is a commonly used technique in industrial processes that allows obtaining a higher biomass concentration when compared with the batch mode of cultivation. The comparison of different feeding strategies, mixed or single, with the objective of obtaining higher concentrations of biomass could be interesting to improve the biomass yield already optimized in this work.

Nowadays, the consumption of products with bioactive properties is increasing exponentially. Frequently, lactic acid bacteria are used in biological processes to synthesize chemicals with interest for the pharma and food industries. This way, the utilization of mutant strains that accommodate the genes necessary to synthesise phenolic compounds will be explored and further optimized.

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ATTACHMENTS

Calibration curves for substrates (glucose and lactose) and lactic acid, as well as, the supplier instructions to the enzymatic kits are described in this section.

A1 - BIOMASS CALIBRATION CURVE

As mentioned in section 3.5, the biomass concentration, $g \cdot L^{-1}$, was determined using a calibration curve, table A1, established for each condition evaluated in the bioreactor experiments.

Table A 1 - Equation of biomass calibration curve for each variable evaluated

Conditions	Range Concentration ($g \cdot L^{-1}$)	Equation : $Abs_{600nm} = (m \pm S_m) \times X + (b \pm S_b)$		R^2
		$(m \pm S_m)$	$(b \pm S_b)$	
Glucose	0.05-0.6	(3.307± 0.149)	(0.015± 0.011)	0.999
Lactose	0.05-0.8	(4.579± 0.013)	(0.019 ± 0.004)	0.997
Non-control of pH	0.02-0.5	(3.499 ± 0.056)	(0.0359 ±0.006)	0.998

Where:

Abs_{600nm} – Absorbance in 600 nm;

m – slope of the calibration curve;

X – biomass concentration ($g \cdot L^{-1}$)

b – y-intercept

S_m and S_b – standard deviation for slope and y-intercept

R^2 – coefficient of determination.

B1 -SUPPLIER INSTRUCTIONS FOR ENZYMATIC KITS

In accordance with section 3.5, glucose and lactic acid concentrations were determined using enzymatic kits. The experiments were made in microplates and the absorbance was measured at a wavelength of 340 nm. The procedures of glucose and lactic acid kits are described below.

- GLUCOSE:
  - Add 100 µL of solution 1 (mixture of triethanolamine buffer, NADH, ATP and magnesium sulfate), 10 µL of sample solution and 190 µL of ultrapure water. In the control (blank) to add 200 µL of ultrapure water since there is not sample solution.
  - Promote the mixture and read the absorbance (A_1) after 3 minutes.
  - Add 2 µL of suspension 2 that contains hexokinase and glucose-6-phosphate dehydrogenase and reaction starts.

- After 15 minutes was read the absorbance (A_2).

Then, was calculated the absorbance differences (A_2 - A_1) for samples and control and substrate the absorbance difference of the sample with the absorbance difference of the control (equation I).

$$\Delta A = (A_2 - A_1)_{sample} - (A_2 - A_1)_{blank} \quad \text{(equation I)}$$

The ΔA was converted in glucose concentration through of calibration curve.

- LACTIC ACID
  - Add 100 µL of solution 1 (mixture of glycylglycine buffer and L-glutamic acid); 20 µL of solution 2 (NAD); 2 µL of suspension 3 (glutamate-pyruvate transaminase); 10 µL of sample solution and 90 µL of ultrapure water. In the control (blank) to add 100 µL of ultrapure water since there is not sample solution.
  - Promote the mixture and read the absorbance of the solutions (A1) after 5 minutes.
  - Add 2 µL of solution 4 that contains L-lactate dehydrogenase and reaction starts.
  - After 30 minutes was read the absorbance (A2).

The absorbance differences (A_2 - A_1) was then calculated for samples (control and substrate) (equation II).

$$\Delta A = (A_2 - A_1)_{sample} - (A_2 - A_1)_{blank} \quad \text{(equation II)}$$

-

The ΔA was converted in glucose concentration through of calibration curve.

C1 - GLUCOSE AND LACTIC ACID CALIBRATION CURVES

As mentioned in section 3.5, glucose and lactic acid were quantified by enzymatic kits. The concentrations of these compounds were calculated through calibration curves, table C1.

Table C 1 - Glucose, lactose and lactic acid calibration curves, obtained by enzymatic kits and HPLC

Conditions	Range Concentration ($g \cdot L^{-1}$)	Equation : $Abs_{340nm} = (m \pm S_m) \times X + (b \pm S_b)$		R^2
		$(m \pm S_m)$	$(b \pm S_b)$	
Glucose	0.05-0.5	(0.801± 0.149)	(0.014± 0.011)	0.999
Lactic acid	0.05-7.5	(0.995± 0.045)	(0.027 ± 0.016)	0.998

D1 - LACTOSE CALIBRATION CURVE

Lactose concentrations were determined by high performance liquid chromatography (HLPC) (section 3.5). With the conditions used, the retention time of lactose is 9.867 minutes. The lactose calibration curve is presented in table D1.

Table D 1 – Equation for lactose calibration curve in HPLC (conditions 60 ºC and 0.5 $mL^{-}min^{-1}$)

Compound	Range Concentration ($g \cdot L^{-1}$)	Equation : $A = (m \pm S_m) \times C + (b \pm S_b)$		R^2
		$(m \pm S_m)$	$(b \pm S_b)$	
Lactose	1-20	(596.49±0.129)	(93.299 ± 0.027)	0.999

Where:

A –peak area ($mV \cdot s^{-1}$)

m – slope of the calibration curve;

C – lactose concentration ($g \cdot L^{-1}$)

b – y-intercept

S_m and S_b – standard deviation for slope and y-intercept

R^2 – coefficient of determination.